Bacteria isolated from hospital, municipal and slaughterhouse wastewaters show characteristic, different resistance profiles

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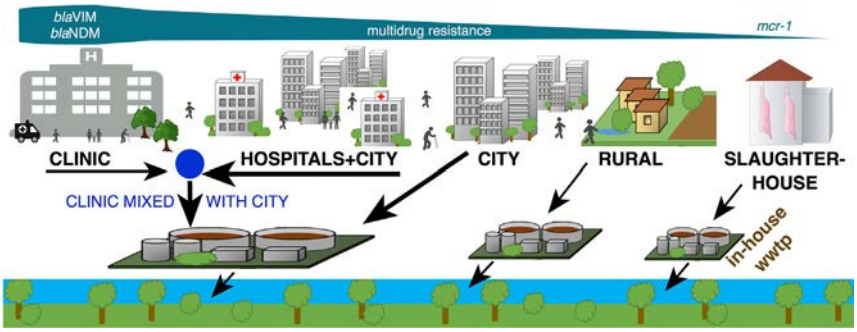
HIGHLIGHTS

- The study compares bacteria isolated from different wastewater biotopes.
- The highest health risks were posed by resistant bacteria from clinical waste water.
- These bacteria were frequently resistant to antibiotics of last resort.
- Hospital wastewater signature was still observed at entry into the treatment plant.
- Bacteria harboring *mcr 1* were isolated only from slaughter house wastewater.

GRAPHICAL ABSTRACT



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ABSTRACT

Multidrug resistant bacteria cause difficult to treat infections and pose a risk for modern medicine. Sources of multidrug resistant bacteria include hospital, municipal and slaughterhouse wastewaters. In this study, bacteria with resistance to 3rd generation cephalosporins were isolated from all three wastewater biotopes, including a maximum care hospital, municipal wastewaters collected separately from a city and small rural towns and the wastewaters of two pig and two poultry slaughterhouses. The resistance profiles of all isolates against clinically relevant antibiotics (including β lactams like carbapenems, the quinolone ciprofloxacin, colistin, and trimetho prim/sulfamethoxazole) were determined at the same laboratory. The bacteria were classified according to their risk to human health using clinical criteria, with an emphasis on producers of carbapenemases, since carba penems are prescribed for hospitalized patients with infections with multi drug resistant bacteria. The results showed that bacteria that pose the highest risk, i. e., bacteria resistant to all β lactams including carbapenems and ciprofloxacin, were mainly disseminated by hospitals and were present only in low amounts in municipal

Abbreviations: cplx, complex; ESBL, extended spectrum beta-lactamase; 3/4MDRO, multidrug-resistant organism, displaying resistance against 3 (3MDRO) or 4 (4MDRO) clinically important antibiotics; MLST, multilocus sequence typing; MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant enterococci; ww, wastewater (figures only); WWTP, wastewater treatment plant.

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Wastewater
Clinic
Slaughterhouses

wastewater. The isolates from hospital wastewater also showed the highest rates of resistance against antibiotics used for treatment of carbapenemase producers and some isolates were susceptible to only one antibiotic substance. In accordance with these results, qPCR of resistance genes showed that 90% of the daily load of carbapenemase genes entering the municipal wastewater treatment plant was supplied by the clinically influenced wastewater, which constituted approximately 6% of the wastewater at this sampling point. Likewise, the signature of the clinical wastewater was still visible in the resistance profiles of the bacteria isolated at the entry into the wastewater treatment plant. Carbapenemase producers were not detected in slaughterhouse wastewater, but strains harboring the colistin resistance gene *mcr* 1 could be isolated. Resistances against orally available antibiotics like ciprofloxacin and trimethoprim/sulfamethoxazole were widespread in strains from all three wastewaters.

1. Introduction

Infections with antibiotic resistant bacteria may be difficult to treat and have demanded an estimated number of 33,000 deaths in 2015 in the EU. The largest effect on deaths could be attributed to 3rd generation cephalosporin resistant *Escherichia coli*, methicillin resistant *Staphylococcus aureus* (MRSA), 3rd generation cephalosporin resistant *Klebsiella pneumoniae* and carbapenem resistant *Pseudomonas aeruginosa* (Cassini et al., 2019).

ESBL (extended spectrum β lactamases, that inactivate 3rd generation cephalosporins and penicillins) producing enterobacteria have spread around the globe since the turn of the century (Woerther et al., 2013) and colonize about 3.5 6.8% of the population in Germany (Idelevich et al., 2016). ESBL producers have been isolated from European wastewaters and wastewater treatment plants (WWTPs), e. g., in Ireland and England (Raven et al., 2019; Smyth et al., 2020), surface waters in Sweden, Norway, and Croatia (Egervarn et al., 2017; Jørgensen et al., 2017; Maravić et al., 2015), wildlife in France (Ngaiganam et al., 2019), and on European as well as imported meat products in Germany and the Netherlands (Evers et al., 2017; A. Müller et al., 2018). In short, ESBL seem to have achieved a ubiquitous dissemination. Severe infections with ESBL producers are treated with carbapenems, piperacillin/tazobactam or ciprofloxacin (Pana and Zaoutis, 2018). However, frequent use of these antibiotics led to the selection of strains resistant to these substances as well (Qu et al., 2019; Yang et al., 2020). These multidrug resistant bacteria often harbor large plasmids that combine several resistance genes and leave few options for choice of antibacterials (Partridge et al., 2018).

