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Antibiotic resistance: the loud pandemic, dissemination and reservoirs

Agnese Lupo

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Habilitation à diriger des recherches

Agnese LUPO

Antibiotic resistance: the loud pandemic, dissemination and reservoirs

Résistance aux antibiotiques: la pandémie bruyante, dissémination et réservoirs

Membres du jury

M. Barraud Olivier*	Maître de Conférences – HDR, UMR INSERM 1092
Mme. Bibbal Delphine	Maître de Conférences – HDR, UMR1436 INRAE
M. Madec Jean-Yves	Directeur de Recherche – HDR, ANSES-Lyon
Mme. Salcedo Suzana*	Directrice de Recherche – HDR, UMR5086 CNRS INSERM
Mme. Vianney Anne	Maître de Conférences – HDR, Université de Lyon 1
M. Zarrilli Raffaele*	Professeur des Universités, Università di Napoli, Italie

*Rapporteur/ice

Acknowledgements

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I am grateful to Prof. Endimiani Andrea for sharing his professional knowledge.

I am grateful to my colleagues of the ANSES-Laboratory of Lyon and in particular to Claire Mottet for providing daily examples of kindness and for her friendship.

Thanks to all the members of the AVB team, they provide always secure and prompt support. Special thanks to Estelle Saras for obtaining many of the results presented in this manuscript.

I am grateful to Jean-Yves Madec and Marisa Haenni for many things, but in particular because they hired a seven-month pregnant woman. I wish that this could serve as example to many. I was granted with an incredible opportunity!

Finally, thanks to all my family for their encouraging words and to Sébastien Coyne, my husband, my colleague, my friend.

FRENCH SUMMARY

La résistance bactérienne aux antibiotiques est un problème majeur tant pour la santé humaine que pour la santé animale. Ses origines sont anciennes et son évolution est inévitable. De nombreux facteurs ont été mis en évidence, qui contribuent à l'évolution de ce phénomène, impliquant autant le réservoir humain que les réservoirs environnemental et animal. De plus, les éléments génétiques mobiles facilitent la propagation des gènes de résistance dans les populations bactériennes.

Pendant ma thèse, j'ai conduit l'analyse moléculaire de la résistance aux macrolides et à la tétracycline dans plusieurs espèces de *Streptococcus*, et particulièrement la caractérisation de l'environnement génétique des gènes appartenant aux familles *mef(E)*, *erm(B)* et *tet(M)* dans une souche clinique de *Streptococcus salivarius*. Le gène *mef(E)* était localisé sur un élément génétique non-conjugatif MEGA (Macrolide Efflux Genomic Assembly) et les gènes *erm(B)* et *tet(M)* co-localisés sur le transposon composite Tn3872, de la famille Tn916, un élément intégratif et conjugatif. Ce dernier et/ou les recombinases propres de l'hôte ont permis la transmission de l'élément MEGA par conjugaison à une souche receveuse de *Streptococcus pneumoniae*. De façon très surprenante, nous avons retrouvé et analysé l'élément MEGA dans des espèces à Gram négatif très éloignées tel que *Haemophilus influenzae*. Récemment nous avons caractérisé un autre élément génétique qui porte des gènes donnant de hauts niveaux de résistance à la gentamicine chez des souches de *Streptococcus dysgalactiae* sbp. *equisimilis* isolées de cheval. Cet élément génétique était capable de transférer chez une souche receveuse de *Streptococcus agalactiae*, une espèce pathogène pour l'Homme. Toutes ces expériences suggèrent que la formation des éléments génétiques composites contribue au développement de la multi-résistance dans les pathogènes à Gram positif et que la diffusion de ces résistances peut se faire à une très large échelle.

A la suite de mon travail de thèse et en considérant l'importance de l'environnement dans le développement et la diffusion de la résistance aux antibiotiques, j'ai rejoint comme PostDoc le Département d'Hydrobiologie à l'Université de Dresde en Allemagne. Mon activité a été conduite au sein du projet IWAS (International Water Alliance Saxony) avec pour objectifs d'améliorer i) l'accès à l'eau potable pour la population et ii) les sources d'eau douce et la gestion des eaux usées en Ukraine, pays sélectionné dû à la faible qualité de ses eaux douces et parce que candidate à devenir membre de l'UE, et devant donc se conformer aux critères Européens de qualité de l'eau. Nous avons analysé la qualité de l'eau de la rivière Bug. Un de ses affluents majeurs est la Poltva, qui prend naissance dans L'viv, recevant ses eaux usées. Nous avons évalué des paramètres biochimiques, physiques et microbiologiques en association avec l'analyse phénotypique et moléculaire de la résistance aux antibiotiques chez des bactéries à Gram négatif. Une haute charge de pollution organique était présente dans la rivière Bug qui provenait de la Poltva. Nous avons trouvé un profil liant une basse qualité de l'eau et la présence de souches multi-résistantes et avons retracé la source de pollution ainsi que la dissémination de clones d'*Escherichia coli* multi-résistants aux antibiotiques. Le transfert horizontal de gènes de résistance a également été obtenu *in vitro* à partir d'une souche isolée de la rivière. Cette étude suggère que les sites particulièrement pollués sont une source non seulement de polluants connus mais aussi de bactéries multi-résistantes, capable de disséminer par clonalité mais également en transférant horizontalement leurs gènes de résistance.

A la suite de ce PostDoc, je me suis engagée à l'Université de Berne en Suisse, dans le département de maladies infectieuses, dans le cadre de RadarGo, projet consistant à élaborer un test de diagnostic moléculaire pour la détection de *Neisseria gonorrhoeae* et la prédiction de la résistance aux antibiotiques associée. En parallèle de ce projet, j'ai eu l'opportunité de mener plusieurs projets visant à étudier la dissémination et l'épidémiologie de bactéries pathogènes humaines et leurs mécanismes de résistance aux antibiotiques. En collaboration avec l'Institut

de Bactériologie Vétérinaire de la Faculté VetSuisse, nous avons trouvé des échantillons de viande vendus en Suisse contaminés par *Acinetobacter baumannii*. Cette bactérie pathogène opportuniste pour l'Homme, connue pour sa capacité à développer des multi-résistances aux antibiotiques, cause des infections sévères avec un choix thérapeutique limité. Le typage moléculaire de ces souches a montré que les isolats retrouvés dans la viande appartenaient au même complexe clonal que des isolats provenant de patients et présentant des résistances multiples aux antibiotiques. Ces résultats suggèrent ainsi que les aliments, la viande crue en l'occurrence, peuvent servir de réservoir de pathogènes humains.

J'ai ensuite continué cet axe de recherche au laboratoire de l'ANSES-Lyon. Actuellement, une étudiante en thèse que je codirige est en charge d'un projet visant à analyser les relations phylogénétiques des souches d'*A. baumannii* retrouvées dans la nourriture avec celles responsables d'infections chez l'Homme. Ainsi, les mécanismes typiques d'adaptation au milieu hospitalier comme la capacité à acquérir des gènes de résistance, à résister aux biocides et à former du biofilm seront étudiés. A l'ANSES-Lyon je participe à l'activité de surveillance moléculaire des bactéries résistantes aux antibiotiques, en particulier d'*Acinetobacter* spp., chez les animaux malades et participe à la caractérisation moléculaire de gènes de résistance dans divers organismes. J'ai également initié des investigations au travers de l'encadrement de deux étudiants en thèse pour comprendre l'impact des antibiotiques sur la composition du microbiote intestinale des animaux et leur capacité à sélectionner des bactéries résistantes. Ces connaissances sont importantes pour continuer à connaître les fonctions du microbiote et les protéger. Mieux connaître les mécanismes de sélection par les thérapies antibiotiques dans le microbiote contribuera à définir des choix thérapeutiques efficaces et limitant la propagation de gènes de résistance au sein de celui-ci et dans l'environnement.

Curriculum vitae

Generalities

Agnese Lupo

Date of birth: 22/04/1979

Nationality: Italian

Country of residence: France

Academic education

1997-2004

Master in Life Sciences, University of Catania, Italy (110/110 cum laude)

Research topic (2 years laboratory practice): genetic elements carrying macrolide resistance genes in streptococci

Supervisors: Prof. Maria Santagati and Prof. Stefania Stefani

2005

Habilitation to the profession of biologist, University of Catania, Italy (158/160)

2005-2008

Doctoral school, University of Catania, Italy

Research topic: integrative and conjugative elements and their mobilization in streptococci

Project and Funding: DRESP 2 (Drug Resistance Spread 2)

Directors: Prof. Stefania Stefani and Prof. Maria Santagati

Publications:

Santagati M., **Lupo A.**, Scillato M., Di Martino A., Stefani S.

Conjugal mobilization of the mega element carrying *mef(E)* from *Streptococcus salivarius* to *Streptococcus pneumoniae*. *FEMS Microbiology Letters*, 2009, 290:79-84.

Borbone S., **Lupo A.**, Mezzatesta M.L., Campanile F., Santagati M., Stefani S.

Evaluation of the *in vitro* activity of tigecycline against multiresistant Gram-positive cocci containing tetracycline resistance determinants. *International Journal of Antimicrobial Agents*, 2008, 31:209-215.

Other activities:

Advisor for master student: genetic elements harboring macrolide resistance in viridans streptococci (Tania Triscari Barberi).

Lectures on antibiotic resistance mechanisms for the cursus of Microbiology Sciences at the Faculty of Life Sciences, University of Catania, Italy

Participation to the DRESP2 reporting meeting, 2005, Budapest, Hungary

Participation to the organization of DRESP2 reporting meeting, 2007, Catania, Italy

Participation to DRESP2 reporting meeting, 2008, Stockholm, Sweden

PostDocs

March-July 2009

Young PostDoc Fellow, Institute Pasteur Paris, France

Research topic: beta-lactams resistance characterization in *Corynebacterium striatum*

Grant: Young Fellow Post-doc training from ESCMID

Advisors: Prof. Patrice Courvalin and Bruno Périchon, PhD

August 2009 - June 2012

PostDoc, Technical University of Dresden, Germany

Research topic: analysis of antibiotic resistance in freshwater highly impacted by human activities

Project and Funding: IWAS (International Water Alliance Saxony); Cost Action TD0803: Detecting evolutionary hot spots of antibiotic resistances in Europe

Director of the unit: Prof. Thomas Berendonk

Publications:

Ertel A. M., **Lupo A.**, Scheifhacken N., Bodnarchuk T., Manturova O., Berendonk T. U., Petzoldt T.

Heavy load and high potential: anthropogenic pressures and their impacts on the water quality along a lowland river (Western Bug, Ukraine). *Environmental Earth Sciences*, 2012, 65:1459-1473.

Lupo A., Coyne S., Berendonk T. U.

Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. *Frontiers in Microbiology*, 2012, 116-134 (Chapter of the book: Analyzing possible intersections in the resistome among human, animal and environment matrices; editor: Stefania Stefani)

Other activities:

Advisor for master student: heavy metal resistance analysis in freshwater bacterial isolates (Claudia Seiler)

Participation to the COST Action DARE meetings (Cyprus, Porto, Switzerland), organization of one meeting in Catania

Lecture for Master Students at Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), University of Oldenburg, Germany

Laboratory on freshwater bacteriology analysis for Master Students at the Technical University of Dresden, Germany

Organization of two sampling campaigns (four weeks, summer 2012, spring 2013) from Dresden to L'viv.

November 2012-December 2013

PostDoc, University of Bern, Switzerland

Research topic: regulation of virulence genes in *Streptococcus agalactiae*

Funding: internal funding

Director of the unit: Prof. Parham Sendi

Publications:

Lupo A., Ruppen C., Hemphill A., Spellerberg B., Sendi P.

Phenotypic and molecular characterization of hyper-pigmented Group B Streptococci. *International Journal of Medical Microbiology*, 2014, 304:717-724

Ruppen C., **Lupo A.**, Decosterd L., Sendi P.

Is penicillin plus gentamicin synergistic against clinical Group B *Streptococcus* isolates?: An *in vitro* study. *Frontiers in Microbiology*, 2016, 7:1680. eCollection.

Other activities:

Building up and managing the research laboratory including budget.

