NARA
Nationales Referenzlaboratorium zur Früherkennung
neuer Antibiotikaresistenzen und Resistenzmechanismen

Centre National de Référence des Résistances Emergentes aux Antibiotiques

Molecular characterizations of antibiotic resistance

Carbapenem-hydrolyzing ß-lactamases of the New Delhi metallo-ß-lactamase (NDM) type belong to the class B ß-lactamases. They are rapidly spreading worldwide including in Switzerland (Findlay et al., 2021).

A series of NDM variants have been identified with 26 currently known NDM derivatives. These variants efficiently hydrolyze all ß-lactams with the exception of the monobactam, aztreonam. NDM-5 has been primarily identified among multidrug-resistant *Escherichia coli* in the United Kingdom (Hornsey et al., 2011).

A combination of aztreonam-avibactam (ATM-AVI) is under development with the aim to efficiently target NDM producers since the monobactam ATM is the only ß-lactam being spared by the hydrolytic activity of NDM and AVI inhibiting the activity of any extended-spectrum ß-lactamase potentially hydrolyzing ATM, considering that ESBLs being often co-produced by NDM producers. However, a series of ATM-AVI resistant NDM-5-producing *E. coli* have been identified. This peculiar resistance pattern is associated with a structural modification of the penicillin binding protein 3 that is a main target of ATM and the co-production of specific plasmid-encoded cephalon-sporinases, such as CMY-42 (Periasamy et al., 2020; Sadek et al., 2020), that latter having high binding affinity to ATM (Sadek et al., 2020).

NDM-5-producing *E. coli* strains are frequently recovered in Switzerland (92 strains collected at the NARA from 2017 to 2021). They belong to several ST types (Chakraborty et al., 2021), but mostly of the ST167 type, and either found from human or food sources (Sadek et al., 2021).

This successful epidemic clone is known to be associated with both multiresistance and virulence traits and is therefore of high public health concern. Plasmids carrying the NDM-5 encoding gene have different backbones and are transferable (Chakraborty et al., 2021).

We showed that those ATM-AVI resistant strains identified in Switzerland are spreading internationally including in France and Germany and their origin are very likely Pakistan (Sadek et al., 2021).

It remains to be determined which is the selecting agent for those strains in Pakistan where the combination of ATM-AVI is not yet available. Identification of this peculiar resistance mechanism underlines that emerging resistance mechanism to a novel antibiotic may spread before its marketing that renders the industrial development of novel antibiotics further complicated.

Aminoglycoside-modifying enzymes, namely phosphotransferases, adenylyltransferases, nucleotidyltransferases, and acetyl-transferases, are known for decades as a common source of acquired resistance to aminoglycoside molecules in Gram negatives. Each of those enzymes may confer resistance to some but not all of the commonly used molecules, namely amikacin, gentamicin, tobramycin, and kanamycin, although the combination of those enzymes in single strains is possible. Lately, 16S rRNA methylases (RMTases) have been identified as a source of acquired resistance to aminoglycosides.