In order to identify key antibiotics that are vital for human medicine and the use of which should be monitored, the WHO has graded antibiotics into three classes (WHO, 2019a). The "access" group includes antibiotics that are active against common susceptible pathogens and show low potential for resistance development. Sulfamethoxazole/trimethoprim, many penicillins, tetracyclines, clindamycin and amikacin belong to this group. In contrast, the "watch" group contains the highest priority antibiotics with a higher potential for selection of resistant strains. The 3rd generation cephalosporins ceftazidime and cefotaxime, piperacillin/tazobactam, ciprofloxacin, the carbapenems and vancomycin belong to this group and are also listed as "Critically Important Antimicrobials for Human Medicine" (WHO, 2019b). The "reserve" group of antibiotics includes "antibiotics of last resort" that should be only given to patients that are infected with multidrug resistant organisms, especially multi drug resistant producers of carbapenemases. Colistin, ceftazidime/avibactam, ceftozolane/tazobactam, intravenous fosfomycin, tigecyline and linezolid belong to this group. These antibiotics should "be protected and priorized as key targets of stewardship programs to preserve their effectiveness" (WHO, 2019a). Ceftozolane/tazobactam and ceftazidime/avibactam have been introduced into clinical medicine in the EU in 2015, only shortly before the study started (EMA, 2015a, 2015b). Tigecycline was approved in 2007 by the EU (EMA, 2007). Amikacin, an aminoglycoside antibiotic, is employed in the hospital as part of a combination therapy and is not available in an oral formulation. Amikacin is the aminoglycoside at least prone to acquired resistance (Ramirez and Tolmasky, 2010). Ciprofloxacin and sulfamethoxazole/trimethoprim belong to the orally available antibiotics and are also prescribed to patients that are not hospitalized, and especially sulfamethoxazole/trimethoprim is frequently used. The oral formulation of fosfomycin is grouped with the "watch" group (WHO, 2019a) and is used in the outpatient setting as a single shot oral treatment of uncomplicated urinary tract infections in women (Gardiner et al., 2019).

Possible sources for the dissemination of antibiotic resistant bacteria are areas where antibiotics are frequently used, such as hospitals and animal breeding, but also colonized members of the community. Especially the use of antibiotics in veterinary medicine and animal production has been much discussed as a major source for antibiotic resistant bacteria (Manyi Loh et al., 2018).

Colistin, cephalosporins, sulfamethoxazole, sulfadiazine, oxacillin and enrofloxacin (which is metabolized to ciprofloxacin in the animal (Cester and Toutain, 1997)) are used in food production in Germany. Especially the use of colistin in food production has a long tradition (Catry et al., 2015) and it was only revived for human treatment when carbapenemase producers started to appear (Li et al., 2006). In contrast, other antibiotics of the "reserve" or the "watch" group are only employed in clinical medicine, e. g., carbapenems, piperacillin/tazobactam, intravenous fosfomycin, tigecycline, the new beta lactam inhibitor combinations (ceftozolane/tazobactam and ceftazidime/avibactam), linezolid, and vancomycin (AG Antibiotikaresistenz am BVL, 2018).

In order to develop criteria that allow hospitals to identify the most critical multidrug resistant strains that cause difficult to treat infections and, therefore, require hygienic precautions, the German Commission for Hospital Hygiene and Infection Prevention (KRINKO) has developed criteria that grade Gram negative bacteria according to their multidrug resistance (KRINKO, 2019) into the categories 3MDRO and 4MDRO (multidrug resistant organism with resistance to three/four clinically important antibiotics). This definition considers clinically relevant antibiotics of the watch group that are normally used against Gram negative rods causing severe life threatening infections (Table 1). The colonization of a patient with a 3MDRO bacterium will require isolation when this patient is located in a ward that houses especially vulnerable immuno compromised patients. Carbapenems are the gold standard for treatment of systemic infections with 3MDRO Enterobacteriaceae. Colonization with a carbapenem resistant 4MDRO requires isolation of the patient and leaves the antibiotics of last resort mentioned above as choice for therapy.

Patients that are colonized with MRSA are isolated as well and infections are treated with vancomycin. In contrast, vancomycin resistant enterococci (VRE) have become endemic in many regions, thus, the necessity of contact isolation of patients that harbor VRE is still being controversially discussed (Vehreschild et al., 2019). These infections are treated with linezolid.

Multidrug resistant bacteria and especially 4MDRO compromise medical procedures (operations, transplantations, chemotherapy) that

Table 1
Definition of 3MDRO and 4MDRO organisms displaying resistance against 3 (3MDRO) or 4 (4MDRO) clinically important antibiotics using either piperacillin or piperacillin/tazobactam for the evaluation:

	3MDRO_Pip[a] (KRINKO, 2019)	3MDRO_Pip/Taz[a] (Baum et al., 2011)	4MDRO[a]
Enterobacterales, A. calcoaceticus-baumannii cplx.			
Acylureidopenicillins	R: piperacillin S: piperacillin/tazobactam	R: piperacillin/tazobactam	R
Ceftazidime and/or cefotaxime	R	R	R
Imipenem and/or meropenem	S	S	R
Ciprofloxacin	R	R	R
P. aeruginosa			
Piperacillin/tazobactam	R: piperacillin, S: piperacillin/tazobactam	R: piperacillin/tazobactam	R
Ceftazidime	R	R	R
Imipenem and/or meropenem	S	One of these two categories still active and no carbapenemase	R
Ciprofloxacin	R		R

[a] The presence of a carbapenemase gene will confer a 4MDRO status to Enterobacteriaceae even if the resistance to carbapenems is not detected in the antibiograms.

have to rely on the availability of effective antibiotics and, therefore, pose the highest risk for healthcare. This study compares the resistance profiles of isolates that were collected simultaneously in a large study between 2016 and 2018 from three different wastewater biotopes, including hospital, two different municipal and pig and poultry slaughterhouse wastewaters. The study aimed to isolate resistant Enterobacterales, *P. aeruginosa*, *A. baumannii*, MRSA and VRE. All isolated strains were analyzed at the same laboratory with the same methods.

Preliminary data about the 3MDRO and 4MDRO status of the Gram negative samples of the clinical system and the wastewater of rural towns, after one year of sampling, have already been published and identified hospitals as the main source of multidrug resistant bacteria (H. Müller et al., 2018). In this study, our aim was to compare the com plete resistance profiles of the Gram negative and Gram positive iso lates from clinical and municipal wastewater to samples obtained from process waters and wastewater of poultry (Savin et al., 2020b) and pig slaughterhouses (Savin et al., 2020b) with emphasis on recently introduced antibiotics and antibiotics of last resort. These data are com pared to the concentrations of resistance genes present in the clinical wastewater samples. Former studies have already shown that antibiotic resistant bacteria are released from hospitals in many countries (Galvin et al., 2010; Jakobsen et al., 2008; Korzeniewska and Harnisz, 2013; Kwak et al., 2015), however, so far, some antibiotics of last resort like ceftazidime/avibactam or ceftozolane/tazobactam have not been ad dressed. In addition our results demonstrate that in our systems the dif ferences between slaughterhouse wastewater, clinically influenced, and municipal wastewater are unusually distinct.