Laboratory training of Corinne Ruppen (PhD student)

December 2013-January 2015

PostDoc, University of Bern, Switzerland

Research topic: development of a molecular screening tool to detect antibiotic resistance in *Neisseria gonorrhoeae*; molecular epidemiology of resistant bacteria from several reservoirs

Project and Funding: Radar-Go (Rapid Diagnosis of Antibiotic Resistance in Gonorrhoeae)

Director of the unit: Prof. Andrea Endimiani

Publications:

Endimiani A., Allemann A., Wüthrich D., **Lupo A.**, Hilty M.

First report of the macrolide efflux genetic assembly (MEGA) element in *Haemophilus parainfluenzae*. *International Journal of Antimicrobial Agents*. 2017, 49:265-266

Donà V., Kasraian S., **Lupo A.**, Guilarte Y.N., Hauser C., Furrer H., Unemo M., Low N., Endimiani A.

Multiplex Real-Time PCR assay with High-Resolution Melting analysis for characterization of antimicrobial resistance in *Neisseria gonorrhoeae*. *Journal of Clinical Microbiology*, 2016, 54:2074-2081

Lupo A., Isis E., Tinguely R., Endimiani A.

Clonality and antimicrobial resistance of *Burkholderia cepacia* complex isolates collected from cystic fibrosis patients during 1998-2013 in Bern, Switzerland. *New Microbiologica*, 2015, 38:281-288

Lupo A., Isis E., Perreten V., Endimiani A.

Raw meat contaminated with epidemic clones of *Burkholderia multivorans* found in cystic fibrosis patients. *Journal of Cystic Fibrosis*, 2015, 14:150-152

Lupo A., Guilarte Y.N., Droz S., Hirzel C., Furrer H., Endimiani A.

In vitro activity of clinically implemented β -lactams against *Aerococcus urinae*: presence of non-susceptible isolates in Switzerland. *New Microbiologica*, 2014, 37:563-566

Lupo A., Vogt D., Seiffert S. N., Endimiani A., Perreten V.

Antibiotic resistance and phylogenetic characterization of *Acinetobacter baumannii* isolates from commercial raw meat in Switzerland. *Journal of Food Protection*, 2014, 77:1976-1981

Seiffert S.N., Carattoli A., Tinguely R., **Lupo A.**, Perreten V., Endimiani A.
High prevalence of extended-spectrum β -lactamase, plasmid-mediated AmpC, and carbapenemase genes in pet food. *Antimicrobial Agents Chemotherapy*, 2014, 58:6320-6323

Guilarte Y.N., Tinguely R., **Lupo A.**, Endimiani A.
Prevalence and characteristics of fluoroquinolone-resistant *Aerococcus urinae* isolates detected in Switzerland. *International Journal of Antimicrobial Agents*, 2014, 43:474-475

Endimiani A., Guilarte Y.N., Tinguely R., Hirzberger L., Selvini S., **Lupo A.**, Hauser C., Furrer H.

Characterization of *Neisseria gonorrhoeae* isolates detected in Switzerland (1998-2012): emergence of multidrug-resistant clones less susceptible to cephalosporins. *BMC Infectious Diseases*, 2014, 14:106-116

Lupo A., Papp-Wallace K.M., Sendi P., Bonomo R.A., Endimiani A.
Non-phenotypic tests to detect and characterize antibiotic resistance mechanisms in Enterobacteriaceae. *Diagnostic Microbiology and Infectious Diseases*. 2013, 77:179-194 (Chapter of book: Antimicrobial Resistance and Food Safety, 2015. Editors: Chen C.Y., Yan X., Jackson C.R.)

Seiffert S.N., Tinguely R., **Lupo A.**, Neuwirth C., Perreten V., Endimiani A.
High prevalence of extended-spectrum-cephalosporin-resistant Enterobacteriaceae in poultry meat in Switzerland: emergence of CMY-2- and VEB-6-possessing *Proteus mirabilis*. *Antimicrobial Agents Chemotherapy*. 2013, 57:6406-6408

Current position

Since October 2015

Researcher, ANSES-Lyon, Lyon, France

Research topic: molecular surveillance of antibiotic resistance in bacterial isolates from diseased animals; analysis of the impact of antibiotic therapies on intestinal microbiota of animals

Director of the unit: Jean-Yves Madec, DVM, PhD (co-director: Marisa Haenni, PhD)

Project and Funding:

- Apramyres: Prévalence et caractérisation moléculaire de la résistance à l'apramycine dans les infections animales en France (EcoAntibio).
- Canibote: Analysis of beta-lactams impact on microbiota of dogs (EcoAntibio).
- EmoxMint: Analysis of beta-lactams impact on microbiota of bovins (SAARA)
- RESVEAM: Analysis of beta-lactams impact on microbiota of bovins, in collaboration with Prof. Prouillac Caroline from VetAgro Sup Institut (EcoAntibio)

Publications:

Ncir S., **Lupo A.**, Drapeau A., Châtre P., Souguir M., Azaiez S., Madec J.Y., Mansour W., Haenni M.

Back to the origin: *bla*_{OXA-204} and *bla*_{NDM-1} genes in *Shewanella* spp. from a Tunisian River. *Microbiol Spectr*. 2022, 10(3):e0160522.

Haenni M., Métayer V., **Lupo A.**, Drapeau A., Madec J.Y.
Spread of the *bla*_{OXA-48}/IncL plasmid within and between dogs in city parks, France. *Microbiol Spectr.* 2022, 10(3):e0040322.

Rochegüe T., Haenni M., Mondot S., Astruc C., Cazeau G., Ferry T., Madec J.Y., **Lupo A.**
Impact of Antibiotic Therapies on Resistance Genes Dynamic and Composition of the Animal Gut Microbiota. *Animals (Basel)*. 2021, 11:3280.

Rochegüe T., Haenni M., Cazeau G., Metayer V., Madec J.Y., Ferry T., **Lupo A.**
An inventory of 44 qPCR assays using hydrolysis probes operating with a unique amplification condition for the detection and quantification of antibiotic resistance genes. *Diagn Microbiol Infect Dis.* 2021, 100:115328.

Osman M., B Halimeh F., Rafei R., Mallat H., Tom J.E., Raad E.B., M Diene S., Jamal S., Atrouni A.A., Dabboussi F., Moudani W., Madec J.Y., Saras E., **Lupo A.**, Haenni M., Rolain J.M., Hamze M.
Investigation of an XDR-*Acinetobacter baumannii* ST2 outbreak in an intensive care unit of a Lebanese tertiary care hospital. *Future Microbiol.* 2020, 15:1535-1542.

Godeux A.S., Svedholm E., **Lupo A.**, Haenni M., Venner S., Laaberki M.H., Charpentier X.
Scarless removal of large resistance island AbaR results in antibiotic susceptibility and increased natural transformability in *Acinetobacter baumannii*. *Antimicrob Agents Chemother.* 2020, 64:e00951-20.

Bourély C., Cazeau G., Jarrige N., Jouy E., Haenni M., **Lupo A.**, Madec J.Y., Leblond A., Gay E.
Co-resistance to amoxicillin and tetracycline as an indicator of multidrug resistance in *Escherichia coli* isolates from animals. *Front Microbiol.* 2019, 10:2288.

Lupo A., Haenni M., Madec J.Y.
Antimicrobial resistance in *Acinetobacter* spp. and *Pseudomonas* spp. *Microbiol Spectr.* 2018, 6(3).

Godeux A.S., **Lupo A.**, Haenni M., Guette-Marquet S., Wilharm G., Laaberki M.H., Charpentier X.
Fluorescence-based detection of natural transformation in drug-resistant *Acinetobacter baumannii*. *J Bacteriol.* 2018, 200:e00181-18.

Poirel L., Madec J.Y., **Lupo A.**, Schink A.K., Kieffer N., Nordmann P., Schwarz S.
Antimicrobial resistance in *Escherichia coli*. *Microbiol Spectr.* 2018, 6(4).

Mani Y., Mansour W., **Lupo A.**, Saras E., Bouallègue O., Madec J.Y., Haenni M.
Spread of *bla*_{CTX-M-15}-producing Enterobacteriaceae and OXA-23-producing *Acinetobacter baumannii* sequence type 2 in Tunisian seafood. *Antimicrob Agents Chemother.* 2018, 62:e00727-18.

Lupo A., Haenni M., Saras E., Gradin J., Madec J.Y., Börjesson S.
Is *bla*_{CTX-M-1} riding the same plasmid among horses in Sweden and France? *Microb Drug Resist.* 2018 May 24.

Haenni M., **Lupo A.**, Madec J.Y.
Antimicrobial Resistance in *Streptococcus* spp. *Microbiol Spectr.* 2018, 6(2)

Lupo A., Saras E., Madec J.Y., Haenni M.
Emergence of *bla*_{CTX-M-55} associated with *fosA*, *rmtB* and *mcr* gene variants in *Escherichia coli* from various animal species in France. *J Antimicrob Chemother.* 2018, 73:867-872.

Haenni M., Beyrouthy R., **Lupo A.**, Châtre P., Madec J.Y., Bonnet R.
Epidemic spread of *Escherichia coli* ST744 isolates carrying *mcr-3* and *bla*_{CTX-M-55} in cattle in France. *J Antimicrob Chemother.* 2018, 73:533-536.

Schultz E., Baucheron S., **Lupo A.**, Saras E., Cloeckart A., Doublet B., Madec J.Y., Haenni M.
Complete sequences of *qnrD*-carrying plasmids in *Proteus* spp. collected from French animals. *J Glob Antimicrob Resist.* 2017, 10:306-307.

Lupo A., Châtre P., Ponsin C., Saras E., Boulouis H.J., Keck N., Haenni M., Madec J.Y.
Clonal spread of *Acinetobacter baumannii* sequence type 25 carrying *bla*_{OXA-23} in companion animals in France. *Antimicrob Agents Chemother.* 2016, 61:e01881-16.

Other activities:

Co-direction of PhD students:

- Tony Rochegue (2018-2021)
- Leila Hamze (since May 2022)
- Morgane Moustaghfir (since November 2022)

Master students advisor: Rochegue T., Rouilly L., Moustaghfir M., Robert M.

Co-coordination of inter laboratory assays of performance for the technique of antibiogram and its interpretation for the veterinary diagnostic laboratories participating to the Resapath (<https://resapath.anses.fr/>)

Formation for veterinary diagnostic laboratories participating to the Resapath

Reviewer for (2022): Journal Antimicrobial Chemotherapy; Microbiology Spectrum; Frontiers in Microbiology; Journal of Global Antibiotic Resistance; BMC Microbiology; International Journal Antimicrobial Agents;

Board member of the Veterinary CA-SFM

Member of the animal welfare in ANSES-Lyon experimental platform

Participation to thesis commissions:

« Caractérisation d'un nouveau mécanisme de résistance aux polymyxines chez *Acinetobacter baumannii* » Jean-Baptiste Vuilleminot, PharmD thesis, Université de Franche-Comté.

« Evaluation d'associations d'antibiotiques par approche PK/PD : efficacité thérapeutique et impact sur le microbiote intestinal » Quentin Valle, PhD thesis, INRAE/ENVET.

Participation to several conferences (> 15 poster presentations ; 3 oral presentation)

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1. Introduction

The ability of bacteria to resist against antibiotics is generating a global health crisis seriously threatening life expectancy. The most recent report from the European Centre for Infectious Diseases Control (ECDC) converged the efforts of 14 European Countries who provided data for estimating the occurrence of blood stream infections sustained by resistant bacteria, attributable death to such infections, and annual health burden in the period 2016-2019. Overall, these events have been significantly increasing in this time lapse with +7,980 deaths in 2019 compared to 2016, when 30,730 deaths attributable to infections caused by resistant bacteria were estimated to occur. In accordance, the number of infections caused by resistant bacteria has increased, with estimated + 180,274 people concerned in 2019 compared to 2016 (685,433 estimated cases) (1). Similar observations have been reported from the United States, according to the 2019 U.S. Centers for Disease Control and Prevention (CDC) report. A worrisome point highlighted by the CDC report is the increasing number of infections sustained by Extended Spectrum Beta Lactamases (ESBL) producers of the Enterobacterales family in the community. This issue concerns also *Neisseria gonorrhoeae* that is responsible of sexually transmitted infection (2).

Bacterial antibiotic resistance has biological basis. Mutation of the bacterial target interacting with the antibiotic is a common resistance strategy towards different classes of antibiotics, like fluoroquinolones (3), and beta-lactams in several Gram-positive (4, 5) and Gram-negative species (6).

