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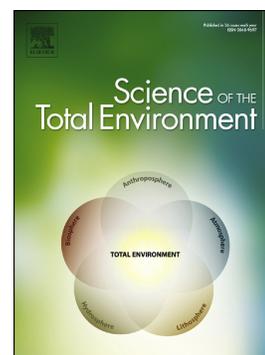
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**Biofilms in hospital effluents as a potential crossroads for carbapenemase-encoding strains**

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**Highlights:**

Bacteria harboring carbapenemase-encoding genes in hospital wastewater biofilms

These genes are carried by conjugative plasmids

Carbapenem in effluent and bacterial resistance are potential hazards

## **Abstract**

Bacterial resistance to carbapenem, which is mainly due to the successful dissemination of carbapenemase-encoding genes, has become a major health problem. Few studies have aimed to characterize the level of resistance in the environment, notably in hospital wastewater, which is a likely hotspot for exchange of antibiotic resistance genes.

In this work, we looked for the presence of imipenem-resistant bacteria and imipenem in the effluent of the teaching hospital of Clermont-Ferrand, France. Selective growth of bacteria from 14-day old biofilms formed in the pipe sewer showed that 22.1% of the isolates were imipenem-resistant and identified as *Aeromonas* (n=23), *Pseudomonas* (n=10), *Stenotrophomonas* (n=4) and *Acinetobacter* (n=1). Fifteen of these strains harbored acquired carbapenemase-encoding genes *bla*<sub>VIM</sub> (n=11), *bla*<sub>OXA-48</sub> (n=2), *bla*<sub>GES</sub> (n=1), *bla*<sub>NDM</sub> (n=1). All isolates also harbored associated resistances to aminoglycosides, fluoroquinolones and/or tetracyclin. S1-nuclease pulsed-field gel electrophoresis analysis of eight selected isolates showed that four of them harbored one to two plasmids of molecular weight between 48.5Kb and 194Kb. *In vitro* transformation assays evidenced the presence of *bla*<sub>VIM</sub> and *bla*<sub>NDM</sub> on plasmids with the *bla*<sub>VIM</sub> harboring 80 Kb plasmid having conjugative capacity.

The predicted environmental concentration of imipenem in the hospital effluent was 3.16 µg/L, suggesting that biofilm bacteria are subjected to sub-MICs of imipenem within the effluent. However, no imipenem molecule was detected in the hospital effluent, probably owing to its instability: *in vitro* assays indicated that imipenem's biological activity was no longer detectable after 45h of storage. However, the predictive value of the hazard quotient relative to the development of resistance was greater than 1.0 (HQR=28.9 ± 1.9), which indicates a possible risk. The presence of carbapenemase-encoding genes in hospital effluent biofilm strains and their ability to transfer are therefore a potential hazard that should not be neglected and points to the need for monitoring antibiotic resistance in hospital wastewater.

## Keywords

Biofilm, Carbapenemase, Hospital effluent, Plasmids, Carbapenem

## 1. Introduction

In the last few decades, antibiotics have been widely used as anti-infectious agents in humans and animals leading to an increase in the percentage of antibiotic-resistant bacteria. Carbapenems are beta-lactam antibiotics with a broad spectrum of activity and are usually considered as the last line of defense against severe infections, mainly those due to multidrug resistant Gram-negative bacteria. In France, carbapenem consumption increased rapidly between 2012 and 2016 from 0.021 to 0.033 dose per 1000 inhabitants per day. This increase was correlated with a rise in the rates of carbapenem-resistant bacteria mostly in a clinical setting (European Surveillance of Antimicrobial Consumption, 2017). Resistance to carbapenems is mainly due to the production of inactivating enzymes, i.e. carbapenemases (Nordmann et al., 2011b, 2011a). A recent survey showed that in Europe, 1.3 patients per 10 000 hospital admissions developed infectious diseases caused by carbapenemase-producing *Klebsiella pneumoniae* or *Escherichia coli*, with the highest incidence found in southern and southeastern Europe (European Surveillance of Antimicrobial Consumption, 2017). Likewise, the World Health Organization recently published a list of antibiotic-resistant pathogens for which new and effective antibiotics are urgently needed (WHO, 2017) with the first priority being focused on carbapenem-resistant bacterial genus. Carbapenem-resistant strains have been detected in human samples but have also been isolated from animal and environmental samples including food, drinking water and wastewater (Jin et al., 2017; Nasri et al., 2017; White et al., 2016; Zurfluh et al., 2017). Such efficient dissemination is probably related to the capacity of the genes encoding the carbapenem-inactivating enzymes to spread within the bacterial world. The carbapenemase-encoding genes most frequently expressed by clinical isolates (KPC, VIM, NDM, GES and OXA-48) are often carried by mobile genetic elements that ensure their maintenance and spread among Gram-negative bacteria (Logan and Weinstein, 2017). Hospital sewage could constitute a privileged place for such dissemination events. First, the bacterial load is heavy, comprising a mixture of antibiotic-resistant clinical pathogens and environmental bacteria aggregated in biofilm structures (Hocquet et al., 2016; Rodriguez-Mozaz et al., 2015), which have a high capacity of colonization and survival in wastewater systems.

Second, antibiotics are present at low concentrations in hospital effluent. After administration to patients, a large part of the native antibiotic molecules and their metabolites are excreted into wastewater, together with the resistant bacteria potentially selected in their digestive tracts

(Chagas et al., 2011; Chang et al., 2010; Längin et al., 2009; Vaz-Moreira et al., 2016). Antibiotics such as fluoroquinolones, sulfonamides and macrolides are frequently detected in wastewater, especially in hospital effluents and surface water (Gros et al., 2013; Le et al., 2016; Lien et al., 2016; Szekeres et al., 2017). In contrast, and despite their intensive use, beta-lactams, owing to their low chemical stability, are rarely detected in hospital effluents (Deshpande et al., 2004). The selective pressure exerted by these molecules is therefore questionable.

In this study, we assessed the presence of carbapenem (imipenem)-resistant bacteria in biofilms formed in the pipes carrying the hospital effluent, and characterized the antibiotic resistance patterns of the isolates. The presence of antibiotics facilitates the emergence of resistance even at sub-MIC concentrations and so we investigated the amount of imipenem theoretically present in the hospital effluent and determined its effective life in laboratory conditions.