2. Materials and methods

2.1. Sampling sites and methods

Samples were taken from the wastewater of a maximum care hospi tal, starting with the building that houses a hemato oncological clinic (designated "ward" in the qPCR measurements) and the part of the hos pital where this building is located, these two sampling sites are sub sumed under the term "building". The hemato oncological clinic has the highest antibiotic consumption in the maximum care hospital. The study then sampled the mixed wastewater of the whole maximum care hospital (1274 beds) including administrative buildings ("clinic") and followed the wastewater to the influent of the local WWTP, where the clinical wastewater has been diluted with municipal waste water ("clinic mixed with city"). However, in this part of the city, addi tional hospitals (comprising 2136 beds) contribute to the wastewater. Calculated from the number of beds, the yearly amount of wastewater produced by the maximum care hospital (225×1000 m^3) and the total yearly influent into the WWTP ($10{,}036 \times 1000$ m^3) through this pipe, the total amount of clinical wastewater constitutes about 6% of the wastewater (603×1000 m^3; dilution 1:16.6) at the sampling point "clinic mixed with city".

Municipal wastewater samples were gained from the wastewater influent of another city district without any hospitals ("city") and from WWTP influents of three small locally separate rural towns ("rural towns") without any hospitals. In this part of the country, crop production is the main agricultural sector (constituting about 42% of land use within the rural catchment area (Schreiber et al., 2017)). All sites were visited between 18 and 22 times during all seasons. For slaughterhouse wastewater, the waste and process waters of two pig and two poultry slaughterhouses, the influent and effluents of the slaughterhouse in house WWTPs and a poultry farm were sampled in another part of Germany ("ww pig" and "ww poultry") (ww: waste water). These slaughterhouses process animals from local farms that are all located within 100 150 km of distance. Each slaughterhouse was visited at least five times.

Samples from the urban and rural WWTP influents were obtained as automated 24 h mixed samples. The samples from the sewer system of the hospital were taken as qualified samples according to the German standard method for the examination of water, wastewater and sludge (DIN 38402 11:2009 02), i. e., five aliquots of the same volume were obtained every 2 min and mixed afterwards.

2.2. Overview of analyses

All samples were analyzed for growth of antibiotic resistant bac teria belonging to Enterobacterales, *P. aeruginosa*, and *Acinetobacter calcoaceticus baumannii* clpx., MRSA, and VRE, which were isolated, identified and tested for resistance phenotypes. Resistance genes in carbapenem and colistin resistant isolates were determined by qPCR of isolated strains. Wastewater samples of the clinical system were analyzed for selected resistance genes by qPCR.

2.2.1. Isolation of strains

All samples were cooled and processed within 24 h. Samples were analyzed as previously described (H. Müller et al., 2018; Savin et al., 2020a; Savin et al., 2020b). In brief, depending on the bacterial density, samples were spread on ESBL, VRE, and MRSA CHROMagar plates (MAST Diagnostica GmbH, Germany) agar plates (1 ml), or concen trated by filtration using 10 or 100 ml samples. Highly concentrated wastewater samples had to be plated in different dilutions (1:10, 1:100, and 1:1000) to enable picking of single colonies. The plates were incubated at an elevated temperature (42 °C) to exclude growth of environmental bacteria. Colonies that showed the appearance of one of the target species (members of the Enterobacterales, *P. aeruginosa*,

the *A. calcoaceticus baumannii* clpx., *E. faecium* as well as *S. aureus*) underwent purification by subculturing on blood agar (H. Müller et al., 2018). Wastewater and process water from the animal production was additionally screened for carbapenemase producers and strains harboring *mcr* genes using enrichment broths and dedicated agar plates (SuperPolymyxin medium) as described previously (Nordmann et al., 2016; Savin et al., 2020b).

2.2.2. Identification of isolates

Selection of target ESBL producing Gram negative bacteria species was carried out on specific agar plates (Chromocult Coliform agar, cetrimide agar and *Acinetobacter* CHROMagar plates) and using clinical routine procedures as oxidase and/or catalase testing, depending on the species. Suspected VRE were sub cultured onto bile esculin agar and MRSA isolates were confirmed as *S. aureus* by coagulase testing. About 6 20 colonies per sample were picked and identified by MALDI TOF MS employing a VITEK® mass spectrometer (bioMèrieux, Marcy l'Etoile, France), VITEK® MS CHCA matrix (# 411071), dispos able targets (# 410893), and the Myla™ software (H. Müller et al., 2018).

2.2.3. Antimicrobial susceptibility testing (AST)

AST was performed by a commercial "dried" broth microdilution (Micronaut S MDR MRGN Screening 3 system (MERLIN, Gesellschaft für mikrobiologische Diagnostika GmbH, Bornheim Hersel, Germany)) for Gram negative samples. This panel tests for resistance against temocillin, piperacillin, piperacillin/tazobactam, cefotaxime, ceftazidime, imipenem, meropenem, amikacin, tigecycline, chloramphenicol, fosfomycin, tri methoprim/sulfamethoxazole, ciprofloxacin, levofloxacin, and colistin, and was used according to the directions of the manufacturers, employing Müller Hinton broth for rehydration of the antibiotics. AST of Gram positive bacteria was performed using the MRSA/GP system (MERLIN), which includes oxacillin, teicoplanin, penicillin G, fusidic acid, linezolid, moxifloxacin, clindamycin, daptomycin, erythromycin, erythromycin/ clindamycin, cefoxitin, ceftaroline, vancomycin, rifampicin, synercid (quinupristin/dalfopristin), and gentamicin.

2.2.4. Typing

Determination of sequence types of Gram negative bacteria was performed by MLST typing for *K. pneumoniae* (Diancourt et al., 2005) or DLST (Basset and Blanc, 2014) and MLST (Curran et al., 2004) for *P. aeruginosa* using the procedures and primers indicated on the websites (http://bigsdb.web.pasteur.fr/klebsiella/, https://pubmlst.org/ paeruginosa/, http://www.dlst.org). DNA was prepared as previously described (H. Müller et al., 2018). *S. aureus* was typed using the *spa* typ ing method (Harmsen et al., 2003) and evaluated with the Ridom StaphType program https://spa.ridom.de/index.shtml (Josten et al., 2013).