Replacement of the target, impairs the efficacy of all beta-lactams in *mec* positive staphylococci (7) and confers resistance to vancomycin in enterococci (8).

Alteration of the antibiotic target by its enzymatic modification is a common strategy as well, and includes the methylation of the ribosomal RNA (rRNA) mediated by Erm proteins

(9). This mechanism confers cross-resistance to macrolides, lincosamides and group b streptogramins (MLSb phenotype) and is widespread among *Streptococcus* spp., *Enterococcus* spp., and *Staphylococcus* spp. (10). Methylation of the 16S rRNA ribosome subunit by Rmt and ArmA methylases confers pan-aminoglycoside resistance (11). Mcr-1 enzymes catalyze the phosphoethanolamine transfer onto bacterial lipid A, causing decreased affinity of colistin to its target (12).

A multitude of acquired enzymes inactivating antibiotics are known and they affect several antibiotic classes like fluoroquinolones (Qnr) (13), aminoglycosides (acetyl-transferases, nucleotidyl-transferases, and phospho-transferases) (14), and beta-lactams (class A-D beta-lactamases) (15). Genes encoding beta-lactamases have had a remarkable dissemination success among Gram-negative pathogens.

Bacteria can impede the antibiotics to achieve a proper concentration at the target by reducing permeability through modification of lipids components of the lipopolysaccharides or through loss or modification of porins (16).

Efflux pumps systems are crucial for cell detoxification. They extrude toxic compounds like organic solvents, heavy metals and biocides and have adapted to extrude many antibiotic classes. Selective pressure exerted by biocide can co-select for antibiotic resistance (17).

From a molecular viewpoint antibiotic resistance disseminates through mechanisms such as conjugation, transformation and transduction mediated by plasmids, integrative conjugative elements, recombinases proper to the bacterial host, and phages. Horizontal gene transfer among bacteria is facilitated in high-density communities like microbiota inhabiting the oral cavity and the gut, but also wastewater treatment plants (18, 19). In these communities bacteria are often submitted to selective pressure by antibiotic therapies or antibiotics' residual favoring emergence and concentration of resistances. Other reactors exist that favor antibiotic resistance

evolution consisting in hospitals and farms, where individuals can share their colonizing or pathogens bacteria easily. Surface water acts like a final collector of all these reactors and as a reactor itself (20). Here, bacteria evolved in humans and animals meet autochthonous species, that are reservoir of antibiotic resistance genes, and exchanges can occur potentially generating novel resistance mechanisms in pathogens (21).

Although any bacterial species is able to develop resistance, in the medical frame some species or families of bacteria are listed by the World Health Organization (WHO) as pathogens that are most critical as global health threat. These include carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, carbapenem-resistant and ESBL-producing Enterobacterales.

Dissemination of carbapenem-resistant *A. baumannii* has different dynamics in Europe. In France, regional disseminations have occurred whereas in seven European countries, including Italy, carbapenem-resistant *A. baumannii* are endemic (22). Most likely, such differences correlate with the different control measures at hospital admission and surveillance systems currently available in each country. Eight internationally disseminated clonal complexes (CC) have been described (23), but CC2 is by far the most prevalent (24).

Dissemination of carbapenem-resistant *A. baumannii* strains is favored by their ability to survive in the hospital environment. The presence of indwelling devices serves as vehicle for the pathogen to the patients (25-28). In turn, the contamination of indwelling devices can occur through hands of hospital personal (29). Furthermore, surfaces of the hospital environment and medical material or equipment can serve as persistent reservoir of *A. baumannii* (30). Strains colonizing these supports could be responsible for the colonization of several patients in the

same ward, generating **outbreaks**¹ (31-33). The ability to persist in hospital environment is often attributed to adaptive features such as resisting to disinfectants (34, 35), producing biofilm (28, 36), surviving to desiccation (37-39), and developing antibiotic resistance (34, 40). The most common mechanism conferring carbapenem resistance in the contemporaneous isolates is the carbapenem-hydrolyzing oxacillinase OXA-23 (24). Carbapenem-resistant *A. baumannii* strains are found also in animals and in France (41). Furthermore, food in particular raw meat is an increasingly recognized reservoir of this bacterium (42). However, the epidemiology of this opportunistic pathogen presents some gaps that hinder the understanding of strains circulation between sectors.

In Enterobacterales families, the most common genes conferring carbapenem-resistance (*bla*_{OXA-48} and *bla*_{NDM-1}) and ESBL phenotype (*bla*_{CTX-M}) are propagated by self-transmissible plasmids whose epidemiology presents typical host and geographical distribution (43, 44).

My research projects have been mostly centered on the molecular characterization of antibiotic resistance and genetic relationships of pathogenic or opportunistic species. These projects have concerned bacteria at the interface between humans, animals and other environmental reservoirs to put the basis for understanding this global and transversal health issue taking into consideration the One Health approach. More particularly, I have been working on antibiotic resistant genes and genetic elements in streptococci and *A. baumannii*. Research on this opportunistic pathogen is still ongoing and in development in my activity. More recently, I have started projects to characterize the impact of antibiotics on the gut microbiota. This ongoing research will provide novel understanding on animal microbiota and selection of its resistance, providing basis to optimize therapies in animals.

¹ Osman M., B Halimeh F., Rafei R., Mallat H., Tom J.E., Raad E.B., M Diene S., Jamal S., Atrouni A.A., Dabboussi F., Moudani W., Madec J.Y., Saras E., **Lupo A.**, et al. Investigation of an XDR-*Acinetobacter baumannii* ST2 outbreak in an intensive care unit of a Lebanese tertiary care hospital. Future Microbiol. 2020, 15:1535-1542.

All together, these approaches are contributing to knowledge for fighting antibiotic resistance, the loud pandemic.

2. Horizontal transfer mechanisms and genetic elements disseminating antibiotic resistance genes

2.1 Insertion sequences and transposons

Although not directly mediating horizontal transfer, transposition significantly contribute to the evolution of antibiotic resistance, for instance by transposing antibiotic resistance genes from the chromosome to self-transferable elements and by providing strong promoters to chromosomal resistance genes physiologically expressed at low level (45). Transposition is mediated by insertion sequences (ISs) and transposons.

ISs transpose from a site to another at intracellular scale. Several ISs families exist that can be classified according to similarities of the amino acid sequence of their transposases and their conserved catalytic domains (46). ISs contribute to bacterial adaptation process at the metabolic level (47) or in the establishment of intracellular lifestyle (46, 48). In *A. baumannii*, interaction of ISs and intrinsic resistance genes, normally expressed at low levels, conducts to resistance development towards several antibiotic classes. *ISAbal* and *ISAbal25* harbor -35 promoter regions at their 3' end that, when inserted upstream of -10 box of *bla_{OXA-51}* and *bla_{ADC}* genes, provide strong promoters for their expression (49). *ISAbal-bla_{OXA-51}* and *ISAbal25-bla_{ADC}* correlate with carbapenem- and ceftazidime resistance, respectively (50-52). Similarly, *ISAbal-eptA* correlates with development of colistin resistance (53). Disruption of outer membrane protein encoding gene *carO* by *ISAbal25* determines carbapenem-resistance (54). Insertion of ISs like *ISAbal* in loci regulating the expression of efflux pumps, for instance *adeR* and *adeS*, cause the overexpression of their regulated operons, leading to multidrug resistance (55, 56).

A key element in the propagation of carbapenem-resistance in several clones of *A. baumannii* is the Tn2006 transposon, constituted by two copies of IS*AbaI* bracketing *bla*_{OXA-23} gene. Tn2006 can be found on conjugative plasmids and/or genomic islands (57).

A particular type of IS is constituted by IS91 or ISCR. These ISs, after integrating in a specific site, are able to co-transpose the adjacent gene at each transposition cycle generating large resistance regions. ISCR1-type fusion with integrons promotes the constitution of complex integrons and mobilization of these latter (58). Integrons possess an integrase constituting the 5' conserved sequence (CS) and mediating site-specific recombination, and a 3'CS, usually constituted by the *qacE* gene mediating resistance to biocides or the *sulI* gene, mediating resistance to sulfonamides. Integrons collect resistance cassettes and provides promotor for cassettes expression but are not self-transmissible (59).

Frequently, Enterobacterales isolates present ISE*cpI* insertion sequence inserted upstream *bla*_{CTX-M} genes (60). ISE*cpI*-*bla*_{CTX-Ms} arrangement, besides providing a strong promoter for *bla*_{CTX-M} genes expression, transposes intra-chromosomally but also between chromosome to conjugative plasmids (60, 61) or among different plasmids type (62).

2.2 Integrative conjugative elements (ICEs) and integrative mobilizable elements (IMEs)

Like transposons, integrative conjugative elements (ICEs) move from a site to another of the chromosome, with molecular mechanisms similar to those of temperate phages. Like plasmids, they are able to transfer from a donor cell to a recipient (63, 64).

ICEs excision from the chromosomal insertion site is mediated by excisionases that also create a circular conjugative intermediate. A relaxase nicks one DNA strand, at the origin of transfer (*oriT*) of the ICE, generating single-stranded DNA (ssDNA) (65). The relaxase

covalently bind to one ssDNA end (66) and translocate the ssDNA to a coupling protein (T4CP) (67) component of the type 4 secretion system (T4SS). Several types of T4SS exist and are classified according to the phylogenetic relationship of its components (68). The ssDNA enters the secretory channel constructed by T4SS (69) and through an extracellular pilus passes from the donor to the recipient cell (70). Once in the recipient cell, the relaxase mediates the circularization of the ICE, and machineries proper to the recipient cell replicate the ssDNA (65). When conjugation is achieved, integration of ICE in the host chromosome occurs by transformation or by site-specific recombination governed by the recombinase of the ICE that can be tyrosine-type integrase (71), serine-type integrase (72) or DDE recombinase (73). These enzymes not only catalyze DNA recombination by different molecular processes but present different prevalence in bacterial genomes.

The most common ICEs harbor a tyrosine-type recombinase (74) and include the families Tn916, CTnDOT, and SXT (75). ICEs of the Tn916 family are most commonly found in Gram-positive bacteria and integrate in regions rich in AT nucleotides (76). CTnDOT-type integrases are present in the genomic island 1 of *Salmonella* (77), whereas SXT-type integrases are found in ICEs harboring different conjugative structures, suggesting the exchange of integrases between ICEs.

Serine-recombinases are found in Tn3-type, Tn5044-type and Tn5397 transposons (72, 78).

A DDE transposase (73) is present in TnGBS2 a widespread conjugative transposon characterized in *Streptococcus agalactiae* (79).

Integrative mobilizable elements (IMEs) or mobilizable plasmids (80) are genetic elements that carry their own recombinase, often a relaxase and/or a coupling protein, but are devoid of all components of T4SS. IMEs can make use of the conjugative machinery of a co-resident

ICEs to transfer from a donor cell to a recipient, largely contributing to the evolution of bacterial genomes. IMEs are important vehicles of genes conferring antibiotic resistance (81, 82).

2.2.1 ICEs and IMEs in streptococci

ICEs and IMEs are important vectors of antibiotic resistance in many species of streptococci. ICEs in streptococci have been classified according their recombinase type and phylogenetic relationship of the T4SS components, the domain of their T4CP, and the relaxase type. Three ICEs super-families have been depicted whose prototype are Tn916, Tn5252, and TnGBS1 (83). Based on the same criteria, two ICEs families can be distinguished in the Tn916 super-family: the Tn916 and the ICES_{t3}. The Tn5252 super-family comprises four families: ICE_{vanG}, Tn1549, TnGBS2, Tn5252. No subfamilies exist for TnGBS1 super-family.

The ICE families are differently distributed among the streptococcal species. For instance, Tn916 is most diffused in *S. suis* and *S. pneumoniae*, whereas ICES_{t3} is most prevalent in *S. dysgalactiae* and *S. gallolyticus*. Elements of the Tn5252 super-family are common in *S. suis*, whereas TnGBS1 super-family members have been found in *S. agalactiae*, *S. anginosus*, *S. intermedius*, and *S. lutetiensis* genomes (83).