## **2. Material and Methods**

### **2.1 Hospital effluent sampling**

The samples were collected three times a day for two weeks from the effluent of the Gabriel Montpied hospital, Clermont-Ferrand (139,500 inhabitants) France (latitude 45.7589632; longitude 3.0941279), which is a medium-size hospital with 1,100 beds that operates 24h a day with a peak of activity from 7a.m to 1p.m. Water consumption is 13,500–16,000 m<sup>3</sup> monthly, between 409 and 478 L/bed/day. The hospital effluent is directly discharged into the urban sewage network without any external connection and then into the municipal WWTP, “Les Trois Rivières”, which receives and treats about 1,700,000 m<sup>3</sup> a month.

The experimental sampling tank (110 cm–50 cm–50 cm) was located on a line receiving exclusively wastewater from the hospital upstream of the connection a pipe that collects rainwater before discharge in the public network wastewater. The physical and chemical characteristics of the hospital effluent have been assessed previously (Ory et al., 2016).

### **2.2 Environmental risk assessment of imipenem**

Measured environmental concentrations (MEC) of imipenem in the effluent were determined with six polar organic chemical integrative samplers (POCIS) containing 200 mg Oasis HLB® (Exposmeter, Tavelso, Sweden). The POCIS were fixed in the effluent tank for 15 days.

Quantitative analysis of imipenem was performed by direct injections of filtered (0.7 µm glass

fiber filter) wastewater on Ultra Performance Liquid Chromatography/Mass Spectrometry system (UPLC/MS/MS, Waters). Due to high instability of carbapenems in presence of organic solvent, no prior sample reconcentration step could be applied. Limit of detection has been evaluated at 2 µg/L (limit of quantification: 5 µg/L).

The predicted environmental concentration (PEC) and ecotoxicological risk (hazard quotient, HQ) were estimated as follows. The consumption of imipenem by patients in Gabriel Montpied hospital during the study period was assessed with PHARMA software (Computer Engineering, Paris, France) and the amount potentially released in the hospital effluent calculated according to pharmacokinetic parameters [www.theriaque.org, www.drugs.com]. The PEC was calculated as previously described (Ory et al., 2016). The environmental risk of imipenem was defined according to its HQ, which is the quotient between PEC and the predicted no effect concentration (PNEC). The PNEC used in this study (78 µg/L) was previously established (Bengtsson-Palme and Larsson, 2016). In addition, the environmental risk of resistance selection in microbial communities was calculated by determining the hazard quotient resistance (HQ<sub>r</sub>), *i.e.* the quotient between PEC and PNEC<sub>MIC</sub>, the latter being calculated from the sample size-adjusted lowest MICs from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) database with an assessment factor of 10 (Bengtsson-Palme and Larsson, 2016).

### 2.3 Measurement of the effective life of imipenem

The stability of imipenem was evaluated by determining its activity on the imipenem-susceptible strain *Escherichia coli* K12 C600 using the agar (Müller Hinton, MH) diffusion test. A stock solution of imipenem (Sigma-Aldrich, Lyon, France) was made at a concentration of 1 mg.mL<sup>-1</sup> and kept at 26 °C throughout the experiment. For each assay, 10 µl of a solution containing 10µg of imipenem were loaded onto sterile 10mm-diameter paper discs, which were then applied onto a bacterial lawn deposited by swabbing a bacterial suspension (McFarland = 0.6) onto MH agar plates. The negative and positive controls consisted of paper discs impregnated with saline and commercialized imipenem disks (10 µg) (Bio-Rad, Ivry-sur-Seine, France) stored at 4°C. The experiment was repeated in triplicate every 6 hours for 54 hours. The diameters of the inhibition zone were measured after 18h of incubation at 37°C.

The stability of imipenem was also measured by determining the bactericidal effect in MH broth medium buffered at pH 7.2. A total of 100 µl of imipenem solution (1 mg.mL<sup>-1</sup>) freshly prepared

was added to 10 ml of *E. coli* C600 suspension (McFarland = 4) in MH broth. After 2 hours of incubation at 37°C under agitation, the number of colony forming units (CFU) was determined by plating serial dilutions of the bacterial suspensions onto MH agar plates further incubated for 18 hours at 37°C. The experiment was repeated every 6 hours for 54 hours and included controls (without antibiotic).

#### **2.4 *In situ* biofilm formation and characterization of imipenem-resistant isolates**

Biofilms were formed on glass slides (75x26 mm) stuck on a rectangular granite slab placed at the bottom of the hospital effluent wastewater tank. The slides were disposed in the direction of the flow and were constantly submerged without any drying period for 15 days (09/04/2014 to 23/04/2014) to allow the development of substantial biofilm biomass. Each glass slide was collected in Falcon tubes filled with 50 mL of saline to rinse the biofilm and remove planktonic cells. Glass slides (n=32) were then scratched with a razor blade and sonicated for 5 min in a total volume of 50 mL saline to disrupt the biofilm (Ory et al., 2016).

Imipenem-resistant bacteria were isolated by spreading serial dilutions (from  $10^{-1}$  to  $10^{-8}$ ) of the biofilm suspension onto R2A-agar medium without and with imipenem at 8 µg/mL and incubated at 30 °C for up to 48 h in aerobic conditions. The resulting colonies were counted, isolated and identified by Gram staining and mass spectrometer analysis (Vitek MS, bioMérieux, La Balme, France) or 16S DNA sequencing with primers 27F-1492R (Lane, 1991). The percentage of imipenem-resistant isolates among the cultivable bacteria was determined by comparing the number of CFU/mL obtained after growth with and without antibiotic in the medium.

The imipenem MICs among the isolates were determined by the broth dilution method (4 to 256 µg/mL) in Mueller-Hinton medium (EUCAST, 2018).