2.2.5. Evaluation of resistance profiles

In order to analyze the composition of the process waters and waste water from slaughterhouses, all samples taken at the connected munic ipal WWTP or swabs taken from animals were excluded from the analysis. Gram negative multidrug resistance profiles were evaluated for Enterobacterales, *P. aeruginosa*, and isolates of the *A. calcoaceticus baumannii* cplx. Other non fermenting bacteria like *Burkholderia*, *Elizabethkingia*, and *Stenotrophomonas* (total of 6 isolates) were ex cluded from the evaluation. Only isolates that showed phenotypic resis tance to cefotaxime and/or ceftazidime (*Pseudomonas*: cefotaxime and ceftazidime) in the Micronaut testing system were included into this evaluation.

Bacterial resistance profiles were evaluated using the EUCAST rules and breakpoints of 2019: 4MDRO and 3MDRO organisms were determined as described in Table 1 by the German Commission for Hospital Hygiene and Infection Prevention (KRINKO), however, including two classes of 3MDRO; "3MDRO_Pip" organisms with resistance to piperacillin only and "3MDRO_Pip/Taz" with resistance to piperacillin/tazobactam. In the case of *P. aeruginosa*, which is in trinsically resistant to cefotaxime, resistance to ceftazidime was al ways required. There were no strains that would have qualified as 4MDRO_Pip, since all 4MDRO harbored a carbapenemase gene or were resistant to piperacillin/tazobactam. The interpretation of an intermediate test results was changed to "susceptible to a high con centration" and was therefore evaluated as a susceptible result ("susceptible, increased exposure") as defined by EUCAST in 2019 (EUCAST, 2019).

In order to observe only the acquired resistance traits, intrinsically re sistant organisms were excluded from the evaluation as follows: Colistin was evaluated excluding *Proteus*, *Providencia*, *Morganella*, and *Serratia*. Tigecycline was evaluated excluding *Proteus*, *Providencia*, *Morganella*, and *P. aeruginosa*. An MIC of 1 μg/ml was evaluated as susceptible. Trimethoprim/sulfamethoxazole was assessed only for Enterobacterales. Ceftazidime/avibactam, amikacin, and ciprofloxacin were evaluated for all species; while temocillin was determined for Enterobacterales and the breakpoint of the BSAC (British Association of Chemotherapy) (susceptible ≤ 32 μg/ml) was used. Ceftozolane/tazobactam was evalu ated for all groups, however following the EUCAST regulations, the breakpoints of 1 μg/ml for Enterobacterales and 4 μg/ml for *Pseudomonas* and *Acinetobacter* were employed. *Acinetobacter* strains were excluded from the evaluation of fosfomycin. All MRSA strains were defined by resis tance to cefoxitin in the antibacterial susceptibility test.

2.2.6. Identification of resistance genes in isolated strains

Resistance genes (carbapenemase genes, *mcr 1*, *vanA*, and *vanB*) in isolated strains were detected by qPCR (TaqMan assays) as previously described (Froeschen et al., 2018; H. Müller et al., 2018). In short, for carbapenemase genes, one to three colonies were picked from fresh blood agar plates, resuspended in 50 μl of water and incubated at 95 °C for 5 min. Carbapenemase genes were then detected by 4Plex PCR employing the Biozyme 2 × qPCR Mastermix (Biozym, Hessisch Oldendorf, Germany) and Mic qPCR Cycler (Bio Molecular Systems, Upper Coomera, Australia). The PCR assay contained 0.25 μM of each primer, 0.125 μM of each probe and 2 μl of the bacterial lysates. The fol lowing cycling conditions were used: 95 °C for 2 min, then 45 cycles at 95 °C for 5 s and 60 °C for 30 s. Only signals that had been detected dur ing the first 30 cycles were counted as positive. Characterized clinical isolates served as positive controls. *mcr 1* was detected with 0.8 μM of the MCR1CLR5_F and MCR1CLR5_R primers in a monoplex reaction using the Biozyme 2 × Sybergreen Mastermix (Biozym) and the follow ing conditions: 2 min at 95 °C, 35 cycles at 95 °C for 5 s and 65 °C for 20 s. *E. coli* J53 V163 was employed as control (Falgenhauer et al., 2016).

For detection of *vanA/B* genotypes, three colonies were touched with a 1 μl loop and resuspended in 500 μl of H_2O and heated to 95 °C for 10 min. 10 μl of Promega GoTaq Probe Mastermix were mixed with 1 μl (10 μM stock) of forward and reverse primer, 0.25 μl (10 μM stock) of labeled probes and 2.5 μl of cooled down template. The end volume of reaction was 20 μl. qPCR was performed on a Bio Rad CFX96 cycler employing 2 min at 98 °C, and 35 cycles at 95 °C for 5 s and 60 °C for 45 s. 16S rRNA genes served as internal controls. All primers are listed in Table S1 (Liu et al., 2016; H. Müller et al., 2018; Swayne et al., 2013; Werner et al., 2011).

2.2.7. Measurement of resistance genes in wastewater by qPCR

For one year, nine wastewater samples (covering all seasons) of the clinical system were also analyzed for resistance genes, utilizing qPCR (CFX96 Touch™ Deep Well Real Time PCR Detection System, Bio Rad, Munich, Germany) in technical duplicates. For further analyses, the mean value of both duplicates was used. Information about wastewater quality is provided in Table S2.

For quantification of 16S rRNA, bla_{NDM}, *sul1*, $bla_{CTX\text{-}M\text{-}15}$, and *mcr* 1 genes, an intercalating Mastermix (Maxima SYBR Green qPCR Master Mix, Thermo scientific, USA) was used. One complete reaction with a

total volume of 20 µl contained 10 µl Maxima Mastermix, 7.2 µl nuclease free water (Ambion, Life technologies, Karlsbad, Germany), 0.8 µl of each corresponding primer stock (5 µM, Sigma Aldrich, Darmstadt, Germany, Table 2), and 1 µl of the sample. The qPCR protocol comprised 10 min at 95 °C for activation of the DNA polymerase and DNA denatur ation followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C for primer annealing and elongation. To determine the specificity of the amplifica tion, a melting curve was recorded by raising the temperature from 60 to 95 °C (1 °C every 10 s).