Coluzzi *et al.* provided a classification for IMEs circulating among streptococci using similar criteria than those used for the categorization of ICEs. In average, IMEs are smaller than ICEs (10kb versus 41kb, respectively), but ICEs and IMEs have similar abundance in the streptococci genomes. IMEs are particularly abundant in *S. anginosus* genomes. IMEs detected in streptococci carried serine and tyrosine recombinases, with tyrosine recombinases largely more represented. Although IMEs do not possess T4SS, half of them were found to harbor a coupling protein. IMEs carry also relaxases frequently hosted by Firmicutes. Several IMEs were found integrated in conserved structures of ICEs, suggesting that ICEs could carry IMEs during

their transfer, although integration of IMEs into ICEs could compromise their conjugative capabilities (81).

2.2.2 ICEs and IMEs carrying macrolide resistance

ICEs and IMEs in streptococci carry and disseminate genes mediating virulence (84, 85) or other physiological properties (86), and genes conferring resistance to antibiotics in particular to macrolides and tetracyclines. **We reviewed²** the prevalence of resistance and occurrence of ICEs in animal streptococci in 2018. Macrolides and tetracycline resistance are mostly reported in *S. suis* isolates from diseased pigs in France (<https://shiny-public.anses.fr/ENresapath2/>).

My first steps in bacteriology research, during the laboratory practice for the master degree at the University of Catania, Faculty of Life Sciences (Italy), were made for the characterization of the Tn2009 transposon in a clinical strain of *S. pneumoniae*. This element consisted in a Tn916 harboring the *tet(M)* gene, conferring tetracycline resistance, and the MEGA element (macrolide efflux genomic assembly). MEGA contains an efflux operon constituted by *mef(E)* and *mel* genes that confers resistance to macrolides, resulting in the so-called M phenotype. Further than the dual efflux operon, MEGA contains other three uncharacterized *orfs*, where *orf5* contains a LexA domain, suggesting its implication in the stress SOS response (87). Originally, Gay and Stephens characterized the MEGA element inserted in several *S. pneumoniae* chromosomal sites. The MEGA element is not surrounded by direct repeats and its propagation from a donor cell to a recipient one was demonstrated to occur by transformation (87). In Tn2009, the MEGA element was inserted into *orf6* of Tn916. Differently from Tn916, the Tn2009 did not show transferability by conjugation to a recipient cell. Instead, Tn2009

² Haenni M., Lupo A., Madec J.Y. Antimicrobial Resistance in *Streptococcus* spp. Microbiol Spectr. 2018, 6(2)

could be transferred by transformation to a Tn2009-free *S. pneumoniae* recipient. Del Grosso *et al.* (88) published similar findings contemporaneously.

I continued to study genetic elements in streptococci during the **PhD thesis**.³ In this frame, I characterized the elements carrying macrolide resistance genes in a *S. salivarius* strain, 3C30, isolated from tonsillitis. The strain was resistant to tetracycline and presented an MLSb phenotype. The strain harbored *mef(E)* and *erm(B)* genes, this latter mediating the cMLSb phenotype, and *tet(M)* gene responsible for tetracycline resistance. The *mef(E)* gene was located on the MEGA element, whereas *erm(B)* and *tet(M)* co-localized on Tn3872, a composite transposon derivative of Tn916 where Tn917 transposon, carrying *erm(B)*, is inserted into *orf9* of Tn916 (89). The MEGA element was inserted in a site displaying homology with the locus *str0653* of *Streptococcus thermophilus* (CNRZ1066). Gene *str0653* was predicted to encode a tRNA methyltransferase. To analyze the transferability of the MEGA element and Tn3872, we implemented a filter mating method in presence of DNase, to exclude transformation, with the *S. salivarius* strain as donor and a novobiocin resistant *S. pneumoniae* strain as recipient. The obtained *S. pneumoniae* trans-conjugants displayed resistance to novobiocin and an M phenotype and carried the gene *mef(E)* on the MEGA element inserted into a gene encoding a tRNA pseudo-uridine synthase. This finding was surprising, considering that the MEGA element lacks recombinases or conjugative machinery and it was known to transfer only by transformation (87). We then realized PCR with divergent primers on the extremity of the MEGA element in the donor *S. salivarius* to detect its excision and circularization. This assay suggested the formation of a MEGA circular intermediate typical of conjugal transfer. When we attempted to transfer the MEGA element from *S. pneumoniae* trans-conjugants to other recipient cells, no circular structures were observed and no transfer of MEGA could be detected.

³ Santagati M., Lupo A., et al. Conjugal mobilization of the mega element carrying *mef(E)* from *Streptococcus salivarius* to *Streptococcus pneumoniae*. FEMS Microbiology Letters, 2009, 290:79-84.

Likely, the MEGA element mobilization and transfer were mediated by the recombinases and conjugative machinery of the Tn3872 transposon. Besides, contribution of recombinases proper to the *S. salivarius* donor into this process cannot be excluded. Overall, such transfer mechanism of MEGA resembles that of IMEs. In this way, the MEGA element could disseminate also in those organisms or species incapable to transform.

In parallel, conjugative transfer on Tn3872 from *S. salivarius* 3C30 was not observed. Previous investigations reported the Tn3872 mobility, although at low frequency from a clinical *Streptococcus defectivus* to a recipient *Enterococcus faecalis* strain (90), but no Tn3872 transfer was observed from a *S. pneumoniae* donor to a recipient *E. faecalis* strain (89), suggesting that the mobility of Tn3872 depends also on the host genomic background. Later, Chaffanel *et al.* (91) analyzing 120 commensal (60 from feces and 60 from saliva of healthy donors) and 92 clinical *S. salivarius* strains from various infections highlighted a high proportion of erythromycin resistant isolates in commensal and clinical strains, 76% and 56% respectively. The predominant element carrying erythromycin resistance genes was Tn3872, which did not produce circular intermediate. Besides, several other genetic elements were present harboring macrolides resistance determinants, including the MEGA element. This study highlighted the importance of *S. salivarius* as reservoir of antibiotic resistance determinants in body sites with high-dense microbial population, the intestine and the oral cavity.

The MEGA element is also present in genomes of Gram-negative species that are component of the flora of the upper respiratory tract or the urogenital mucosa, like *Neisseria subflava* (92), *Haemophilus parainfluenzae* (93), and *Haemophilus influenzae* (94).

During the Postdoc at the University of Bern, Department of Infectious Diseases, Switzerland, **I contributed**⁴ to characterize the *H. parainfluenzae* AE-2096513 strain, resistant to macrolides (95). The strain harbored *tet(M)* gene adjacent to the MEGA element carrying the *mef(E)* gene (KJ545575). This genetic structure, inserted in a phosphodiester glycosidase encoding gene, was not part of a typical Tn2009. Most likely, the acquisition of this genetic fragment has occurred by transformation and donor could potentially be streptococci resident in the same habitat.

The presence of MEGA in Gram-positive and Gram-negative species phylogenetically distant, corroborate the observation that besides phylogenetic relatedness between donor and recipient that favor recombination events, major driver of horizontal gene transfer is the ecological niche where bacteria cohabit (96).

This characterization was conducted in parallel to the main subject of the postdoc developed in the frame of the RADAR-Go project, funded by SwissTransMed. The aim of RADAR-Go (Rapid Diagnosis of Antibiotic Resistance in Gonorrhoea) was the development of a multiplex qPCR based on generic dye (SYBRGreen) and high-resolution melting analysis (HRM) to detect the most **common mechanisms of antibiotic resistance**⁵ in the sexually transmitted pathogen *Neisseria gonorrhoeae*. **I initiated**⁶ the design of the qPCR strategy and analysis of opportune and discriminative molecular targets predicting resistance. The method could be an effective and rapid screening tool.

⁴ Endimiani A., Allemann A., Wüthrich D., **Lupo A.**, Hilty M. First report of the macrolide efflux genetic assembly (MEGA) element in *Haemophilus parainfluenzae*. International Journal of Antimicrobial Agents. 2017, 49:265-266

⁵ Endimiani A., Guilarte Y.N., Tinguely R., Hirzberger L., Selvini S., **Lupo A.**, et al. Characterization of *Neisseria gonorrhoeae* isolates detected in Switzerland (1998-2012): emergence of multidrug-resistant clones less susceptible to cephalosporins. BMC Infectious Diseases, 2014, 14:106-116

⁶ Donà V., Kasraian S., **Lupo A.**, et al. Multiplex Real-Time PCR assay with High-Resolution Melting analysis for characterization of antimicrobial resistance in *Neisseria gonorrhoeae*. Journal of Clinical Microbiology, 2016, 54:2074-2081

2.2.3 ICEs and IMEs and aminoglycoside resistance in streptococci

In my current position at ANSES-Lyon, I contribute to the activities of surveillance of antibiotic resistance in bacteria isolated from diseased animals. Such surveillance is realized through the Resapath consisting in a coordinated network of veterinary diagnostic laboratories (n= 101 in 2021) which realize diagnostic and antibiograms on veterinary clinical bacterial isolates. The results of antibiograms (n= 62,070 in 2021) are transmitted to ANSES-Lyon. These data, collected at the national scale from different animal hosts and diseases, are analyzed and a report is issued yearly (97).

Through Resapath, during 2017-2022, we observed the emergence of *Streptococcus dysagalactiae* sbsp. *equisimilis* (*SDSE*) presenting high-level gentamicin resistance (HLGR) (n= 27) in the genital tract of mares screened before *in vitro* fecundation. HLGR is an uncommon phenotype among streptococci and has clinical importance because it impedes synergy between aminoglycosides and beta-lactams, a combination used to treat invasive infections caused by *Streptococcus* spp. The HLGR *SDSE* isolates were collected at ANSES-Lyon. We analyzed, by next generation sequencing, the genetic relationship of the isolates and the genetic basis underlying HLGR.

The 27 *SDSE* isolates shared large genomic similarity and a probable inter-stud transmission occurred. In one *SDSE* strain for which full-assembled genome was obtained, the investigation lead to the characterization of a novel ICE derived from a Tn1549-type and containing an IME carrying *aph(2'')*-*Ib* and *aac(6')*-*Iam* genes, conferring HLGR. These genes carried on the IME were present in all HLGR isolates of our study. The Tn1549-type ICE was able to transfer to an erythromycin resistant *S. agalactiae* isolate. Similarity searches in sequences databases revealed that the IME characterized in these *SDSE* and harboring HLGR genes, was present in a *Haemophilus influenzae* strain, inserted in a Tn6686-type transposon. Furthermore, this IME with HLGR genes was also located on plasmids of Enterobacterales

strains. In all the hosting elements, the IMEs were inserted in a *topB* gene, encoding a DNA topoisomerase III.

In another *SDSE* strain, the IME carrying HLGR genes was integrated in a *topB* gene in turn inserted in a complex mosaic region including a copy of Tn*GBS*-type transposon. We did not detect *in vitro* transfer to a recipient cell of Tn*GBS*-type element.

IME containing HLGR genes, by targeting the *topB* gene of pre-existing genetic elements, could favor its dissemination by the hosting ICE or plasmid. Furthermore, integration in pre-existing elements could overcome the limitation of horizontal gene transfer in multiple phylogenetically distant species, thus granting the necessary homology for the integration. The manuscript on these results is in preparation (**Annex 1**).

Rochegüe Tony, PhD student, whose thesis was co-directed by Prof. Tristan Ferry and myself, initiated the HLGR *SDSE* characterization as a side project of his thesis.

The work on the analysis of the ICE and IME carrying HLGR was particularly important for developing my professional competences. In this frame, I acquired knowledge on novel technologies such as genome sequencing by short- and long-reads. It was also a good opportunity for beginning professional collaboration in the French scientific tissue. In fact, Sandrine Hughes and Benjamin Gillet from the Institute of Genomic Functionality of Lyon contributed with the long-reads sequencing and Philippe Glaser from the Pasteur Institute in Paris was a precious collaborator for these genome analysis.

HLGR streptococci will continue to be monitored through Resapath, because it is a phenotype of **clinical concern**⁷ and has demonstrated to transfer *in vitro* to the **human pathogen**⁸ *S. agalactiae*.

⁷ Ruppen C., **Lupo A.**, Decosterd L., Sendi P. Is penicillin plus gentamicin synergistic against clinical Group B *Streptococcus* isolates?: An in vitro study. *Frontiers in Microbiology*, 2016, 7:1680. eCollection.