The antibiotic resistance pattern of the isolates was determined by agar diffusion susceptibility testing (EUCAST Guidelines): ampicillin (Amp, 10 µg), ticarcillin (Tic, 75 µg), piperacillin + tazobactam (Ptz, 30/6 µg), Amoxicillin-clavulanic acid (Amc, 20-10 µg), cephalosporin (Czn, 30 µg), cefalexin (Cn, 30 µg), cefoxitin (Fox, 30 µg), cefotaxime (Cox, 5 µg), ceftazidime (Czd, 10 µg), cefuroxime (Cxn, 30 µg), cefixime (Fix, 5 µg), cefepime (Fep, 30 µg), mecillinam (Mec, 10 µg), ertapenem (Etp, 10 µg), aztreonam (Atm, 30 µg), temocillin (Tem, 30 µg), gentamicin (Gmn, 10 µg), netilmicin (Ntm, 10 µg), tobramycin (Tmn, 10 µg), amikacin (Amk, 30 µg), nalidixic acid

(Nal, 30 µg), norfloxacin (Nxn, 10 µg), ofloxacin (Ofx, 5 µg), ciprofloxacin (Cip, 5 µg), chloramphenicol (Chl, 30 µg), trimethoprim and sulfamethoxazole (Stx/Tmp, 25 µg), tetracycline (Tet, 30 µg), 200 µg), rifampicin (Rif, 30 µg) and colistin (Col, 10 µg). (Bio-Rad, Ivry-sur-Seine, France). The breakpoint values were interpreted according to the EUCAST guidelines (2018).

### 2.5 Detection and analysis of carbapenemase-encoding genes

All imipenem-resistant isolates were screened by simplex PCR for the presence of carbapenemase genes with primers specific for *bla*<sub>VIM</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>GES</sub>, *bla*<sub>KPC</sub> and *bla*<sub>NDM</sub> (Table 1). The PCR products were subjected to gel electrophoresis in 1% (w/v) agarose gel and sequenced by external Sanger sequencing services (GATC Biotech, Germany).

### 2.6 Transfer capacities of carbapenemase-encoding genes from effluent isolates

Plasmid DNA from biofilm isolates was extracted with the miniprep plasmid DNA method (Birnboim and Doly, 1979) and analyzed by electrophoresis in a 0.7% agarose gel. Purified plasmid DNA was subjected to electroporation into *E. coli* Top10 according to the manufacturer's instructions (Bio-Rad, Ivry-sur-Seine, France). Bacterial suspensions were plated onto LB agar plates containing 2 µg/ml imipenem. Resulting colonies (transformants) were checked by performing PCR with carbapenemase-specific primers and their phenotypical antibiotic resistance pattern determined by agar diffusion testing as described above.

Mating experiments were performed with *E. coli* C600 as a recipient strain, only resistant to rifampicin. Briefly, the imipenem resistant donor (wild-type effluent isolates or transformants) and recipient strains were grown in LB medium overnight at 37°C and then mixed (1:1 ratio) in 10 ml of LB fresh medium. After 2h of incubation at 37°C, transconjugants were selected by plating serial dilutions of the suspensions onto LB agar plates supplemented with imipenem and rifampicin (2 µg/ml and 75 µg/ml, respectively). The presence of carbapenemase-encoding genes in selected transconjugants was assessed by specific PCRs and by determination of the phenotypical antibiotic resistance pattern as described above. The conjugative characteristics were determined by calculating the capacity of transfer (ratio transconjugant/donor CFU/ml).

### 2.7 Plasmids analysis by S1-nuclease PFGE

The plasmid content from wild-type effluent strains, transformants, and transconjugants was analyzed by pulse field gel electrophoresis (PFGE) after S1 nuclease digestion (Barton et al., 1995). All PFGE reagents used were from Bio-Rad (CHEF Genomic DNA Plug Kits). Genomic DNA agarose blocks were digested with S1endonuclease (New England Biolab) for 90 min at 37°C, and then loaded into 1% electrophoretic agarose gel and run with the CHEF-DR II system (Bio-Rad), with a specific program 1-10s,18h, 6V/cm (voltage, pulse time, and electrophoretic time) at 14°C. The gels were then stained with Gel Star® (Lonza, Rockland, ME USA) and analyzed by FPQuest® software (BioRad, Marne la Coquette, France).

### 3 **Results**

#### 3.1 **Assessment of imipenem in the hospital effluent and determination of its *in vitro* stability**

The chemical and physical parameters of the hospital effluent were as follows : pH  $8 \pm 0.2$ ; temperature  $26.2 \pm 0.7$  °C; conductivity  $873.2 \pm 66.2$   $\mu\text{Sm}/\text{Cm}$  ; Total Organic Carbon  $216.8 \pm 47.5$  mg/L; Dissolved Organic Carbon  $193.6 \pm 42.4$  mg/L; Total Carbon  $229.7 \pm 40.9$  mg/L (Ory et al., 2016). Analysis of POCIS incubated in the hospital effluent for 15 days evidenced no imipenem. The *in-vitro* stability of imipenem at 26°C was determined by its biological activity, which indicated that its half-life was about 26h. No inhibitory effect was evidenced after 45h of storage of the antibiotic stock solution (Figures 1 and 2).

The amount of imipenem potentially released in the hospital wastewater was calculated (Table 2). The imipenem HQ was equal to  $0.046 \pm 0.003$  whereas the calculated HQr value was equal to 28.9, indicating a potential risk of antibiotic resistance selection (Table 3).

#### 3.2 **Characterization of biofilm imipenem-resistant isolates**

Culture of effluent biofilm suspensions on non-selective medium led to the isolation of  $2.85 \times 10^5$  CFU/mL. The addition of imipenem to the medium resulted in the selection of about  $6.3 \times 10^4$  CFU/mL, *i.e.* 22.1% of the cultivated bacteria. We selected 38 imipenem resistant isolates on the basis of colony characteristics (size, color, aspect). Their identification indicated that they belonged to 8 different bacterial genera, mostly within *Gamma-Proteobacteria* (Table 4). Members of the *Aeromonadaceae* family were the most prevalent (60.5%), followed by *Pseudomonaceae* (26.3%).

Antibiotic susceptibility testing indicated that half of the isolates had multiresistant phenotypes including five antibiotic classes, betalactams, fluoroquinolones, tetracyclin, aminoglycosides, and trimethoprim-sulfamethoxazole (Table 4). The MIC of imipenem was higher than 32 µg/ml for 89.5% of the isolates (n=34/38) and higher than 256 µg/ml for 23.7% (n=9/38). *Pseudomonas* spp. and *Stenotrophomonas* spp. isolates had the highest MICs (Table 4).

Detection of the carbapenemase-encoding genes by PCR and sequencing showed that 15 strains harbored at least one of the following carbapenemase-encoding genes, *bla*<sub>VIM</sub> (11), *bla*<sub>NDM</sub> (1) and *bla*<sub>OXA-48</sub> (2); three strains harbored *bla*<sub>GES</sub> encoding genes, one carbapenemase and two extended-spectrum beta-lactamase genes (Table 4).