The detection of the carbapenem resistance gene bla_{VIM} was per formed in a primer/probe approach. The reaction mix contained 10 µl PCR Mastermix (TaqMan universal PCR Mastermix, Applied Biosystems, USA), 0.4 µl FAM labeled probe (Sigma Aldrich, Darmstadt, Deutsch land), 0.6 µl corresponding primers (5 µM), 12.5 µl nuclease free water, and 1 µl sample. Due to the utilization of a probe, the temperature gradient was altered to: initial incubation of 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 1 min at 60 °C.

Data acquisition was performed using the Bio Rad CFX Manager software. For calculation of gene copies from the C_T value, individual, linear calibration curves were used. Each calibration curve consists of a serial dilution over at least 5 log steps of a reference strain containing the respective gene. Known genome sizes of the reference bacteria were used to calculate gene copies in each reaction utilizing the amount of DNA in each dilution step of the calibration curve. Eq. (1) was used to create a correlation between the amount of DNA in the calibration solu tions and the corresponding gene copies. It utilizes an average molecu lar weight for one base pair of about 650 g/mol, Avogadro's number with 6.022×10^{23} molecules/mol, and a converting factor of 10^9 ng/g.

$$\text{gene copies} \quad \frac{(amount\ of\ DNA\ [ng]) * 6,022 * 10^{23}}{(average\ size\ of\ genome\ [bp]) * 10^9 * 650} \tag{1}$$

Table 3 summarizes all information relevant for the qPCR quantification. Based on the C_T value of each individual sample, the gene copy number in one ml sample was calculated using (Eqs. (2) (4)).

$$\frac{gene\ copies}{reaction} \quad 10^{\frac{'CT\ \ value'\ \ n}{m}} \tag{2}$$

$$\frac{gene\ copies}{\mu L\ sample\ DNA} \quad 10^{\frac{'CT\ \ value'\ \ n}{m}}/V_R \tag{3}$$

$$\frac{gene\ copies}{mL\ sample} \quad \frac{gene\ copies * V_E}{V_S} \tag{4}$$

Here V_R is the sample volume of the DNA extract used for one reac tion, V_S the original sample volume, and V_E represents the total volume of the eluate after DNA extraction.

To further evaluate the proportion of antibiotic resistance genes on the complete population, a normalization to the 16S rRNA gene of each sample was performed based on the gene copies in 100 ml sample of 16S rRNA and the respective gene.

2.2.8. Statistical analysis

The proportions of resistant isolates or different species obtained during the project were compared pairwise for different sampling sites using the chi square test. The number of strains tested is shown in Table 2 and N was at least 200 in case of the staphylococci and 333 1050 for the Gram negative bacteria. The bar charts of the qPCR deter minations represent the mean values of nine samplings covering all sea sons in one year and the error bars represent their respective standard deviation calculated as sample variance (s_n). For all qPCR determina tions, significance was calculated using the Mann Whitney *U* test.

3. Results

3.1. Species

Numbers of isolates and sampling sites are described in Table 3. Fig. 1 shows the distribution of species with resistance to 3rd generation ceph alosporins at the different sampling sites. *Citrobacter* and *Enterobacter* species ($p < 0.05$), and *Klebsiella* species ($p < 0.001$) were detected sig nificantly more frequently than *E. coli* in the wastewater of the clinical system, whereas *E. coli* was more prevalent in slaughterhouse wastewa ter ($p < 0.001$) and in municipal wastewater ($p < 0.001$) than in clinical wastewater. In addition, the clinical wastewater harbored also high pro portions of *Pseudomonas* ($p < 0.001$) compared to the slaughterhouse and municipal wastewater.

3.2. Multidrug resistance profiles of Gram negative isolates

A comparison of the phenotypical multidrug resistance profiles of all isolates showed, that the strains isolated from wastewater containing hospital effluents were characterized by a considerably higher percent age of multidrug resistant 4MDRO isolates and carbapenemase pro ducers than strains from the other sampling sites as previously described (H. Müller et al., 2018). Eighty percent of all carbapenemase producers of the clinical system harbored a metallo β lactamase gene (bla_{NDM}, bla_{VIM}, bla_{GIM} or bla_{IMP}). An elevated level of 4MDRO strains was still detected at the influent of the mixed wastewater at the WWTP ("clinic mixed with city") (Fig. 2A). The second sampling site at the WWTP receiving municipal wastewater of another city district without hospitals was characterized by significantly less 4MDRO and only a few carbapenemase producers ("city"). In contrast, the food pro duction wastewater contained neither 4MDRO nor carbapenemase pro ducers; even a more sensitive cultivation method with an enrichment

Table 2
Primers used for qPCR of resistance genes in wastewater samples.

Target	Primer sequence	Calibration equation curve	Amplicon size	Efficacy	R^2	LOD	Reference strain	Literature
Eubacteria (16S rRNA)	Fwd: TCCTACGGGAGGCAGCAGT Rev: ATTACCGCGGCTGCTGG	F(x) = 3406x + 36,360	195 bp	96.6%	1.000	10,600	*E. coli* pNORM	(Rocha et al., 2020)
$bla_{CTX\ M\ 15}$	Fwd: CGCTTTGCGATGTGCAG Rev: ACCGCGATATCGTTGGT	F(x) = 3504x + 34,255	551 bp	92.9%	1.000	93	*E. coli* pNORM	(Paterson et al., 2003; Rocha et al., 2020)
bla_{VIM}	Fwd: GAGATTCCCACGCACTCTCTAGA Rev: AATGCGCAGCACCAGGATAG Probe: ACGCAGTGCGCTTCGGTCCAGT	F(x) = 3829x + 40,868	61 bp	82.5%	0.999	118	*P. aeruginosa* PA49	(van der Zee et al., 2014)
*van*A	Fwd: TCTGCAATAGAGATAGCCGC Rev: GGAGTAGCTATCCCAGCATT	F(x) = 3541x + 33,078	376 bp	91.6%	1.000	43	*E. faecium* B7641 *vanA*	(Klein et al., 1998)
mcr-1	Fwd: GGGCCTGCGTATTTTAAGCG Rev: CATAGGCATTGCTGTGCGTC	F(x) = 3386x + 35,349	183 bp	97.4%	0.999	8	*E. coli* NRZ-14408	(Hembach et al., 2017)
bla_{NDM}	Fwd: TTGGCCTTGCTGTCCTTG Rev: ACACCAGTGACAATATCACCG	F(x) = 3293x + 35,877	82 bp	101.2%	0.999	66	*K. pneumoniae* ATCC BAA-2146	(Paterson et al., 2003)

LOD: limit of detection.