⁸ **Lupo A.**, Ruppen C., Hemphill A., Spellerberg B., Sendi P. Phenotypic and molecular characterization of hyper-pigmented Group B Streptococci. *International Journal of Medical Microbiology*, 2014, 304:717-724

2.3 Resistance islands in *Acinetobacter baumannii*.

Resistance islands in *A. baumannii* are derived from transposons backbones carrying at least five genes encoding structures involved in transposition and modules carrying antibiotic resistance genes (98).

The first characterized resistance island was AbaR1, in the strain AYE belonging to a global disseminated clonal complex (CC1) (99). AbaR1 is 86 kbp large and contains 25 genes conferring resistance to multiple antibiotics. Furthermore, AbaR1 carries operons conferring resistance to heavy metals (mercuric, arsenic and cadmium), and four complex class 1 integrons. AbaR1 has terminal 26 bp inverted repeats and was found inserted into the *comM* gene surrounded by 5bp direct repeats generated by the duplication of the insertion site, overall suggesting that acquisition took place by transposition. AbaR1 derive from AbaR0, which in turn is derivative of Tn6019 transposon. Numerous variants of AbR0 have been reported, mostly arising by recombination events mediated by IS26. These variants are integrated into the *comM* gene, like AbaR1, and are widespread among isolates belonging to CC1 (100, 101).

In *A. baumannii* isolates belonging to CC2, two other genomic islands are disseminated consisting in AbGRI-1 (102) and AbGRI-2 (103). In CC1 and CC2 isolates, AbaR4 island, derived from Tn6022 carrying a copy of Tn2006 and inserted in *comM* locus, is increasingly reported (98).

Genes encoding structures dedicated to transposition in AbaR4 presented low similarity with genes reported from other previously characterized transposons (98), suggesting that transposition could not have been the mechanism of acquisition. Recently, Godeaux *et al.* (104) has provided experimental evidences that AbaR4 is able to disseminate from a donor cell to a recipient through transformation.

In order that transformation could take place, exogenous DNA needs to be up taken by the recipient cell. The physiological status during which bacteria can uptake DNA is named competence. Several bacterial families and genera are naturally competent, including *S. pneumoniae* (105), *H. influenzae* (106) and *Acinetobacter* spp. (107).

Double-strand DNA (dsDNA) uptake in *A. baumannii* is tightly linked to the production of a type four pilus (T4P), which also confers twitching motility on wet surfaces (108). When arriving into the periplasmic space, a strand of the DNA is digested and the obtained single-strand DNA (ssDNA) crosses the inner cellular membrane through a channel (109). In the bacterial cell, the ssDNA is complexed by single-strand-binding proteins and RecA that mediate the recombination process, assisted by ComM (110). The RecA-ssDNA complex searches for homologous regions on the dsDNA of the recipient cell. If a homology extending for 8-20 nucleotides is recognized, exchange of the newly acquired DNA strand replacing the existing in the recipient cell, takes place (111).

The ability of *A. baumannii* to uptake exogenous DNA was demonstrated several years ago (112). Following reports highlighted the importance of this mechanism in the acquisition of antibiotic resistance genes. ST25 *A. baumannii* strains collected in our laboratory through Resapath and isolated from diseased animals were able to transform, acquiring an engineered DNA fragment encoding a superfolder green fluorescent protein, which allows visualization of transformants by **flow-cytometry**⁹. Isolates belonging to other sequence types did not produce transformants at the detectable threshold. Furthermore, the assay highlighted that presence of Ca⁺ increased the frequency of transformation in the model strain *A. baumannii* AB5075, while presence of bovine serum albumin decreased such frequency. Results obtained with albumin were in opposite direction from what has been observed in strain *A. baumannii* A118 that was

⁹ Godeux A.S., **Lupo A.**, et. al. Fluorescence-based detection of natural transformation in drug-resistant *Acinetobacter baumannii*. J Bacteriol. 2018, 200:e00181-18.

however not evaluated with the same methodology (113). Nevertheless, a strain dependent behavior cannot be excluded. Several factors impact the frequency of transformation, including the **integrity of the *comM* locus**,¹⁰ the extend of homology between transforming DNA and the chromosome of the recipient cell, but also the presence of motifs for epigenetic DNA modification which favor the acquisition of DNA fragments from kin isolates (109).

Transformation is one horizontal mechanism at the base of AbaR acquisition in the *A. baumannii* chromosome that afterwards could disseminate vertically (104, 114). Such acquisition could be responsible for the expansion of multidrug resistant clones that constitute a global menace for human and animal health (115).

2.4 Plasmids

Plasmids are extra-chromosomal genetic elements able to self-replicate and in most cases to self-transfer to a recipient host. Their role in the dissemination of antibiotic resistance genes is well known and especially in members of the Enterobacterales family (116, 117).

2.4.1 A snapshot on plasmid repertoire in *Acinetobacter baumannii*

If the characterization of plasmids, their associated resistance genes, and their global epidemiology is extensively investigated in Enterobacterales and in transversal sectors, including humans, animals and environmental sources, the characterization of plasmids in *A. baumannii* and their importance for resistance genes dissemination is less understood. Probably, this discrepancy is linked to the lack of a commercialized method for plasmids classification in *Acinetobacter* genus compared to Enterobacterales. Furthermore, antibiotic resistance genes important for therapy in humans have been characterized mostly borne in the chromosomes of

¹⁰ Godeux A.S., Svedholm E., **Lupo A.**, *et al.* Scarless removal of large resistance island AbaR results in antibiotic susceptibility and increased natural transformability in *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2020, 64:e00951-20.

clinical isolates. Nevertheless, Bertini *et al.* (118) was the first to propose a classification system based on the amplification and sequencing of *rep* and *oriT* sequences. The system was based on six multiplex PCR reactions on 27 genes. A group was then determined according to a threshold of nucleotide identity, that Bertini proposed at 75%. With these criteria, 32 homology groups (GR) could be defined. In the continuity with the methodology proposed by Bertini, Castro-Jaimes *et al.* (119) proposed an update of the typing system using full sequenced plasmids (n= 494) available in the NCBI database in 2020. The three most prevalent replicases belonged to the Rep_3 family, followed by PriCT_1 type, and MeR_SF type. Among plasmids carrying these replicases, authors could characterize 24 additional homology groups compared to the Bertini's scheme. Plasmids belonging to GR50 were present in several *Acinetobacter* spp. and plasmids belonging to GR38, GR42, and GR43 had a broad host-range, being present in several bacterial genera such as *Vibrio*, *Klebsiella* and *Pseudomonas* (119). More recently, Lam *et al.* (120) has reviewed the *A. baumannii* plasmid classification, using available sequences in NCBI at the beginning of 2021 (n= 621). Their analysis was convergent with that from Castro-Jaimes finding replicase Rep_3 as the most prevalent, followed by PriCT_1 type. Lam *et al.* found Rep_1 replicase as the third most common. Using a 95% cut off for nucleotide similarity, Lam *et al.* identified several sub-groups belonging to these three major families. In both the Rep_1 and PriCT_1 families, six sub-types were identified. Plasmids carrying Rep_3 replicase, besides being the most abundant, presented also the highest diversity including 69 subtypes. Plasmids carrying Rep_1 replicases were smaller than 3kb and did not harbor antibiotic resistance genes whereas plasmids carrying PriCT_1 replicase included well known plasmids such as pACICU2, previously classified as GR6 and carrier of *bla*_{OXA-23} and *aphA6* genes. The nucleotide sequences used for the classification were made publicly available in GitHub, facilitating the implementation of plasmids typing in *Acinetobacter* spp. Although an equivalence with previously recognized homology groups was provided, the parallelism

between the new nomenclature proposed by Lam and those proposed in previous schemes might not be trivial for users.

Recently, we have characterized ST25 *A. baumannii* strains isolated from diseased animals. Strains isolated from dogs and cats presented carbapenem-resistance and carried *bla*_{OXA-23}. Due to the importance of this clone among diseased animals in France, we undertook an analysis based on genome sequencing of these isolates. For some of them, we obtained full-assembled genomes exploiting short- and long-reads sequencing technology. We analyzed ST25 *A. baumannii* strains from French animals in regards to human strains and to genomes publicly available from the National Centre Biological Institute database. The manuscript presenting these results has been submitted (**Annex 2**).

Overall, 20 full-assembled ST25 *A. baumannii* were available and belonged to four phylogenetic sub-clades (TableS2 of Annex 2). These 20 full-assembled genomes were analyzed for their plasmid content. All of them contained a large plasmid (100163-232318 nucleotides) and several strains contained multiple plasmids (Table S2 of Annex 2). We used the Castro-Jaimes scheme for classification. Some large plasmids (5/20) belonged to broad host-range GR38 whereas the remaining were non-typable. Among the ST25 strains, plasmids belonging to the same homology group harbored similar genetic elements (Table S2 of Annex 2). Similarity of resistance genes carried by Tn6183 and the multi-resistance island RI-PER7, both located on GR38 plasmids, suggests a possible evolutionary link between the two genetic elements. Non-typable large plasmids carried an AbGRI-1 resistance island similar to that found in globally diffused multi-resistant clones. Furthermore, both GR38 and non-typable large plasmids carried a IS91-composed transposon, harboring *acc(3)-IIa* and *acc(6')-Ian* genes. This composite transposon is also on plasmids of *K. pneumoniae* (pKp 1050-4; CP023420.1) and *Serratia marcescens* (p1163; AP01411.1). Beside antibiotic resistance genes, the largest plasmids present in each isolate carried loci encoding regulators of chromosomal

genes involved in virulence (121, 122) a factor that probably contribute to their maintenance into the cell. When we aligned the sequences of large non-typable plasmids with GR38 ones, the overall similarity mostly reflected clade grouping of the chromosomes then plasmid typing suggesting a co-evolution between genomes and these plasmids (Figure 3B of Annex 2).

ST25 *A. baumannii* strains harbored GR6 plasmids, like p51877-2 that carried a *bla*_{OXA-23} gene located on a Tn2008 and a *aph*(3')-*Via* gene located TnAphA6, similarly to pACICU2 (Figure 3A, Annex 2). We detected the *in vitro* transfer of the GR6-p51877-2 plasmid to a recipient rifampicin resistant *A. baumannii* (BM4547).

The role of *A. baumannii* plasmids in the dissemination and evolution of antibiotic resistance has been probably underestimated. In the ST25 isolates, plasmids have proved to disseminate horizontally *bla*_{OXA-23}, one of the most threatening resistance mechanism that currently circulates among clinical pathogens. Furthermore, they serve as platform for genetic elements that can be exchanged with distantly related species, like the *IS91*-mediated transposon carrying *acc*(3)-*Ila* and *acc*(6')-*Ian* genes that is found also in Enterobacterales. Recently, Rafei *et al.* (123) has reported the characterization of a clinical human ST25 *A. baumannii* strain, unveiling the role of its plasmid as platform for the evolution of AbGRI-1 resistance island, crucial element for resistance dissemination in major *A. baumannii* clones.

The characterization of the plasmid content in *Acinetobacter* spp. deserves further investigation to understand their role in antibiotic resistance dissemination but also in the pathogen physiology. Other *Acinetobacter* spp. than *A. baumannii* carry emergent plasmids with relevant antibiotic resistance determinants. For instance, we recently detected the emergence among diseased animals of *Acinetobacter lwoffii* with determinants conferring resistance to tigecycline, a phenotype common in Asia but unreported in France so far. Tigecycline resistance genes were plasmid located and their preliminary analysis will be presented (poster) in June at the 13th International Symposium on the Biology of *Acinetobacter*.