### 3.3 Plasmid analysis of carbapenem-resistant isolates

Plasmid DNA was extracted from eight wild-type strains belonging to different genera (*Aeromonas* spp., *Stenotrophomonas* spp. and *Pseudomonas* spp.) harboring various carbapenemase-encoding genes (*bla*<sub>VIM</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>NDM</sub> and *bla*<sub>GES</sub>) and with imipenem MICs greater than 32 µg/ml. Transformants *E. coli* resistant to imipenem were obtained with each plasmid extract and up to four phenotypic profiles were observed for each set of transformants according to their resistance profiles (Table 4). The presence of *bla*<sub>VIM</sub> and *bla*<sub>NDM</sub> was evidenced in two transformants. Using the transformants harboring *bla*<sub>VIM</sub> as donor strains, mating experiments gave rise to imipenem-resistant transconjugants at a frequency of  $2.25 \times 10^{-5}$  per input donor. No transconjugant was obtained when transformants harboring *bla*<sub>NDM</sub> as donor strains were used despite multiple attempts.

Plasmid analysis by S1-nuclease PFGE evidenced at least two plasmids in only four out of the eight selected wild-type strains (Table 4). The wild-type *Aeromonas hydrophila/sobria* VIM-19 (GEMH7) harbored two plasmids, around 97kb and 194kb. Only the smaller of the two was evidenced in the respective imipenem resistant *E. coli* transformants in which, however, no carbapenemase-encoding gene was detected by PCR (Table 4). Two plasmids (molecular weight around 97kb and 200kb) were also evidenced in both the wild-type *Aeromonas hydrophila* VIM-19 (GEMH9) and its transformants and transconjugants, where *bla*<sub>VIM-19</sub> was detected by PCR (Table 4). The two other wild-type strains (GEMH52 and GEMH13) gave rise to transformants harboring plasmids but no carbapenemase encoding genes were detected (Table 4).

## Discussion

The sources of antibiotic resistance in the environment tend to be multifactorial. However, excessive human, veterinary, and agricultural use of antimicrobial products together with the low efficacy of sewage treatment are major causative factors. Antibiotics and their metabolites released into WWTP persist in treated effluents and contaminate surface waters (Korzeniewska et al., 2013; Osińska et al., 2016; White et al., 2016). One of the main environmental problems caused by hospital effluents is due to their discharge in urban sewerage systems without preliminary treatment of the high loads of pharmaceuticals and antibiotic resistant bacteria that they contain. However, the likelihood of active molecules being detected in the effluents depends on the compound's initial concentration, its stability and the sensitivity of testing methods. Beta-lactams are the most commonly prescribed antibiotics worldwide but they are unstable and few studies have addressed the presence of penicillin and cephalosporin in wastewater (Dinh et al., 2017; Gros et al., 2013; Nielsen et al., 2013; Szekeres et al., 2017). Carbapenems are beta-lactams mainly used in hospitals as the last line of defense against severe infections. To our knowledge only a few studies, performed in Romania and Belgium, have reported the presence of imipenem in hospital wastewater (Haller et al., 2018; Szekeres et al., 2017). In our study, we focused on imipenem because it is the carbapenem molecule most frequently used in the Clermont-Ferrand teaching hospital, with a PEC of  $3.51 \pm 0.24 \mu\text{g/L}$ . The detection limit of our analytical method was  $2 \mu\text{g/L}$  and therefore the passive sampling device used for collecting molecules over time should have allowed quantification of imipenem in the hospital wastewater samples. However, we were unable to detect the molecule in the hospital effluent even after 15 days of collection, owing probably to the high instability of the molecule in wastewater. We did not search for non-active metabolites. In addition, there could be a weak capacity of accumulation of imipenem in POCIS device. New analytical developments, more robust and sensitive need to be investigated and the real capacity of POCIS device to accumulate carbapenems needs to be assessed. Evaluation of the *in-vitro* biological effectiveness of an imipenem solution stored at  $26^\circ\text{C}$ , the average hospital effluent temperature, showed a rapid and complete loss of activity within a few hours (Figures 1-2). Imipenem is thus probably rapidly

degraded in non-active metabolites in the effluents because of the high temperature of the wastewater and its alkaline pH (pH8), which enhances beta-lactam degradation (Elragehy et al., 2008; Smith et al., 1990). However, the daily discharge of imipenem in the hospital effluent, despite its instability, could exert a continuous selective pressure.

Because it has rarely been detected in complex environments, imipenem has never been associated with risk. The environmental risk of toxic substances is usually assessed on the basis of the hazard quotient (HQ), which is the ratio between the PEC and the highest concentration of the substance without any harmful effect on the environment (PNEC). In our study, the effluent hospital HQ of imipenem was low (=0.046), suggesting no potential toxicity for the environment. However, the environmental risk assessment of resistance selection in microbial communities (HQ<sub>r</sub>) was greater than 1 (=28.9), indicating the capacity to select resistant species. The PNEC<sub>MIC</sub> widely used to calculate HQ<sub>r</sub> derives from the study of clinical strains and might not be strictly applicable to environmental species such as *Aeromonas* spp (Bengtsson-Palme and Larsson, 2016). It is noteworthy that sub-MICs of antibiotics can also exert selective pressure on local microorganisms and thus contribute to the emergence of resistance (Karkman et al., 2017; Murray et al., 2018). Hospital wastewaters contain a variety of antibiotics of variable lifetime (Le et al., 2016), which gives rise to hazardous mixtures in which imipenem could contribute to a synergic toxic effect.

In our study, 22.1% of the culturable cells from biofilms formed in the hospital effluent were resistant to imipenem. The most frequent species was *Aeromonas* spp, which is often intrinsically resistant to carbapenem owing to chromosome-encoded beta-lactamase production (Ko et al., 1998; Rasmussen and Bush, 1997; Rossolini and Walsh, Timothy amicosante, 1996; Stunt et al., 1998; Walsh, 2008). Carbapenem resistance-related impermeability mechanisms have also been described in such environmental strains (Yordanov and Strateva, 2009). However, we were able to detect carbapenemase-encoding genes such as *bla*<sub>OXA48</sub>, *bla*<sub>GES</sub>, *bla*<sub>NDM</sub> and *bla*<sub>VIM</sub> in the effluent biofilm isolates of this study, some of which were plasmid-encoded (*bla*<sub>VIM</sub> and *bla*<sub>NDM-6</sub>). The ability of plasmids harboring *bla*<sub>VIM</sub> to widely spread (Queenan and Bush, 2007; Tato et al., 2010) could explain the high frequency of *bla*<sub>VIM</sub> among the hospital effluent isolates. The carbapenemase-encoding genes *bla*<sub>NDM</sub>, *bla*<sub>VIM</sub> and *bla*<sub>KPC</sub> have already been detected in *Aeromonas* spp isolated in aquatic samples (Hughes et al., 2016; Libisch et al., 2008; Subirats et al., 2017; Walsh et al., 2011).