Table 3
Sampling sites and number of isolates.

Target species	Sampling site	Designation of sampling sites in Figs. 1–6	Number of Gram-negative strains with resistance to cefotaxime and/or ceftazidime *Enterobacteriaceae*, *P. aeruginosa*, *A. calcoaceticus-baumannii* clpx.	Number of Gram-positive isolates	
				MRSA	VRE
Clinical wastewater Gram-negative 759 Isolates	Wastewater of hemato-oncological clinic and wastewater of hemato-oncological clinic mixed with 6 additional clinics	"Building"	345	0	59
	Mixed wastewater of maximum care hospital	"Clinic"	218	14	65
	Mixed wastewater of clinic and city including 10 other hospitals (sampled at the influent into WWTP)	"Clinic mixed with city"	196	13	46
Municipal wastewater 815 Gram-negative isolates	Mixed municipal wastewater of another city district (influent into WWTP)	"City"	200	18	33
	Wastewater of four little rural towns in an agricultural area (influent into WWTP)	"Rural towns"	615	22	131
Slaughterhouse wastewater 1049 Gram-negative isolates	Wastewater and process water of two different pig slaughterhouses	"Ww pig"	386	162	0
	Wastewater and process water of two different poultry slaughterhouses and one small broiler raising farm	"Ww poultry"	663	86	1

broth did not yield any carbapenemase producers (Savin et al., 2020a; Savin et al., 2020b) (Fig. 2A).

Comparing 3MDRO bacteria, remarkably different results were ob tained employing piperacillin (3MDRO_Pip) (KRINKO, 2019; Wendt et al., 2012; Table 1) versus piperacillin/tazobactam (3MDRO_Pip/Taz) (as defined by Baum et al. (2011) and Magiorakos et al. (2012)) in the evaluation (Fig. 2B): There were significantly lower numbers of 3MDRO_Pip/Taz than 3MDRO_Pip in the slaughterhouse wastewaters as well as in municipal wastewaters (sampling points "city", "rural towns" and also in "clinic mixed with city"). In addition, the number of 3MDRO_Pip/Taz species was significantly lower in slaughterhouse wastewater than in clinically influenced wastewater (sampling point "city mixed with clinic") and in the municipal wastewaters (Fig. S1).

3.3. Susceptibility of Gram negative isolates to antibiotics of last resort and the newly introduced antibiotic temocillin

The highest percentage of colistin resistance (percent of colistin resistant isolates among the strains resistant to 3rd generation cephalosporins) was present in the wastewater from the clinical sampling points (Fig. 2C), and again there was a significant differ ence between the two influents of the WWTP. However, some iso lates from slaughterhouse and the municipal wastewater ("city", "rural towns") also showed colistin resistance. The transferable gene *mcr* 1 could not be found in the isolates from clinical or munic ipal sampling points. In contrast, *mcr* 1 was detected in the isolates from the slaughterhouses (Savin et al., 2020b). A part of these iso lates was obtained using a more sensitive selection procedure and they did not show resistance to 3rd generation cephalosporins.

High percentages of the isolates obtained from all sampling points with undiluted clinical wastewater ("building" and "clinic") were also re sistant to ceftazidime/avibactam, ceftozolane/tazobactam, and temocillin. In contrast, resistance levels were significantly lower in the slaugh terhouse wastewater (Fig. 3A C). As observed for the 4MDRO and 3MDRO_Pip/Taz phenotypes and colistin, significant differences between the two influents at the WWTP were detected for ceftozolane/tazobactam and temocillin. A high susceptibility among isolates from all systems was observed for the protein biosynthesis inhibitor tigecyline (Fig. 3D).

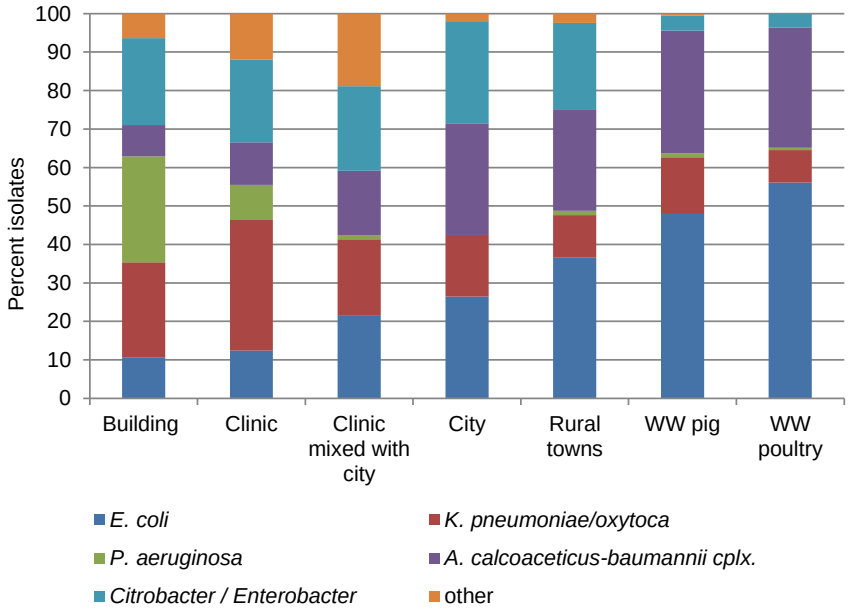

Fig. 1. Species distribution of isolates with resistance to 3rd generation cephalosporins from different sampling sites in this study. "Other" comprises the genera *Serratia*, *Kluyvera*, *Pantoea*, *Salmonella*, and *Morganella*.