2.4.2 A glance on plasmids harboring multiple resistance genes in Enterobacterales

In Enterobacterales, conjugative plasmids are important vehicles for dissemination of genes conferring resistance to several antibiotic classes. They frequently carry genes conferring ESBL phenotype (e.g. *bla*_{CTX-M₅ and *bla*_{SHV12}), or encoding carbapenamases (e.g. *bla*_{OXA-48} and *bla*_{NDM-1}), resistance to colistin (*mcr* genes) and pan-aminoglycosides resistance (e.g. *rmtB* and *armA*) (43). Like ICEs, plasmids can be classified. Categorization of plasmids is epidemiologically useful to trace antimicrobial resistance dissemination geographically and through sectors. So far, the most common used method consists in the categorization of sequences of the genes constituting their replicons (*inc/rep*) (116, 124). Nowadays, 28 Inc type plasmids circulate among Enterobacterales. Distribution of plasmids type differs according to host and geographical origin of the isolates (44).}

An example is provided by a study **we conducted**¹¹ on *E. coli* isolates, presenting ESBL phenotype, collected from horses in France (n= 41) and Sweden (n= 33). Most of isolates (54/74, 73%) carried a *bla*_{CTX-M-1} gene. Other ESBL encoding genes were less common (*bla*_{CTX-M-9}, n= 1; *bla*_{CTX-M-14}, n= 1; *bla*_{CTX-M-2}, n= 7; *bla*_{CTX-M-15}, n= 7; *bla*_{SHV-12}, n= 4). Gene *bla*_{CTX-M-1} was mostly located on IncHI plasmids (42/54). Proportions of *E. coli* carrying IncHI plasmids were similar between France and Sweden. However, a deeper analysis demonstrated that, in the two countries, two IncHI variants were circulating: pST9 in France, and pST2 in Sweden, suggesting a specific geographical distribution. IncHI plasmids have wide host-range. So far, they have principally diffused in Europe, in isolates of environmental origin (44, 125) and in *E. coli* from horses (126-128). Probably, the presence of a *fos* operon on IncHI plasmids, involved

¹¹ **Lupo A.**, Haenni M., Saras E., Gradin J., Madec J.Y., Börjesson S. Is *bla*_{CTX-M-1} riding the same plasmid among horses in Sweden and France? Microb Drug Resist. 2018 May 24.

in fructose metabolism, justifies the successful dissemination of this plasmid type among this animal host.

IncI1-type plasmid was the second most common carrying *bla*_{CTX-M-1} in *E. coli* from horses, but especially present in France (nine isolates) and sporadically in Sweden (one isolate). At a deeper characterization, these plasmids were heterogeneous. IncI1 plasmids have narrow host-range, often harbor *bla*_{CTX-M-1} and are mostly spread in Europe, where they have successfully disseminated among ESBL-*E. coli* from poultry and bovines (44).

In another study, **we found**¹² IncI1 plasmids carrying *bla*_{CTX-M-55}, a variant of *bla*_{CTX-M-1} that in the early 2000 was exclusively spread in Asia, in *E. coli* isolates from French diseased animals. However, IncI1 plasmids were less common among *E. coli*-*bla*_{CTX-M-55} carrier (2/21). Gene *bla*_{CTX-M-55} was mostly located on IncF plasmids (in 19/21 isolates) of different subtypes. The IncFII plasmids were found in non-related animals and in multiple *E. coli* clones, suggesting their ability to transfer horizontally. Often, IncFII-*bla*_{CTX-M-55} plasmids carried other resistance genes, such as *rmtB*, *fosA* and *mcr-3* genes conferring pan-aminoglycoside, fosfomycin and colistin resistance, respectively. In parallel to this study, **we demonstrated**¹³ that *bla*_{CTX-M-55} associated to *rmtB* and *mcr-3* genes on IncFII plasmids propagated through an epidemic *E. coli* clone (ST744) among French cattle. IncFII plasmids are confined to Enterobacterales, in particular to *E. coli* where, nevertheless, they are the most disseminated plasmids carrying *bla*_{CTX-M} genes (44).

A broad-spectrum plasmid type is the IncL/M, which is the main vector of the *bla*_{OXA-48} gene encoding the third most diffused carbapenemase among Enterobacterales (129, 130). **We**

¹² **Lupo A., et al.** Emergence of *bla*_{CTX-M-55} associated with *fosA*, *rmtB* and *mcr* gene variants in *Escherichia coli* from various animal species in France. J Antimicrob Chemother. 2018, 73:867-872

¹³ Haenni M., Beyrouthy R., **Lupo A., et al.** Epidemic spread of *Escherichia coli* ST744 isolates carrying *mcr-3* and *bla*_{CTX-M-55} in cattle in France. J Antimicrob Chemother. 2018, 73:533-536.

found¹⁴ that this gene, located on IncL/M plasmids, was present in *K. pneumoniae* and *E. coli* strains isolated from feces laying in urban resting areas for dogs in Lyon and surrounding villages. Interestingly, IncL/M-*bla*_{OXA-48} was found in one isolate of *K. pneumoniae* and one of *E. coli* in the same feces sample, suggesting the dissemination of the plasmid in the gut of the dog. Laying, not picked up feces might be source of contamination with worrisome resistance mechanisms, especially for dogs frequently doing coprophagia. Dogs could afterwards transmit the acquired bacterium to cohabiting humans.

Several other incompatibility groups exist among Enterobacterales plasmids, such as the IncK that, similarly to IncL/M-*bla*_{OXA-48}, is strongly associated to *bla*_{CMY-2} or *bla*_{CTX-M-14} beta-lactamases encoding genes. Another example of antibiotic resistance gene association and plasmid type is provided by the IncX4 and genes *mcr-1* and *mcr-2* conferring non-susceptibility to colistin (44).

Overall, all these reports corroborate the importance of plasmids as vectors of genes conferring major phenotypes in isolates of animal origins. Although certain plasmid types occur with geographical tropism and principally in animal and environmental sectors, others with broad-range host are threatening antibiotic resistance spreader for animals and humans. Surveillance of these phenotypes is crucial to build measures aiming to limiting further spread.

3. Reservoirs of antibiotic resistance

3.1 Freshwater and hotspots of antibiotic resistance spread

Environmental compartments are intrinsically connected to the antibiotic resistance issue (131). Already in 2008, Baquero *et al.* (20) pointed to the four reactors of antibiotic resistance

¹⁴ Haenni M., Métayer V., Lupo A., *et al.* Spread of the *bla*_{OXA-48}/IncL plasmid within and between dogs in city parks, France. *Microbiol Spectr.* 2022, 10(3):e0040322.

evolution: i) the human and animal intestinal microbiota hosting high density bacterial population often submitted to antibiotic selective pressure; ii) crowded habitats where humans or animals co-habit for long time (e.g. hospitals, caring facilities, farms); iii) the wastewater treatment plant where mixing of bacteria from different individuals and sectors mix and undergo to selective pressure by antibiotics, biocides, and heavy metals; iv) surface water that serves as collector of all the other reactors and industrial pollution. Furthermore, water habitat hosts indigenous bacterial species that are reservoirs of antibiotic resistance mechanisms for pathogens. The dissemination of progenitors of *bla*_{CTX-M} genes from *Kluyvera* spp., usually inhabiting water (132, 133) to human Enterobacterales pathogens has been demonstrated. Other resistance mechanisms have their progenitors in water or environment inhabiting bacteria, like for instance *qnr* genes (134) and *bla*_{OXA-48} (135). **I reviewed**¹⁵ this aspect and occurrence of antibiotic resistant bacteria in water bodies in 2012.

Water autochthonous species can also be recipients of antibiotic resistance genes from pathogens. Recently, **we have described**¹⁶ the circulation of the *bla*_{OXA-48-like} and *bla*_{NDM-1-like} genes among pathogen and freshwater isolates, collected in a river of the Mediterranean basin. *Shewanella* spp. are progenitors of *bla*_{OXA-48} gene, encoding carbapenem-hydrolyzing oxacillinase. A freshwater *Shewanella xiamenensis* isolate harbored a *bla*_{OXA-204} gene surrounded by a C15 peptidase-encoding gene and a *lysR* in its chromosome (136). *ISEcpI-bla*_{OXA-204-lysR} genetic segment has been found in a clinical *K. pneumoniae* isolate. We reported *ISEcpI-bla*_{OXA204-lysR} on a plasmid of a freshwater *S. xiamenensis* isolate, suggesting the shuffling of *bla*_{OXA-204} between environmental and pathogenic species. In a freshwater *Shewanella bicestris* isolate, the carbapenemase-encoding gene *bla*_{NDM-1}, together with *bla*_{OXA-}

¹⁵ **Lupo A., et al.** Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. *Frontiers in Microbiology*, 2012, 116-134

¹⁶ Ncir S., **Lupo A., et al.** Back to the origin: *bla*_{OXA-204} and *bla*_{NDM-1} genes in *Shewanella* spp. from a Tunisian River. *Microbiol Spectr.* 2022, 10(3):e0160522

¹⁰, *sull*, and *aadA1* genes, was surrounded by two copies of IS91. This composite transposon was plasmid located and homologies searches in public databases revealed that part of it was present on plasmids of other *Shewanella* spp., suggesting that this genus contributes to nourish *bla*_{NDM-1} dissemination.

Through surface water, antibiotic resistant bacteria can enter drinking water sources, thus surface water quality is important to prevent this propagation chain. Furthermore, rivers can transport antibiotic resistant bacteria amplifying the problem of point source pollution. For instance, we **found**¹⁷ that seafood farmed in sites influenced by wastewater was colonized with multiple bacteria harboring resistance mechanisms of high clinical concern. Seafood is often manipulated and consumed uncooked, thus possibility that these resistance mechanisms integrates into microbiotas of consumers is elevated.

My first PostDoc was at the Technical University of Dresden, in Germany. Here, I built up the facilities needed for the bacteriology analysis in the department of hydrobiology, and I worked for the project IWAS (International water alliance Saxony). This project aimed to improve access of the population to drinking water, as well improve management of wastewater in Ukraine to meet European Union directive quality standards. The first phase of the project was to evaluate the quality of the surface water in order to understand the impact of point and diffused pollution sources. The analysis integrated several parameters: water quality criteria (chemical and physical), heavy metal contamination (running water and sediments), bioindication (using macroinvertebrates), hydromorphology field surveys, and microbiology (***E. coli* enumeration and antibiotic resistance analysis**)¹⁸.

¹⁷ Mani Y., Mansour W., **Lupo A.**, *et al.* Spread of *bla*_{CTX-M-15}-producing Enterobacteriaceae and OXA-23-producing *Acinetobacter baumannii* sequence type 2 in Tunisian seafood. Antimicrob Agents Chemother. 2018, 62:e00727-18.

¹⁸ Ertel A. M., **Lupo A.**, *et al.* Heavy load and high potential: anthropogenic pressures and their impacts on the water quality along a lowland river (Western Bug, Ukraine). Environmental Earth Sciences, 2012, 65:1459-1473.

The region under investigation was the Western Bug River catchment that constitutes the border between Ukraine and Poland. The first segment of the Bug River is impacted by rural activities. Downstream this area, there is the Poltva confluence. The Poltva tributary receives the wastewater of L'viv city. Downstream the Poltva confluence, the water reservoir Dobrotivir was built, and further downstream a large, open air, disused mining area was present.

Upstream Poltva confluence, *E. coli* isolates were heterogeneous and carried single resistance determinants (e.g. *bla*_{TEM-1} penicillinase) and other water quality parameters were acceptable. The confluence of Poltva dramatically decreased all the water quality parameters. In the Poltva and downstream its confluence in the Bug river, multidrug resistant *E. coli* were found, all of them belonging to the globally distributed ST131 and with an indistinguishable profile according to genome macro restriction typing. Isolates carried *bla*_{CTX-M-15} and were resistant to fluoroquinolones. The water basin, Dobrotivir reservoir, improved the microbiological parameters and multidrug resistant strains were not detected. Pollution by mining activity did not correlate with antibiotic resistance occurrence, at least in *E. coli* isolates. In the Bug, even in proximity of the Poltva confluence, recreational activities (fishing, bathing, laundry) were frequent.

These results highlight the necessity of risk assessment of antibiotic resistance occurrence in the environment for short term and long-term consequences for human health and emphasize the need of water sanitization for mitigating antibiotic resistance dissemination.

3.2 Animals and food reservoirs of *Acinetobacter baumannii*

While many reports and genome sequencing are helping to draw the picture of carbapenem-resistant *A. baumannii* molecular epidemiology, the knowledge on the carbapenem-susceptible isolates is scarce. Analyzing 3,575 genomes available in the NCBI repertoire in 2019, Hamidian

and Nigro (24) showed that ST2 isolates were by far the most represented (2,191/3,575) and that the majority carried an acquired carbapenem-resistance gene (1,678). The second most diffused sequence type was ST1 (173 genomes) with 109 carrying an acquired carbapenem-resistance gene. In addition, this analysis highlighted that genomes available in the repository are geographically biased, coming mostly from US, and that genomes from Europe were relatively few. Although genomes devoid of acquired carbapenem-resistance genes were found (24), metadata on these latter were not discussed. Nevertheless, it appears that available genomes from non-hospital sources are few (137). The interest in the extra-hospital epidemiology of this species is rising, because of the occurrence of rare but severe community acquired infections. These events concern mostly individuals with co-morbidities (diabetes, alcoholism, or smokers) (138-140). Furthermore, strains causing hospital-acquired infections and unrelated to epidemic clones are reported as well (141), suggesting an influx into the hospital from outer sources. Carriage of *A. baumannii* in the human community is variable, being more frequent in wet region of the globe, like South East Asia (142), than Central Europe (143). Strains circulating in the community are genetically more heterogeneous than hospital strains and often susceptible to antibiotics (143).