The high number of imipenem resistant strains detected in the effluent was not related to the rate of resistance observed in the local hospital setting. Indeed, very few clinical strains harboring carbapenemase were isolated in our hospital from 2012 to 2017. They harbored mainly *bla*<sub>OXA-48</sub> (1 strain in 2012, 1 in 2013, 2 in 2014, 1 in 2015, 3 in 2016, 1 in 2017) and more rarely *bla*<sub>NDM</sub> (1 strain in 2014, 1 in 2016 and 1 in 2017) and *bla*<sub>VIM</sub> (1 strain in 2016 and 1 in 2017) (data not shown). In addition, these strains belong mostly to *Escherichia coli* and *Klebsiella pneumoniae*, genera not detected in the hospital biofilm effluent. We cannot exclude the possibility that mobile genetic elements were exchanged between environmental and clinical strains despite our failure to obtain transconjugants *in vitro* mating assays using wild-type strains harboring carbapenemase-encoding conjugative plasmids as donors. It is noteworthy, however, that transconjugants were obtained when *E. coli* transformants harboring imipenem *bla*<sub>VIM</sub>-encoding plasmid were used as donors. Similar observations have been made by other workers (Kieffer et al., 2016; Ramoul et al., 2016), suggesting that conjugation events are promoted under specific conditions encountered *in situ* and that only the most environmentally adapted species such as *Aeromonas* would survive in the long term within the highly competitive community of biofilms. The bacterial biofilm content of hospital effluent would thus not mirror the ecology of circulating clinical strains in a low prevalence context of carbapenemase-encoding clinical strains such as that encountered in our hospital. However, persistent aquatic communities in the environment are potential crossroads for antibiotic multi-resistance strains. Indeed, analysis of the antibiotic resistance phenotype of the imipenem-resistant transformants obtained in this study showed that additional resistances had been co-transferred, indicating a co-localization of antibiotic resistance genes on plasmids, as reported elsewhere (Samuelsen et al., 2009; Xie et al., 2017).

In conclusion, while specific treatment of hospital wastewater is often recommended to limit the dissemination of anti-microbial resistance mechanisms, our study shows that these treatments should also target the biofilm cells aggregated on the pipe surfaces.

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ACCEPTED MANUSCRIPT

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**Table 1.** Oligonucleotides used in this study

	Primers' names	Sequence (5'–3')	Ref *	Hybridation (° C)	Expected product size (bp)
Carbapenemase- encoding gene	<i>bla</i> <sub>KPC</sub> KPC-F	ATG TCA CTG TAT CGC CGT CT	This work	54	881
	KPC-R	TTA CTG CCC GTT GAC GCC CA			
	<i>bla</i> <sub>NDM</sub> preNDM A	CAC CTC ATG TTT GAA TTC GCC	This work	60	832
		preNDM B			
	<i>bla</i> <sub>GES</sub> GES-F	GGA ATT CCA TAT GCG CTT CAT TCA CGC ACT A	This work	60	891
		GES-R			
	<i>bla</i> <sub>VIM-1</sub> VIM-1A NdeI	GGA ATT CCAB TAT GTT AAA AGT TAT TAG TAG T	Robin et al.,	60	829
		VIM-1B NotI			
	<i>bla</i> <sub>VIM-2</sub> VIM-F	GGA ATT CCA TAT GTT CAA ACT TTT GAG TAA G	This work	60	828
		VIM-R			
<i>bla</i> <sub>OXA-48</sub> OXA-F bis	GGG GAC GTT ATG CGT GTA TT	This work	60	780	
	OXA-R bis				GAG CAC TTC TTT TGT GAT GGC

\*For the design of new carbapenemase detection primers used in this work, sequences were downloaded from the GenBank database and aligned using the ClustalX software (version 2.0). The different primers were designed using the FastPCR software.

Robin, F., Aggoune-Khinache, N., Delmas, J., Naim, M., Bonnet, R., 2010. Novel VIM metallo- $\beta$ -lactamase variant from clinical isolates of *Enterobacteriaceae* from Algeria. *Antimicrob. Agents Chemother.* 54, 466–470. <https://doi.org/10.1128/AAC.00017-09>.

PCR cycles were as follows : 95°C 15s, then 30 cycles : 95°C 30s, hybridation temperature 1min30, 72°C 30s; final elongation 72 °C 10min

**Table 2.** Consumption of imipenem at the Gabriel Montpied hospital over the 2 week-analysis and pharmacokinetic parameters used to estimate the amount of imipenem potentially released in the hospital wastewater.

Molecule	Route of administration	Form	Quantity (mg)	Consumption	q= Quantity consumed ( $\mu\text{g}$ )	% dose urinary excretion (min-max)	% dose fecal excretion	Q = Quantity of unchanged drug eliminated by organism ( $\mu\text{g}$ )
Imipenem and cilastatin	Intravenous	Infusion solution	500	152	$7.6 \cdot 10^{+07}$	70-80%	<1%	$5.70^{+07} \pm 3.8^{+06}$

**Table 3.** Predicted Environmental Concentration (PEC), Predicted No-Effect Concentrations (PNEC), Predicted No-Effect Concentrations according to Minimum Inhibitory Concentration (MIC) and determination of the Hazard quotient (HQ) and Hazard Quotient resistance (HQr) for Imipenem (IMP). The PEC was calculated as previously described (Ory et al., 2016) and the PNEC and PNEC<sub>MIC</sub> were previously established (Bengtsson-Palme and Larsson, 2016).