All of them methylate the target of aminoglycosides, namely the 16S rRNA, and consequently confer high-level and broad-spectrum resistance to all clinically-relevant aminoglycosides. They are mostly encoded by plasmids and found among many different Gram-negative bacteria. To date, ten RMTases have been identified (namely ArmA, RmtA-H, and NpmA, including a series of variants within these groups) (Doi et al. 2016). The recent emergence and spread of those genes encoding RMTases is of significant concern as they are often acquired by *Enterobacterales* that produce ESBLs or carbapenemases, and more specifically isolates producing metallo-ß-lactamases of the NDM type. We have retrospectively analyzed and characterized the association of carbapenem- and aminoglycoside-resistant clinical isolates in Switzerland during a 3.5-year period between January 2017 and June 2020 (Fournier et al., 2022). A total of 103 pan-aminoglycoside- and carbapenem-resistant clinical isolates were recovered at the NARA. Over the 991 *Enterobacterales* collected during this period, 103 (10.4%) were resistant to both carbapenems and all aminoglycosides. Among these 103 isolates, 35 isolates produced NDM-like carbapenemases, followed by OXA-48-like (*n* = 23), KPC-like (*n* = 21), or no carbapenemase (*n* = 13), OXA-48-like and NDM-like co-production (*n* = 7), and VIM-like enzymes (*n* = 4). The RMTases ArmA, RmtB, RmtC, RmtF, RmtG, and RmtB + RmtF were identified among 51.4%, 13.6%, 4.9%, 24.3%, 1%, and 1%, respectively. Plasmid co-localization of the carbapenemase and the RMTase encoding genes was found among ca. 20% of the isolates. A high diversity was identified in terms of the nature of associations between RMTase- and carbapenemase-encoding genes, of incompatibility groups of the corresponding plasmids, and of strain genetic backgrounds, highlighting heterogeneous importations rather than clonal dissemination. The annual rate of RMTases among carbapenemase-producing *Enterobacterales* showed an increasing trend over the time, from 7.5%, 10.7%, 11.2% to 13% from 2017 to 2020. This resistance rate questions about the clinical interest of plazomycin marketed in the US but not in Europe, taking in account that RMTase producers are also resistant to plazomycin (a novel aminoglycoside).

Diagnostic tests

Accurate and rapid detection of ESBL-producing *Enterobacterales* is critical in different clinical contexts, either when considering treatment of different types of infections (and especially bloodstream infections) and therefore optimizing antibiotic stewardship interventions, or for epidemiological purposes. ESBLs known to widely spread among community isolates are mostly of the CTX-M type. However, producers of non-CTX-M-type ESBLs have also been reported as sources of infections or/and outbreaks, including strains producing TEM, SHV, PER and VEB derivatives. Those enzymes have all the capacity to hydrolyze and consequently significantly compromise the efficacy of penicillins, aztreonam and cephalosporins.

Three methods have been compared to detect ESBL producers from blood cultures (Boattini *et al*, 2022).

The Rapid ESBL NP test (Liofilchem, Roseto degli Abbruzzi, Italy) is a colorimetric cefotaxime-hydrolysis assay detecting the presence/absence of any type of ESBL, of the presence of an enzyme or combination of enzymes hydrolyzing cefotaxime but being not inhibited by the addition of tazobactam (i.e. AmpC, ESBL+ AmpC, carbapenemase with or without ESBL) (Nordmann et al., 2012).

The NG CTX-M Multi assay (NG Biotech, Guipry, France) is a lateral-flow immunoassay including monoclonal antibodies specifically detecting CTX-M-type ESBLs only.

The E-test (bioMérieux, Marcy l'Etoile, France) is an antimicrobial gradient method that combines the principle of dilution methods with that of diffusion methods to determine the MIC value. Both ESBL E-test ceftazidime +/- clavulanic acid and cefotaxime +/- clavulanic acid may allow to identify ESBL production. To confirm the production of ESBLs among isolates overproducing AmpC ß-lactamases, it is recommended that an additional ESBL confirmation test shall be performed with cefepime as the indicator cephalosporin, considering that this ß-lactam antibiotic is usually poorly hydrolyzed by AmpC ß-lactamases. Over the studied period, a total of 242 blood cultures were processed with 58.7% associated with *K. pneumoniae* or *E. coli.* The Rapid ESBL NP test, the NG-CTX-Multi and the E-test all showed good performance for detecting ESBL production. Of note, the Rapid ESBL NP test and the NG-CTX-M test both offer the significant advantage of a turn-around time of 15-45 min, while the direct E-test technique necessitates an 18-h incubation time. Noteworthy, the Rapid ESBL NP test possesses the additional advantage to detect any type of ESBL.