Although the proper reservoir of *A. baumannii* has not yet been unveiled (144), strains have been reported from several extra-human sources, including animals and food products, and their analysis is crucial to understand the global epidemiology of this opportunistic pathogen.

3.2.1 *Acinetobacter baumannii* in animals

Reports of *A. baumannii* strains from animals are continuously increasing. **We reviewed**¹⁹ the literature on the occurrence in animals of *A. baumannii* and its resistances in 2018. Molecular typing of *A. baumannii* isolates from animals has highlighted, in most cases, the

¹⁹ **Lupo A.**, Haenni M., Madec J.Y. Antimicrobial resistance in *Acinetobacter* spp. and *Pseudomonas* spp. Microbiol Spectr. 2018, 6(3).

presence of globally expanded clones, especially those belonging to CC2, suggesting a transmission from humans to animals. Dissemination of *A. baumannii* strains among hospitalized animals has been reported (145) and presence of indwelling devices is a risk factor for infection with this opportunistic pathogen (146), similarly to what is described in humans.

In the frame of Resapath surveillance activity, we have noted the importance of *A. baumannii* ST25, a clone emerging globally in human clinic (147). ST25 *A. baumannii* is often responsible for urinary tract infections in pets (cats and dogs) and includes carbapenem resistant isolates, producing OXA-23. **We reported**²⁰ the clonal diffusion of these isolates in 2016 and we have continued the surveillance of this clone.

We undertook a deeper genomic analysis of ST25 *A. baumannii* (n= 33). To appreciate a possible epidemiological link with isolates collected from humans, 11 isolates provided by colleagues of the CNR Antibiotic resistance in Besançon were added, and we collaborated for the analysis. The paper describing this research has been submitted (**Annex 2**). The genomes of the isolates of this study were analyzed together with genomes belonging to the ST25 available in the NCBI database. Overall, 141 ST25 *A. baumannii* genomes were included, highlighting their clustering in four phylogenetic clades (CI-CIV). Clades CI and CIII have been diffusing exclusively in South America. The other two clades, CII and CIV, contained genomes from several continents. Genomes included in clade CIV had an amount of resistance genes significantly higher than that observed in genomes of other clades. Furthermore, clade CIV contained the most of isolates from animals. Intriguingly, carbapenem-resistant ST25 *A. baumannii* shared large genomic similarity with isolates from humans. Recently, Jacobmeyer *et al.* (148) analyzed 29 carbapenemase-producing *A. baumannii* isolates collected from animals attending a veterinary clinic. Nine of these isolates were from seven veterinary clinics

²⁰ **Lupo A., et al.** Clonal spread of *Acinetobacter baumannii* sequence type 25 carrying *bla*_{OXA-23} in companion animals in France. *Antimicrob Agents Chemother.* 2016, 61:e01881-16.

from France and five of them belonged to ST25. Carbapenemase-producing ST25 isolates were also found among German and Italian pets, but less frequently (3/16 and 1/3, respectively). These results further corroborated the general importance of ST25 circulation among companion animals, particularly in France. This epidemiological situation is surprising, given that most of human carbapenem-resistant isolates belong to ST2 (24). However, epidemiological data in humans derive from the hospital sector and colonization of human population by carbapenem-resistant or susceptible *A. baumannii* outside nosocomial settings remains largely unknown. Hérivaux *et al.* (149) screened dogs and cats (n= 150) attending a preventive veterinary clinic. Four dogs (2.7%) with no history of antibiotic exposure or previous hospitalization were colonized with *A. baumannii* isolates and two were ST25 OXA-23 producers, demonstrating that pets can be a reservoir of carbapenem-resistant *A. baumannii* in the community. The origin of carbapenem-resistant *A. baumannii* in pets is unknown. Carbapenems for treating infections in animals are forbidden in France, thus selective pressure in this sector seems not to be the driver of this emergence. A spill over from humans cannot be excluded.

The surveillance in *Acinetobacter* spp. isolates is continuing through Resapth. The transmission of clones of bacterial species at the interface between humans and animals, especially pets living in close contacts with humans, is a key topic of the research unit to which I belong. This research is important for identifying crucial spots for antibiotic resistance dissemination and, when necessary, put in place control measures. I intend to continue to contribute to this research line focusing on *A. baumannii*. Although the prevalence of carbapenem or multidrug resistant *A. baumannii* is quite low in France compared to other European countries (<5% in 2019), increasing travelling from and to neighboring countries, where carbapenem resistant *A. baumannii* is endemic, might contribute to change the local situation. Thus, surveillance of antibiotic resistance of concern for human and animal therapy

and research on this species is necessary. In the near future, the analysis of *A. baumannii* isolates belonging to CC2 will be undertaken similarly to that conducted on ST25 isolates.

Furthermore, I believe that for a better understanding of the epidemiology of *A. baumannii* in diseased animals, the gap of knowledge in the extra-hospital human community should be filled. The synergy between researchers in the human and animal sectors is necessary, even at international scale.

3.2.2 *Acinetobacter baumannii* in food products

Food products can host resistant bacteria. The risk that these bacteria represent in the frame of antibiotic resistance dissemination is debated. For food products that are consumed cooked, the risk of colonization of manipulators with food-borne bacteria is linked to manipulation and poor hygiene. However, **resistant bacteria**²¹ or **clones**²² common to strains responsible for infections in humans are frequently observed in food products. Beside animals, *A. baumannii* isolates are increasingly being reported from food products, including fresh retailed vegetables (150, 151) and raw meat (152). These isolates could disseminate in the community through food manipulation, raising the probability to enter hospital settings.

During my Postdoc at the University of Bern, Switzerland, in collaboration with Prof. Perreten (Institute of Veterinary Bacteriology at the University of Bern), **I characterized**²³ a group of *A. baumannii* isolates collected from retailed raw meat in the frame of a screening campaign conducted during November 2012-May 2013. We determined the sequence type and antibiotic resistance profile of these isolates that demonstrated heterogeneity, belonging to

²¹ Seiffert S.N., Tinguely R., **Lupo A.**, *et al.* High prevalence of extended-spectrum-cephalosporin-resistant Enterobacteriaceae in poultry meat in Switzerland: emergence of CMY-2- and VEB-6-possessing *Proteus mirabilis*. Antimicrobial Agents Chemotherapy. 2013, 57:6406-6408

²² **Lupo A.**, *et al.* Raw meat contaminated with epidemic clones of *Burkholderia multivorans* found in cystic fibrosis patients. Journal of Cystic Fibrosis, 2015, 14:150-152

²³ **Lupo A.**, *et al.* Antibiotic resistance and phylogenetic characterization of *Acinetobacter baumannii* isolates from commercial raw meat in Switzerland. Journal of Food Protection, 2014, 77:1976-1981

several sequence types and antibiotic susceptibility. Some of the detected sequence types belonged to CC3.

In the frame of a project investigating the presence of *K. pneumoniae* in raw retailed chicken meat and vegetables conducted in the AVB unit during December 2018-July 2019 (153), we investigated in parallel the presence of *A. baumannii* isolates. While no isolate was detected in vegetables, a high prevalence was detected in raw chicken meat with 22 out of 40 samples positive (55%). We determined the sequence type of these isolates and some of them shared the same sequence type of isolates from raw meat analyzed in Switzerland. This result was surprising, because of the large time lapse that occurred between the sampling campaigns and the different geographical origin of the meat samples. Prof. Perreten made available the isolates analyzed in the first sampling campaign in Switzerland and a deeper analysis of these isolates together with those collected in France was designed. Leila Hamze, PhD student that I am supervising since May 2022 currently develops this project. The genomes of isolates from raw meat (72 in total) were sequenced. Preliminary analysis of these isolates focused on the determination of their sequence types and antibiotic resistance profiling. We found 51 different sequence types among the 72 strains, suggesting genetic heterogeneity in this group of isolates. Two isolates carried genes conferring streptomycin resistance, whereas 13 carried *tet(39)* gene, conferring tetracycline resistance and presence of the genes corresponded to the expected phenotype.

We performed a phylogenetic analysis of the 72 isolates on their core-genomes has been performed including genomes available from the NCBI database with identical sequence type or their single locus variants. Isolates from raw meat share extensive genomic similarity with isolates causing infections in humans and generating outbreaks. Genomes from clinical isolates with identical sequence types to those found in raw meat harbor acquired resistance genes

conferring carbapenem resistance. These results will be presented by Leila in a poster to the 9th ARAE symposium, Tours, France (July).

To explore the potential of *A. baumannii* isolates from raw meat to acquire resistance genes *in vitro*, these isolates will be tested as recipient for transforming DNA. Their ability to acquire plasmids bearing multiple antibiotic resistance genes will be evaluated in conjugation assays using opportune donors. Furthermore, adaptive features usually attributed to persistence in clinical environment such as ability to form biofilm, survival after exposure to biocides used in hospital settings and in food-processing industry will be investigated.

The source of contamination with *A. baumannii* for raw meat is unknown. More recently, Schmitz *et al.* (154) have reported high prevalence of *A. baumannii* in the meconium of one-day-old turkey reared for meat production (80%, n= 118). These data could suggest that poultry could be a particular reservoir for *A. baumannii*. Prevalence in adults, estimated by boot swab, resulted very low. This dynamic suggests a transient presence of *A. baumannii* in reared poultry and raise questions on the source of *A. baumannii* in one-day-old turkey. Wilharm *et al.* (155) detected two *A. baumannii* isolates from avian wildlife and livestock sharing the same sequence types with the isolates we detected in raw meat.

Poultry sector requires further investigation to elucidate its role in the *A. baumannii* epidemiology, starting from the farming environment and including surfaces of food-processing industry. Besides, raw meat is confirmed to host multiple *A. baumannii* clones. It is important to investigate the features of these isolates to understand if they could contribute to nourish antibiotic resistance evolution of this opportunistic pathogen.

4. A novel research axe: impact of antibiotic therapies on intestinal microbiota

The importance of the intestinal microbiota for human health has been appreciated since decades (156). This complex ecological consortium is an organ fundamental for host homeostasis. Intestinal microbiota provides nutrients through the digestion of fibers whose metabolites (Short Chain Fatty Acids) have a beneficial role on the health of the host enhancing susceptibility to insulin that in turn regulates lipids and sugar metabolism (157). Intestinal microbiota is involved in vitamins production, absorption of cations (158) and provides a barrier against pathogens. Many bacterial species produce butyrate, which is involved in the regulation of genes encoding tight junctions of epithelial cells, regulation of immune system cells, and inhibition of cytokines (159, 160). More recently, a role of chemical signals produced in the intestinal microbiota in the modulation of the nervous system and link with neurodegenerative diseases has been investigated (161).

The functions of intestinal microbiota are conserved, but bacterial composition is unique to each individual, although a core at the phylum level can be recognized among mammals consisting in Bacteroidetes and Firmicutes, the most abundant, followed by Actinobacteria, Verrucomicrobia, Proteobacteria and Fusobacteria (162). Diversity and richness are indexes frequently used to describe the status of the intestinal microbiota. High diversity and species richness correlate with good health status of the host (163).

Construction of the intestinal microbiota composition in humans is influenced by early life events such as modality of birth (vaginal *versus* cesarean section) (164) and pre-weaning nutrition (breastfeeding *versus* milk formula) (165). Furthermore, birth seems to be a crucial moment for the colonization of the gut with resistant bacteria, often transmitted by the mother or from the environment (166). Intestinal microbiota is highly dynamic in childhood, and the introduction of solid food in diet constitutes a major event for its maturation (167). Physiological modification then occurs by aging conducting to a loss of diversity and richness

(168). In adulthood, composition of intestinal microbiota is stable but several environmental perturbations can affect its composition and functions (169). Antibiotic therapies can disrupt the ecology of the microbiota, inducing dysbiosis characterized by high abundance of Proteobacteria and decrease of species diversity (170). These alterations can favor the emergence of pathogen species, such as *Clostridioides difficile* (171).