		Values	Standard Deviation
Concentration ( $\mu\text{g/L}$ )	PEC	3.61	0.24
	PNEC	78	
	PNEC <sub>MIC</sub>	0.125	
HQ= PEC /PNEC		0.046	0.003
HQr= PEC/PNEC <sub>MIC</sub>		28.9	1.92

**Table 4.** Antibiotic resistance pattern of wild-type (WT) strains isolated from effluent biofilm and their *E. coli* transformants (T) on agar plate containing imipenem. The minimum inhibitory concentrations (MICs) of imipenem are indicated for each isolate. The antibiotic resistance pattern was determined by agar diffusion susceptibility testing after 18h of incubation at 37°C. The carbapenemase/extended-spectrum beta-lactamase (ESBL)-encoding genes detected by PCR and sequence analysis are indicated for each isolate: bla<sub>VIM</sub>, bla<sub>OXA-48</sub>, bla<sub>GES</sub> and bla<sub>NDM</sub>.

Bacterial species (name of strain)	Imipenem MIC g/L	Carbapenemase / ESBL encoding gene	Antibiotic resistance phenotype: wild-type (WT) and <i>E. coli</i> transformants (T)																								
			W	A	T	P	A	C	C	F	C	C	C	F	M	I	E	T	G	N	T	C	Tm	T			
<i>Aeromonas hydrophila/caviae</i> (GME H1)	32	bla <sub>VIM-19</sub>	W	m	i	t	m	z	C	n	F	C	C	C	F	M	I	E	T	G	N	T	C	Tm	T		
			p	c	z	c	n	n	x	x	d	n	x	c	p	p			m	n	m	n	h	p/S	e		
																							l	tx	t		
<i>Aeromonas hydrophila/sobria</i> (GME H7)	128	bla <sub>VIM-19</sub>	W	m	i	t	m	z	C	n	F	C	C	C	F	M	I	E	T				C	Tm	T		
			p	c	z	c	n	n	x	x	d	n	x	c	p	p			m	n	N	N	O	C	C	Tm	T
																							l	p/S	e		
																							h	tx	t		
			T	m	i	t	m		C	n	F	C	C	C	F	F	M	I	E	A	T	G		C	Tm	T	
			p	c	z	c			n	x	x	d	n	x	p	c	p	p	m	m	n	n		h	p/S	e	
																							l	tx	t		
			T	m	i	t	m		C	n	F	C	C	C	F	M	I			T						T	
			p	c	z	c			n	x	x	d	n	x	c	p				m						e	
																										t	
			T	m	i	t	m		C	n	F	C	C	C	F	M	I	E	A	T						Tm	
			p	c	z	c			n	x	x	d	n	x	c	p	p	m	m						p/S	tx	

		T <sub>4</sub>	A m p	T i c	P t z	A m z	C n	F o x	C o x	C z d	C x n	F i x	M e c	I m p	T e m	G e m	N t m	T m n	C h l	Tm p/S tx	T e t								
<b><i>Aeromonas hydrophila/caviae</i></b> (GME H8)	32	W T	A m p	T i c	P t z		C n	F o x	C o x	C z d	C x n	F i x	M e c	I m p	E e p	A t p	T t m												
<b><i>Aeromonas hydrophila</i></b> (GME H9)	32	<i>bla</i> <sub>VIM-19</sub> W T	A m p	T i c	P t z	A m z	C n	F o x	C o x	C z d	C x n	F i x	M e c	I m p	E e p	A t p	T t m	T m n	N a l	C i p	Tm p/S tx	T e t							
		T <sub>1</sub>	A m p	T i c		A m c							M e c	I m p	E e p	A t p	T t m	G m n		C h l	Tm p/S tx	T e t							
		<i>bla</i> <sub>VIM-19</sub> T <sub>2</sub>	A m p	T i c	P t z	A m z	C n	F o x	C o x	C z d	C x n	F i x	M e c	I m p															
<b><i>Aeromonas hydrophila/sobria</i></b> (GME H10)	64	<i>bla</i> <sub>NDM-6</sub> W T	A m p	T i c	P t z	A m z	C n	F o x	C o x	C z d	C x n	F i x	M e c	I m p	E e p	A t p	T t m	G m n	N m n	T m n	A m k	N x p	O f i	C p/S tx	Tm p/S tx	T e t	C o l	R i f	
		T <sub>1</sub>	A m p	T i c		A m c							M e c	I m p	E e p	A t p	T t m												
		T <sub>2</sub>	A m p	T i c	P t z	A m z	C n	F o x	C o x	C z d	C x n	F i x	M e c	I m p		A t p	T t m												
		<i>bla</i> <sub>NDM-6</sub> T <sub>3</sub>	A m p	T i c	P t z	A m z	C n	F o x	C o x	C z d	C x n	F i x				T e m	G e m	N t m	T m n	A m k									



<i>ria</i> (GME H25)																										
<i>Aeromonas hydrophila</i> (GME H31)	256	<i>bla<sub>OXA-48</sub></i>	W T					C z n	C n	F o x	C o x	C z d	C x n	F i x	F e p	M e c	I e p	E m p		T e m						
<i>Aeromonas hydrophila</i> (GME H32)	64	<i>bla<sub>GES</sub></i>	W T	A m p	T i c	P t z	A m c	C z n	C n	F o x	C o x	C z d	C x n	F i x	M e c	I e p	E m p	A t m	T e n	G e m	T m n	N a l	C i p	Tm p/S tx	C o l	
<i>Aeromonas hydrophila/caviae</i> (GME H34)	256		W T	A m p	T i c			C z n	C n	F o x	C o x		C x n	F i x	M e c	I e p	E m p	A t m	T e n			N x n	O f x	C i p	T e t	C o l
<i>Aeromonas hydrophila/caviae</i> (GME H35)	256		W T			A m c			C n		C o x	C z d		F i x	M e c	I e p	E m p	A t m	T e n			N x n			Tm p/S tx	
<i>Aeromonas hydrophila/caviae</i> (GME H36)	4		W T	A m p		P t z		C z n	C n	F o x	C o x		C x n	F i x	M e c	I e p	E m p	A t m	T e n			N t m			C i p	



H50)