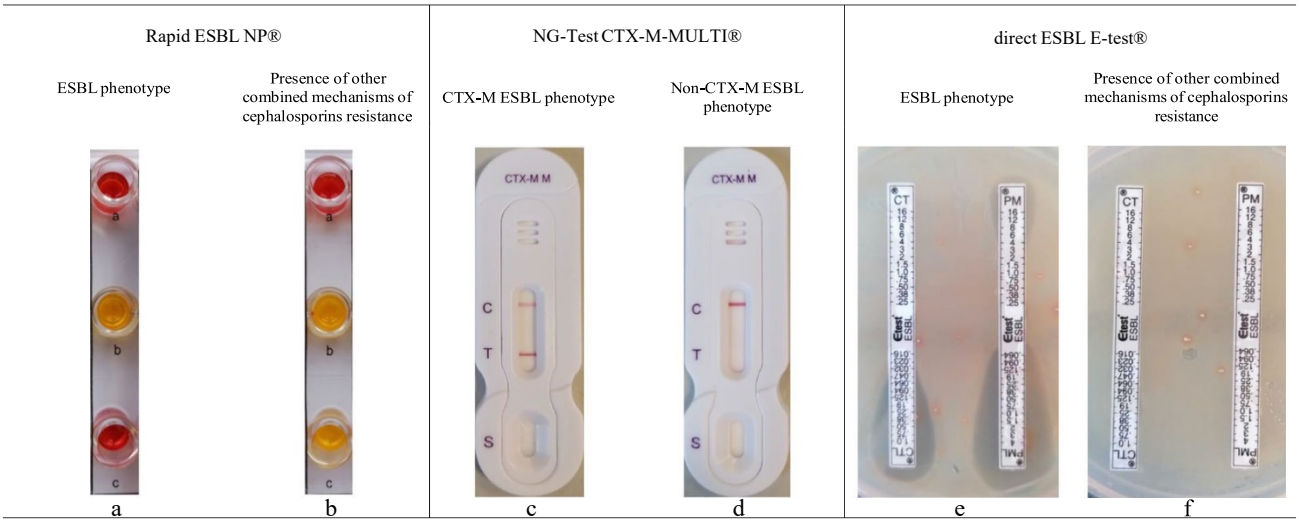

Fig. Several phenotypic test results obtained directly for K. pneumoniae- or E. coli-positive blood cultures. Rapid ESBL NP; (a) ESBL phenotype and (b) presence of other combined mechanisms of cephalosporin resistance. NG test CTX-M MULTI; (c) producer of a CTX-M ESBL and (d) non-CTX-M ESBL producer. Direct ESBL E -test; (e) ESBL producer and (f) isolate being resistance to cephalosporins due to other combined mechanisms.

Similar results were obtained by using those tests from cultures grown on selective media for detecting ESBL producers for epidemiological purposes (Blanc et al., 2021). Another test, the ß-Lacta test (Bio-Rad, Marnes-La-Coquette, France) includes a chromogenic cephalosporin that turns from orange to red upon hydrolysis. It detects well any type of cephalosporin resistance but lacks specificity since it may detecting ESBLs, overproducers of cephalosporinases as well as carbapenemase producers (Poirel et al., 2016).

A MALDI-TOF MS based approach can be used for detecting ESBLs (Jung et al., 2014). However, it requires the corresponding equipment and careful adaptation for its implementation in routine microbiology. A direct inactivation method based on inactivation of hydrolysis of a cephalosporin can be also used (Bianco et al., 2019) but it may lack of reproducibility to its unique home-

made version and necessitates a turn-around time of several hours.

Novel molecules

Recently, the efficacy of new antibiotics has been evaluated against multidrug-resistant Gram-negative bacteria. Among them, combinations of known ß-lactams with recently developed ß-inhibitors such as ceftolozane-tazobactam (C/T), ceftazidime-avibactam (CZA), meropenem-vaborbactam (MEB) and imipenem-relebactam (I/R) (Doi, 2019). C/T does not possess significant activity against carbapenemase producers although other combinations usually do. None of those inhibitors has significant activity against carbapenemases of the metallo-enzyme types, i.e. NDM, IMP, and VIM types. Avibactam and relebactam belong to the same group of inhibitors, i.e. diazabicyclooctane group where as vaborbactam is a boronic acid derivative. Avibactam is active against carbapenemases of Ambler class A (KPC) and Ambler class D (OXA-48-like) while vaborbactam and relebactam are active against carbapenemases of class A only. Taking in account the inhibitory properties of the molecules and the intrinsic activity of the ß-lactam that is combined with the inhibitors, MEB was more effective (84% versus 63%) than CZA from a collection of 150 carbapenemase-producing isolates representtative of the strains received at the NARA (P. Nordmann, personal communication).