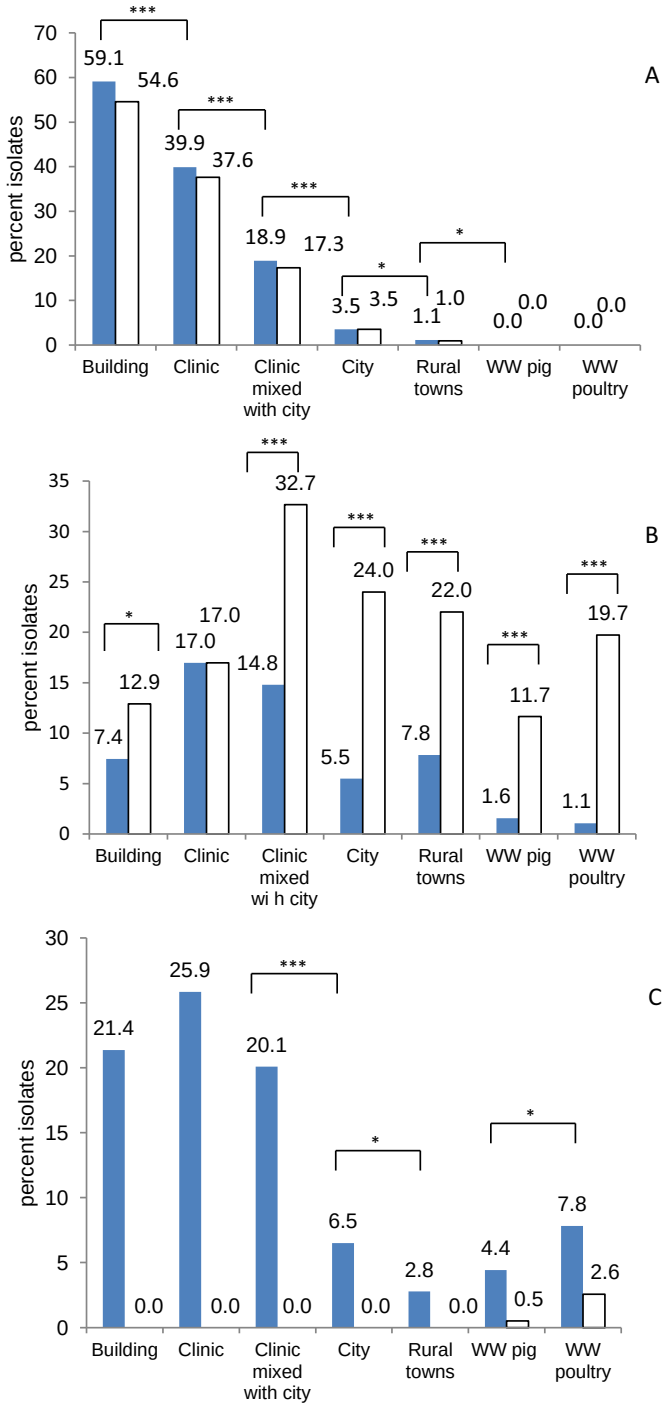

Fig. 2. A) Percentage of 4MDRO isolates (filled bars) and carbapenemase producers (white bars) among strains with resistance to 3rd generation cephalosporins; B) percentage of 3MDRO_Pip/Taz (filled bars) and 3MDRO_Pip (white bars) isolates among strains with resistance to 3rd generation cephalosporins; C) percentage of colistin resistant isolates (filled bars) and strains harboring *mcr-1* (white bars) among strains with resistance to 3rd generation cephalosporins from different sampling sites.

3.4. Resistance of Gram negative isolates to established and frequently used antibiotics

The overall resistance burden to amikacin was low; however, the influence of the hospital wastewater was discernible (Fig. 4A). The percentages of ciprofloxacin resistant bacteria were high from all sampling sites, with hospital isolates showing the highest resistance. This difference was still highly significant at the influent of the clinically influenced wastewater into the WWTP (Fig. 4B). The resistance rates were lowest in pig slaughterhouses.

For fosfomycin the highest resistance rate was observed among the hospital isolates (Fig. 4C). In the community, resistance was also commonly found and lowest percentages were present in the samples from the slaughterhouses. The resistance distribution resembled that of ciprofloxacin: Again the signature of the clinical wastewater was still detected at the influent into the WWTP and even lower percentages of resistant bacteria were isolated from the slaughterhouse wastewater.

For trimethoprim/sulfamethoxazole the overall resistance burdens were high. The difference between pig and poultry slaughterhouses was significant (Fig. 4D).

3.5. Gram negative XDR isolates showing susceptibility to only one antibiotic substance

Extensively drug resistant (XDR) strains with susceptibility to only one antibiotic substance were isolated exclusively from the clinically influenced sampling sites. All three *P. aeruginosa* were susceptible to colistin and all *K. pneumoniae* strains remained susceptible to tigecycline or fosfomycin (Table 4). Pan resistant bacteria were not detected.

3.6. Gram positive resistant isolates

MRSA isolated from wastewater of the different sampling points were also compared (Fig. 5). Here only low levels of resistance against the 5th generation cephalosporin ceftaroline were present. For the quinolone moxifloxacin, the resistance of the pig isolates was significantly lower than that of isolates from poultry slaughterhouse. An opposite tendency was found for resistance to trimethoprim/sulfamethoxazole. MRSA typing (Fig. 6) showed that hospital associated *spa* types belonging to clonal complexes (CC) CC1 (t127), CC5 (t002, t003, t014, t045, t688, t1282), CC6 (t304), CC8 (t008, t1767), and CC22 (t016, t032, t223, t463, t608, t8934), were found in clinical and municipal wastewater. In contrast, livestock associated *spa* types (CC9 (t2922, t1430, t13177) and CC398 (t011, t034, t899, t1793, t2011, t2576, t8100, t8588, t9266)) were detected in the slaughterhouses. The only exceptions were t127 (CC1) and t001 (CC5) that were found for four isolates from wastewater of the pig slaughterhouses. Here it has to be kept in mind that the sampled clinical and municipal wastewaters were obtained from an area with crop production and little livestock farming. This might explain the absence of livestock associated MRSA from the clinical and municipal wastewater systems.

VRE strains from the hospital influenced wastewater as well as from the municipal systems belonged to the species *Enterococcus faecium*. Most strains showed the *vanB* genotype (clinical system 90% *vanB*, municipal wastewater (city: 97% and rural towns: 99%)). Only two strains that showed resistance to linezolid were recovered from the mixed wastewater of the clinic. No strain was resistant to all antibiotics tested. The only VRE recovered from slaughterhouse wastewater harbored *vanA* (Savin et al., 2020b).