<i>Aeromonas hydrophila/caviae</i> (GME H51)	128	W	A	T	P	A	C	C	F	C	C	C	F	F	M	I	E	A	T	T	N	O	C	C	Tm	T						
			m	i	t	m	z	n	n	x	x	d	n	x	p	c	p	p	m	m	m	n	x	f	i	h	p/S	e				
			p	c	z	c																	n	x	p	l	tx	t				
		T <sub>1</sub>	A	T	P	A		C	F	C	C	C	F	F	M	I	E	A	T								T					
			m	i	t	m		n	n	x	x	d	n	x	p	c	p	p	m	m								e				
			p	c	z	c																						t				
		T <sub>2</sub>	A	T		A									M	I	E	A	T													
			m	i		m										e	m	t	t	e												
			p	c		c										c	p	p	m	m												
<i>Aeromonas hydrophila/caviae</i> (GME H52)	256	<i>bla<sub>GES-2</sub></i>	W	A	T	P	A	C	C	F	C	C	C	F	F	M	I	E	A	T	T	N	O	C	C	Tm	T	C				
				m	i	t	m	z	n	n	x	x	d	n	x	p	c	p	p	m	m									e	o	
				p	c	z	c																	n	x	p	l	tx	t	l		
		T <sub>1</sub>		T		A			F		C	C	F	F	M			A										T				
				i		m			x		o		z	x	i	e	e		t										e			
				c		c							d	n	x	p	c		m										t			
<i>Pseudomonas indologydans</i> (GME H2)	64	<i>bla<sub>VIM-44</sub></i>	W	A	T		A	C	C	F	C	C	C	F		M	I	E		T	G	N	T	A	N	O	C	C	Tm	T		
				m	i		m	z	n	n	x	x	d	n	x			e	m	t		e	m	t	m	m	a	f	i	h	p/S	e
				p	c		c											c	p	p		m	n	m	n	k	l	x	p	l	tx	t
		T <sub>1</sub>	A	T	P	A		C	F	C	C	C	F	F	M	I	E	A	T										T			
			m	i	t	m		n	n	x	x	d	n	x	p	c	p	p	m	m										e		
			p	c	z	c																							t			
		T <sub>2</sub>	A	T	P	A		C	F	C	C	C	F	F	M	I	E	A	T													
			m	i	t	m		n	n	x	x	d	n	x	p	c	p	p	m	m												
			p	c	z	c																										
<i>Pseudomonas oleovorans</i> (GME	64	<i>bla<sub>VIM-44</sub></i>	W	A	T	P	A	C	C	F	C	C	C	F		M	I	E		T	G	N	T		N	N	O	C	C	Tm		
				m	i	t	m	z	n	n	x	x	d	n	x			e	m	t		e	m	t	m	a	x	f	i	h	p/S	
				p	c	z	c											c	p	p		m	n	m	n	l	n	x	p	l	tx	

H12)

<i>Pseudo monas oleovor ans / mendoc ina</i> (GME H13)	128	<i>bla<sub>VIM</sub>- 30</i>	W	A	T	A	C	C	F	C	C	C	F	M	I	E	T	G	N	T	N	C	C	Tm	T		
			T	m	i	m	z	n	n	x	x	d	n	x	c	p	p	e	m	t	m	a	i	h	p/S	e	
			p	c	c	n	n	x	x	d	n	x	c	p	p	m	n	m	n	l	p	l	tx	t			
			T	A	T	P	A		C	F	C	C	C	F	F	M	I	E	A	T				T			
			1	m	i	t	m		n	x	x	d	n	x	p	c	p	p	m	m					e		
				p	c	z	c																	t			
			T	A	T		A		C	F	C	C	C	F	F	M	I	E	A	T							
			2	m	i		m		n	x	x	d	n	x	p	c	p	p	m	m							
				p	c		c																				
<i>Pseudo monas pseudo alcalige nes</i> (GME H18)	128	<i>bla<sub>VIM</sub>- 41</i>	W	A	T	P	A	C	C	F	C	C	C	F	M	I	E	T		N	T		O	C	Tm		
			T	m	i	t	m	z	n	n	x	x	d	n	x	c	p	p	e	t	m		f	i	p/S		
				p	c	z	c	n	n	x	x	d	n	x	c	p	p	m	m	n		x	p	tx			
<i>Pseudo monas oleovor ans</i> (GME H19)	128	<i>bla<sub>VIM</sub>- 17</i>	W	A	T		A	C	C	F	C	C	C	F	M	I	E	T	G	N	T	N	O	C	Tm		
			T	m	i		m	z	n	n	x	x	d	n	x	c	p	p	e	m	t	m	a	f	i	p/S	
				p	c		c	n	n	x	x	d	n	x	c	p	p	m	n	m	n	l	x	p	tx		
<i>Pseudo monas stutzeri</i> (GME H26)	256		W	A	T	P	A	C	C	F		C	C	F	M	I	E	A	T		T	A		C	Tm	T	
			T	m	i	t	m	z	n	n	x		d	n	x	c	p	p	m	m		n	k		i	p/S	T
				p	c	z	c	n	n	x		d	n	x	c	p	p	m	m		n	k		p	tx	t	l
<i>Pseudo monas spp.</i> (GME	256		W	A	T	P	A	C	C	F	C		C	F	M	I	E	A	T	G	T		N	C	Tm	T	
			T	m	i	t	m	z	n	n	x		x	n	x	c	p	p	m	m	n	n		x	i	p/S	e
				p	c	z	c	n	n	x		x	n	x	c	p	p	m	m	n	n		n	p	tx	t	

H45)

<i>Pseudo monas oleovorans</i> (GME H41)	128		W T	A m p	T i c	P z	A m z	C n	C <sup>n</sup>	F o x	C o x	C z d	C x n	F i x	F e p	M e p	I e p	E m p	A t m	T e m	G m n	T m n	N l	N n	O x	C p	C l	Tm p/S tx	T e t	C o l
<i>Pseudo monas oleovorans</i> (GME H42)	64		W T	A m p	T i c		A m c	C n	C <sup>n</sup>	F o x	C o x	C z d	C x n	F i x		M e c	I e p	E m p	T e m	G m n	N m	T m	A m	N k	N l	O n	C p	C l	Tm p/S tx	T e t
<i>Pseudo monas oleovorans</i> (GME H44)	128	<i>blav<sub>VM</sub></i>	W T	A m p	T i c	P z		C n	C <sup>n</sup>	F o x	C o x	C z d	C x n	F i x	F e p	M e p	I e p	E m p	T e m	G m n	N m	T m	N l		O x	C p		Tm p/S tx		
<i>Stenotr ophom onas maltop hilia</i> (GME H14)	128		W T	A m p	T i c	P z	A m z	C n	C <sup>n</sup>	F o x	C o x	C z d	C x n	F i x		I m p	E m p		T e m	G m n	N m	T m	N l			C p		Tm p/S tx		
<i>Stenotr ophom onas maltop hilia</i> (GME H33)	128		W T	A m p	T i c	P z	A m z	C n	C <sup>n</sup>	F o x	C o x	C z d	C x n	F i x	F e p	M e p	I e p	E m p	A t m	T e m	G m n	T m n	A n	N k	O l	C p	C l	Tm p/S tx	T e t	C o l