Condition of the intestinal microbiota is crucial also for animal health, participating to disease prevention and animals' performance (172, 173). Investigations are focusing on the possibility to manipulate bovine's microbiota for improving general health of animals but also to increase meat production, sparing CO₂ and CH₄ emission (174). Microbiota in newborn animals are dynamic and are influenced by the same factors relevant for human microbiota. For instance, modality of birth delivery affects the initial colonization of calves' gut (175, 176). Feeding methods of pre-weaned calves are diverse (177) and colostrum feeding promotes colonization of the gut by beneficial bacteria for weight gain and intestinal development (e.g. villus circumference, height and epithelial cell proliferation) (178, 179).

A dramatic effect of antibiotic therapies on intestinal microbiota, both in humans and animals, is the selection of antibiotic resistant bacteria by eliminating susceptible clones and facilitating horizontal gene transfer (18, 180). Once excreted in the environment, these selected resistant bacteria could spread further, colonizing other hosts. This dissemination mechanism is particularly concerning for livestock. In the farm environment, the propagation from one individual to another is facilitated as well as the dispersion in the surrounding spaces through pasture.

Among food-producing animals in France, bovines and calves represent a particular concern for the issue of antibiotic resistance. In these hosts, proportion of multidrug resistant *E. coli* is equal to 20% (97). In the past, feeding calves with waste-milk containing antibiotics residues was common (181). This practice selects resistant bacteria in calves' intestine. Dupouy

et al. investigated the selective power of cefquinome (4th generation cephalosporin) administered to calves colonized by different amount of ESBL-producing *E. coli*. The administration mimicked residual concentration found in waste milk (2 mg/L) and that of udder milk of treated cows (20 mg/L). Cefquinome had a selective effect, irrespective of the administrated concentration, on all calves colonized by ESBL-producing *E. coli* prior to antibiotic exposure (182). European regulations exist since 2015 discouraging this practice. In calves, antibiotic treatments are more frequent than in adult bovines (183), probably because of more frequent digestive and respiratory disorders occurrence. Calves are subject to stressful situation, like long lasting transport from farms to feedlots centers. Here, sometimes calves are hosted at high density favoring diseases transmission. These factors, probably explain higher resistance rates in young bovines than adult ones (97).

The impact of antibiotic therapies on animals' microbiota, including calves, has been **reviewed**²⁴ by Tony Rochegüe in 2021, during his PhD thesis that I co-directed with Prof. Tristan Ferry. The review focused on the main food-producing and companion animals and highlighted that irrespective of antibiotic classes and animal hosts, antibiotic therapies decreased species diversity and richness favoring the bloom of potential enteropathogens and the selection of antibiotic resistance. Studies analyzing impacts of sub-therapeutic dosages reported the augmentation of taxa involved in sugar metabolism, suggesting a link with weight gain. This result should not be interpreted positively, considering that parallel information on antibiotic resistance selection was rarely reported and selection of antibiotic resistance is known to occur also at low antibiotic concentration.

²⁴ Rochegüe T., Haenni M., Mondot S., Astruc C., Cazeau G., Ferry T., Madec J.Y., **Lupo A.** Impact of Antibiotic Therapies on Resistance Genes Dynamic and Composition of the Animal Gut Microbiota. *Animals (Basel)*. 2021, 11:3280.

During his thesis, Tony Rochegüe analyzed the impact of intra-muscular administration of amoxicillin in a cohort of calves treated for umbilical infections, compared to a cohort of healthy calves, of the same age of treated ones and not receiving amoxicillin. By qPCR, using hydrolytic probes designed and validated in the frame of **his study**,²⁵ we observed a dramatic increase of *bla*_{TEM} gene, encoding a penicillinase able to hydrolyze amoxicillin, in feces of treated calves at the end of the treatment. One week after amoxicillin withdrawal, level of *bla*_{TEM} gene copies decreased at levels comparable to the pretreatment. Other resistance genes, like *tetA*, *strA* and *strB*, and *intI1* followed the same dynamic, suggesting that amoxicillin co-selected resistance to other antibiotic classes (tetracycline and streptomycin) of therapeutic importance in veterinary sector.

In the frame of this project, DNA extracted from calves' feces was also sequenced by a shotgun approach. Analysis of these data has been started by Thibault Destanque, bio-informatician in the AVB unit since November 2022. Preliminary analysis demonstrated increase of relative abundance of Bacteroides and Proteobacteria phyla in treated calves along with a decrease of species diversity. Analysis of metabolic pathways and abundance of resistance genes is ongoing. Potentially, these data could reveal which microbiota functions are affected by amoxicillin therapy providing the basis for eventual prebiotic supplementation in treated animals, avoiding unintended effects of amoxicillin.

In the continuity with this project and in collaboration with Prof. Caroline Prouillac from VetAgro Sup Institute, we are investigating the effect of amoxicillin-clavulanic acid (AMC) combination at therapeutic dosages, for treating umbilical infections, on calves' microbiota.

²⁵ Rochegüe T., Haenni M., Cazeau G., Metayer V., Madec J.Y., Ferry T., **Lupo A.**

An inventory of 44 qPCR assays using hydrolysis probes operating with a unique amplification condition for the detection and quantification of antibiotic resistance genes. *Diagn Microbiol Infect Dis.* 2021, 100:115328.

Morgane Moustaghfir, PhD student, is developing the project under Prof. Prouillac and myself direction.

The AMC combination is an important antibiotic for animals and humans, used to treat a variety of infections especially in pediatric and elderly population. AMC is used also after therapeutic failure with amoxicillin or when an ESBL-producing pathogen is detected (184). However, during AMC treatment, side effects (diarrhea) seem to be more frequent than what is observed for amoxicillin. Furthermore, proportion of AMC-resistant *E. coli* is increasing globally (185) and longitudinal selective ability of AMC needs to be investigated. Beside analyzing the effect of the therapy on the microbiota composition and antibiotic resistance selection, the project aims to compare the effects of oral versus intramuscular administration. In parallel to microbiota analysis by shotgun sequencing and cultivation, the exposure of the microbiota to AMC will be evaluated by quantification of the antibiotic concentration in calves' feces and pharmacokinetics analyzed. To understand the source of resistant bacteria present in calves' feces, their presence will be investigated in the environment of the farms hosting the calves and in calves' mothers. Overall, this project will provide information if one administration route could avoid negative effects, such as antibiotic resistance selection, on microbiota more effectively than the other one. Analyzing the response of microbiota to AMC could provide the basis to develop strategies to mitigate undesired effects.

Research on antibiotic resistant bacteria in intestinal microbiota in animals has been previously conducted in the research unit, mostly based on cultivation methods. The development of novel molecular approaches developed in the frame of Tony's thesis, like quantification of resistance genes by qPCR and analysis by shotgun sequencing, can open to a more comprehensive picture of the selection process and functionality of the microbiota.

5. Conclusions

In the recent years, antibiotic resistance crisis has been called as “silent pandemic”, probably because its spread occurs underhand without appreciable events in the daily life of most of people, in contrast to acute pandemic such as the Sars-CoV2 one. However, the viewpoint raised by Mendelson *et al.* (186) sounds pertinent: the communication strategy that associates the AMR crisis with “silent” might be counterproductive. This was emphasized by Dame Sally Davies at a recent BSAC Spring Conference who said: “Calling antimicrobial resistance a “silent pandemic” doesn’t work. Politicians can ignore a silent pandemic. AMR is a grand pandemic. It can’t be ignored”. Antibiotic resistance crisis is indeed there, speaking loud with 35,000 attributable death per year in EU and 4.95 M deaths worldwide in 2019 (184, 187).

This global health issue has reached the agenda of the G7 2021 spring summit. Several aspects have been discussed by health ministers and possible interventions to tackle antibiotic resistance prioritized. Besides antibiotic stewardship and policies for a rationale and controlled use of antibiotic, investment on novel antibiotics development is crucial, and the strategy of national subscription programs has been discussed. **Rapid diagnostic**²⁶ of antibiotic resistance is important for targeted therapy and its relevance in point-of-care settings has been highlighted together with the development of diagnostics in low and medium income countries. Indeed, economically affordable technologies are needed for adoption in any region of the globe. Infection and prevention control of infectious diseases is of priority both in the community and the hospital settings. In line with this principle, development of vaccine to prevent infectious diseases is urgently needed too. Finally, yet importantly, all compartments should be

²⁶ **Lupo A.**, Papp-Wallace K.M., Sendi P., Bonomo R.A., Endimiani A. Non-phenotypic tests to detect and characterize antibiotic resistance mechanisms in Enterobacteriaceae. *Diagnostic Microbiology and Infectious Diseases*. 2013, 77:179-194

considered, from human to animal to the environment. A general agreement came out on the necessity of a One Health approach to find solution for the antibiotic resistance crisis, considering the health of humans, animals and environment intrinsically connected. Antibiotic resistance is indeed an ecological issue connected to other major crisis such as climate changes. Future availability of safe drinking water seems seriously compromised and major flooding increase the risk of water-borne diseases. More frequent viral epidemics could cause an increase, justified or not, of antibiotics prescription, as observed during the COVID-19 pandemics, worsening the selection and dissemination of antibiotic resistance.

In this panorama, it is crucial to maintain strategies already in place, such as the surveillance of antibiotic resistances, following the One Health perspective. Programs are already in place and future integrated One Health surveillance approaches are in evolution (188). Furthermore, surveillance programs can benefit of next-generation sequencing approaches that are becoming increasingly accessible from financial and analytical viewpoint. Such surveillance and molecular characterization of resistance genes and genetic vectors can contribute to prolong the effectiveness of antibiotics, calling for opportune recommendations for stewardship, intervention when necessary, and overall increased awareness of the antibiotic resistance crisis outside the scientific community.

In line with these aspects, I wish to continue focusing on two main research topics in the AVB unit at Anses-Lyon: i) surveillance of resistance in *Acinetobacter* spp. and its molecular epidemiology; ii) impact of antibiotic treatment on the microbiota.

Investigations on *Acinetobacter* spp. are necessary to understand the current epidemiological situation in France and more globally, especially after our studies that demonstrated ability of emerging clones to colonize both humans and animals. The presence of carbapenem-resistant strains in companion animals is surprising, considering that this antibiotic class is not used in veterinary clinic. Sources of carbapenem-resistant *A. baumannii* for pets

need to be identified. A clear picture of *A. baumannii* prevalence in the human community along with the determination of antibiotic susceptibility and molecular typing could help understanding the propagation of this species among hosts. Understanding extra-hospital as well as carbapenem-susceptible human epidemiology of *A. baumannii* could contribute to evaluate the risk that the presence of multiple *A. baumannii* clones, also found in food products, represent. Investigating the ability to acquire antibiotic resistance, resist biocides, and resist to environmental stresses by biofilm, or survive to desiccation could in fine contribute to unveil potential target to inhibit the pathogen growth and predict the emergence of successful clones from these reservoirs.

Intestinal microbiota is a major organ for mammals' health maintenance, but it is also a reservoir of antibiotic resistant bacteria. Antibiotic therapies could select antibiotic resistant bacteria, favoring their blooming, and eliminate susceptible ones, decreasing diversity. This loss of diversity in the intestinal microbiota could favor genetic exchanges between closely related bacteria, enhancing antibiotic resistance transfer or multidrug resistance development. However, platforms for horizontal gene transfer such as broad-host range plasmids or IMEs like those reported from Gram-positive and Gram-negative species that exploit conserved regions of other genetic elements to broaden their host spectrum are able to overcome genetic barriers to recombination or transfer thus further increasing the risk of dissemination. Resistant bacteria selected in the intestinal microbiota, once released in the environment can colonize other hosts and/or encounter other species to whom the resistance can be transferred. Studying the effect of clinically relevant antibiotic therapies is important since these studies, coupled with pharmacokinetics measurements, could pave the principles for effective antibiotic therapies protecting the intestinal microbiota and sparing massive resistance selection.

If antibiotic resistance is unavoidable, many accelerators of this process could be mitigated. These studies integrate in the necessary steps to fight antibiotic resistance and contribute dampen the loud pandemic.

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Annex 1