Actually, although vaborbactam is not active against OXA-48-like enzymes, many OXA-48 like producers and NDM remain susceptible to meropenem. Currently, none of the clinically-available inhibitors are active against carbapenemases of the metallo-ß-lactamase type, and are therefore urgently needed since there is a growing number of isolates producing NDM enzymes both in nosocomial and community settings.

One-Health antibiotic resistance

The aim of a study performed in 2019 was to prospectively evaluate the prevalence of intestinal carriage of colistin-resistant and extended-spectrum β-lactamase (ESBL)-producing *Enterobacterales* among 81 pigs from a Swiss farm (Canton of Fribourg) attending an animal health and antibiotic stewardship program (aiming to reduce as much as possible the use of antibiotics as antimicrobial agents) and to determine the associated mechanisms of resistance (Fournier et al., 2021).

Of note, those pigs had no contact with outside environment. A total of 38 ESBL-producing *Escherichia coli* and a single ESBL-producing *Enterobacter cloacae* were recovered from those 81 pigs, corresponding to a high prevalence of 50%, no other β-lactamase producer being identified. Among the 38 ESBL-producing *E. coli*, all belonged to sequence type (ST) ST10, except two ST34 and ST744 isolates. Among the ST10-$bla_{\text{CTX-M-1}}$ isolates, three subclones (n = 22, n = 13, and n = 1, respectively) were identified according to the genotyping analysis. The most commonly identified IncI1 plasmid harboring the $bla_{\text{CTX-M-1}}$ gene was 143 kb in size and co-harbored other resistance genes encoding resistance to tetracycline and sulfonamides. Only three colistin- resistant *Enterobacterales* isolates were recovered, namely two *K. pneumoniae* isolates and a single *E. cloacae* isolate. Screening for the plasmid-borne *mcr-1* to *mcr-9* genes encoding resistance to polymyxins gave negative results. The two *K. pneumoniae* isolates were clonally related, belonged to ST76, and harbored a truncated *mgrB* chromosomal gene being the source of colistin resistance. A high prevalence of fecal carriage of ESBL-

producing *E. coli* was found, being mainly caused by the spread of a clonal lineage within the farm. By contrast, a low prevalence of colistin-resistant *Enterobacterales* was found.

The high rate of CTX-M-1 producers (being commonly identified in animals) among fattening pigs and sows would suggest the presence of selective pressure in the farm. However, the absence of antibiotic usage in these fattening pigs did not support an antibiotic-related one. The maintenance of the CTX-M-1-encoding plasmid might, however, be explained by the presence of a toxin-antitoxin system preventing the bacterial strain from plasmid loss. The presence of toxin–antitoxin systems on plasmids is known to be responsible for the post-segregational killing of daughter cells that do not contain the plasmid after the cell division completion (Smith et al., 2004).

International spread

There is currently some debate about whether the Covid19 pandemics induced increased selective pressure with last-resort antibiotics, including carbapenems, and even the ultimate ß-lactam/ß-lactamase inhibitor combinations such as ceftazidime/avibactam (CZA). The fear is that an increased and sometimes inappropriate usage of those antibiotics might have been generated by this crisis, especially in ICUs. Consequently, selection of nosocomial isolates being almost panresistant to antibiotics might have occurred in specific settings. This is examplified by the outbreak that occurred in a Covid-19 ICU in Italy, involving a CZA-resistant and KPC-producing *K. pneumoniae* (Bianco et al., 2022).

The outbreak involved two distinct clones, the most commonly identified producing a point-mutant derivative of KPC-2 conferring resistance to CZA. Noteworthy, those isolates showed a restored susceptibility to carbapenems, since this KPC-2 derivative almost lacked its ability to hydrolyze carbapenems. Likewise, the occurrence of novel KPC variants among ST307 high-risk *K. pneumoniae* clones occurred in Spanish ICUs, having again almost totally lost their carbapenemase property (Hernandez-Garcia et al., 2022).