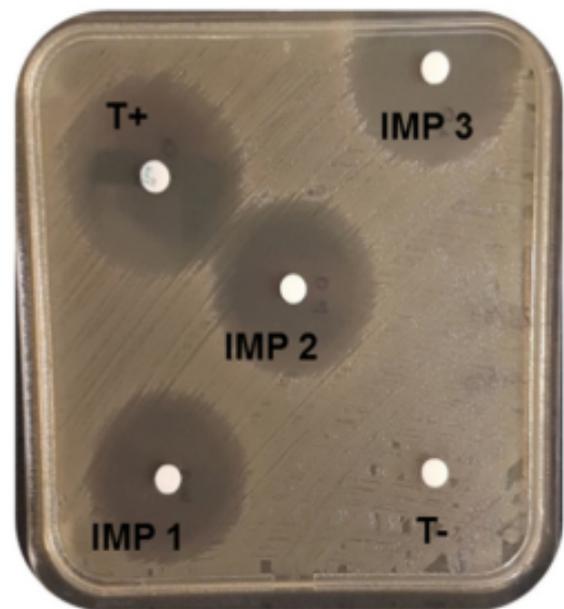
<i>Stenotrophomonas maltophilia</i> (GME H47)	256	<i>bla</i> <sub>OXA-48</sub>	W	A	T	P	A	C	C	C	C	F	F	I	E	A	T	G	T	N	N	O	C	C	Tm	T	C				
			T	m	i	t	m	z	C <sub>n</sub>	o	z	x	i	e	m	t	t	e	m	m	m	a	x	f	i	h	p/S	e	o		
				p	c	z	c	n	x	d	n	x	p	p	p	m	m	n	n	l	n	x	p	l	tx	t	l				
			T	A	T		A							M	I	E	A	T													
			l	m	i		m							e	m	t	t	e													
				p	c		c							c	p	p	m	m													
<i>Stenotrophomonas maltophilia</i> (GME H48)	256		W	A	T		A	C	F	C	C	C	F	F	M	I	E	A	T	G						C					
			T	m	i		m	z	o	o	z	x	i	e	e	m	t	t	e	m						i					
				p	c		c	n	x	x	d	n	x	p	c	p	p	m	m	n						p					
<i>Acinetobacter baumannii</i> complex (GME H28)	256		W	A	T	P	A	C	F	C	C	C	F	F	M	I	E	A	T	G	N	T	A	N	N	O	C	C	Tm	T	C
			T	m	i	t	m	z	o	o	z	x	i	e	e	m	t	t	e	m	t	m	m	a	x	f	i	h	p/S	e	o
				p	c	z	c	n	x	x	d	n	x	p	c	p	p	m	m	n	m	n	k	l	n	x	p	l	tx	t	l

Ampicillin (Amp, 10 µg), ticarcillin (Tic, 75 µg), piperacillin +tazobactam (Ptz, 30/6 µg), Amoxicillin-clavulanic acid (Amc, 20-10 µg), cephazolin (Czn, 30 µg), cefalexin (Cn, 30 µg), cefoxitin (Fox, 30 µg), cefotaxime (Cox, 5 µg), ceftazidime (Czd, 10 µg), cefuroxime (Cxn, 30 µg), cefixime (Fix, 5 µg), cefepime (Fep, 30 µg), mecillinam (Mec, 10 µg), imipenem (Imp, 10 µg), ertapenem (Etp, 10 µg), aztreonam (Atm, 30 µg), temocillin (Tem, 30 µg), gentamicin (Gmn, 10 µg), netilmicin (Ntm, 10 µg), tobramycin (Tmn, 10 µg), amikacin (Amk, 30 µg), nalidixic acid (Nal, 30 µg), norfloxacin (Nxn, 10 µg), ofloxacin (Ofx, 5 µg), ciprofloxacin (Cip, 5 µg), chloramphenicol (Chl, 30 µg), trimethoprim and sulfamethoxazole (Stx/Tmp, 25 µg), tetracycline (Tet, 30 µg), rifampicin (Rif, 30 µg) and colistin (Col, 10 µg) (Bio-Rad, Ivry-sur-Seine, France)

**Figure 1.** Determination of the stability of imipenem using the agar (Müller Hinton, MH) diffusion test and the imipenem-susceptible strain *Escherichia coli* K12 C600. Agar diffusion tests were performed in triplicate (IMP1, IMP2 and IMP3) by loading imipenem (10µg) stored at 26°C for different lengths of time (T=0h, T=30h and T=48h) on paper discs. The negative (T-) and positive (T+) controls consisted of paper discs impregnated with saline and commercial imipenem disks (10µg), respectively.

**Figure 2.** Percentage of imipenem activity determined by measurement of its bactericidal effect on *E.coli* after different storage times at 26°C (● agar diffusion test and ▲ broth medium test)

**T0h**



**T30h**



**T48h**

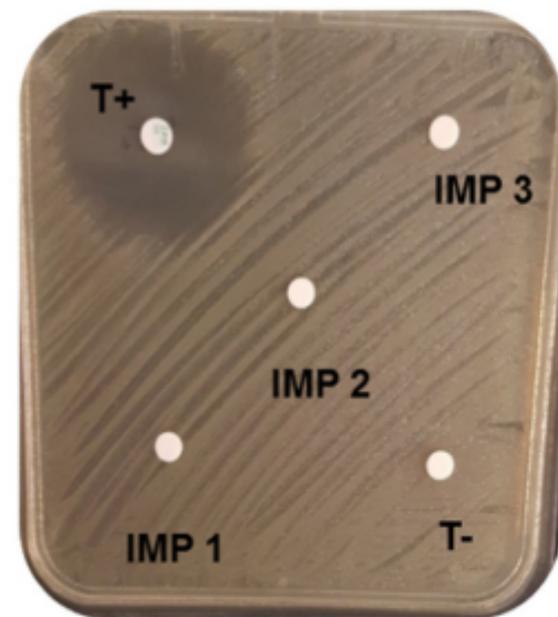


Figure 1