The subsequent spread of such clonal strains is worrying, since those KPC producers for which reversion to carbapenem resistance might occur are difficult to recognize and detect, owing the lack of selectivity of the carbapenem-based selective media commonly used in clinical laboratories for screening. These observations in other European countries might be considered as a significant alert for possible importation in Switzerland. Clinical microbiologists therefore need to be informed that such weird resistance phenotype might be observed and that corresponding surveillance is therefore required.

Antibiograms of clinical interest

E. coli producing OXA-48 and CTX-M-15

Decreased susceptibility to carbapenems mostly seen with ertapenem. Resistance to temocillin. Co-production of an extended-spectrum ß-lactamases (ESBL) observed in 80% of the cases, that may explain the resistance observed for ceftazidime, cefotaxime, and aztreonam. Susceptibility to ceftazidime-avibactam with avibactam well inhibiting CTX-M-15, while OXA-48 does not hydrolyze ceftazidime.

AM, Ampicillin (10 µg); TIC, Ticarcillin (75 µg); PRL, Piperacillin (30 µg); TPZ, Piperacillin/Tazobactam (30/6 µg); CZA, Ceftazidime/Avibactam (14 µg); CAZ, Ceftazidime (10 µg); TIM, Ticarcillin/Clavulanate (75/10 µg); IPM, Imipenem (10 µg); CTX, Cefotaxime (5 µg); AMC, Amoxicillin/ Clavulanate (20/10 µg); FEP, Cefepime (30 µg); ETP, Ertapenem (10 µg); FOX, Cefoxitin (30 µg); ATM, Aztreonam (30 µg); TEM, Temocillin (30 µg); MEM, Meropenem (10 µg)

***E. coli* producing KPC-3**

Resistance to carbapenems of variable level depending on the carbapenem molecule.
Susceptibility to the ceftazidime/avibactam combination.
Reduced susceptibility but not full resistance to temocillin.

AM, Ampicillin (10 µg); TIC, Ticarcillin (75 µg); PRL, Piperacillin (30 µg); TPZ, Piperacillin/Tazobactam (30/6 µg); CZA, Ceftazidime/Avibactam (14 µg); CAZ, Ceftazidim (10 µg); TIM, Ticarcillin/ Clavulanate (75/10 µg); IPM, Imipenem (10 µg); CTX, Cefotaxime (5 µg); AMC, Amoxicillin/ Clavulanate (20/10 µg); FEP, Cefepime (30 µg); ETP, Ertapenem (10 µg); FOX, Cefoxitin (30 µg); ATM, Aztreonam (30 µg); TEM, Temocillin (30 µg); MEM, Meropenem (10 µg)

***K. pneumoniae* producing NDM-1**

High-level resistance to all ß-lactams with the exception of aztreonam.

AM, Ampicillin (10 µg); TIC, Ticarcillin (75 µg); PRL, Piperacillin (30 µg); TPZ, Piperacillin/Tazobactam (30/6 µg); CZA, Ceftazidime/Avibactam (14 µg); CAZ, Ceftazidime (10 µg); TIM, Ticarcillin/ Clavulanate (75/10 µg); IPM, Imipenem (10 µg); CTX, Cefotaxime (5 µg); AMC, Amoxicillin/Clavulanate (20/10 µg); FEP, Cefepime (30 µg); ETP, Ertapenem (10 µg); FOX, Cefoxitin (30 µg); ATM, Aztreonam (30 µg); TEM, Temocillin (30 µg); MEM, Meropenem (10 µg)

Further meetings

Swiss Society Microbiology Meeting; Lausanne, Tuesday 30th of August to Thursday 1st of September 2022

Réunion Interdisciplinaire de Chimiothérapie Anti-Infectieuse (RICAI); Paris, Monday 13th of December to Tuesday 14th of December 2022

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