3.7. Concentrations of resistance genes in hospital wastewater

The above results indicate which percentage of the isolates with resistance to 3rd generation cephalosporins was multidrug resistant and/or resistant to other antibiotics, but they do not give absolute concentrations of resistant bacteria, especially carbapenemase producers. In order to determine the concentration of resistance genes in the clinical wastewater, the copy numbers of several resistance genes were measured in the clinical and municipal wastewater by qPCR. In the wastewater of the ward and clinic, concentrations of carbapenemase genes were high with 10^5 10^6 copies/ml of bla_{NDM} and bla_{VIM}, surpassing the concentration of the ESBL gene $bla_{ctx\text{-}M\text{-}15}$. These high concentrations decreased by one to two log steps by dilution with wastewater of the city. In spite of this, the concentrations of the genes bla_{NDM}, $bla_{ctx\text{-}M\text{-}15}$,

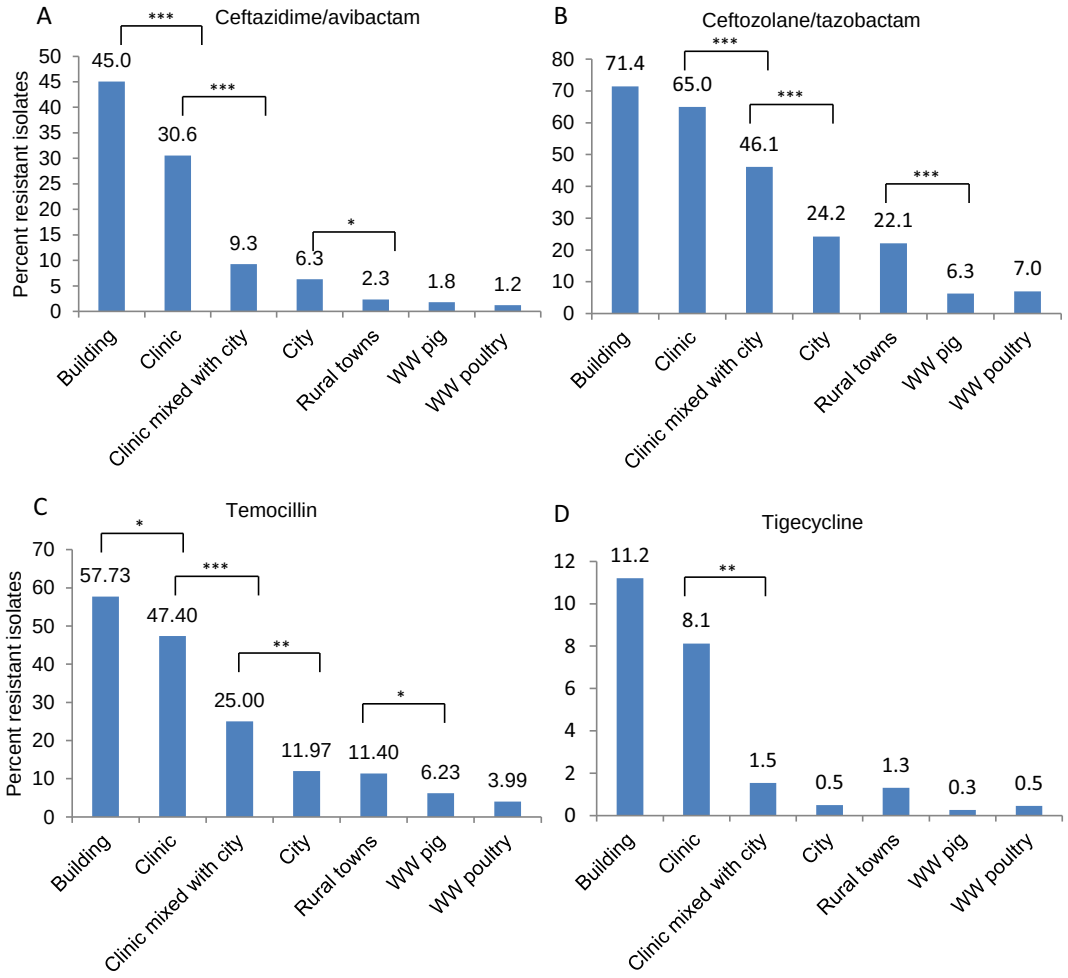

Fig. 3. Percentage of A) ceftazidime/avibactam, B) ceftozolane/tazobactam, C) temocillin, and D) tigecycline resistant isolates among strains with resistance to 3rd generation cephalosporins from different sampling sites.

and *sul1* were still significantly higher in clinically influenced wastewa ter at the entry into the WWTP (Fig. 7). In the WWTP, about 99 99.9% of the remaining genes were removed; however, as the initial concentra tions had been high, resistance genes were still detected in the effluent. The concentrations of the transferable colistin resistance gene *mcr* 1 were low. The measurement of 16S rRNA copies showed that the waste water of the ward carried tenfold less bacteria than the other samples and that most bacteria were removed in the WWTP (Fig. S2). Normali zation of the resistance genes to 16S rRNA concentrations demonstrated the dilution of resistant bacteria in the wastewater path. The apparently higher concentrations of carbapenemase gene copies/16S rRNA in the ef fluent of the WWTP compared to the "city" wastewater were not signifi cant (Fig. S3). The highest concentrations of carbapenemase genes/16S rRNA were detected for bla_{VIM} (1.27×10^{-1}/16S rRNA in the wastewater of the ward and 7.7×10^{-3}/16S rRNA in the wastewater of the clinic); the concentrations of bla_{NDM} (4.85×10^{-2}(ward) and 4.1×10^{-4} (clinic)) were lower.

3.8. Daily loads of resistance genes

It might be argued that, even if the clinical wastewater carries a high concentration of carbapenemase genes and multidrug resistant organ isms, it contributes only a small volume to the wastewater that reaches the local WWTP. This plant receives an average of 27,498 m^3 wastewater per day from the part of the city that harbors all clinics ("clinic mixed with city"), whereas the second influent receives an average of 23,389 m^3 wastewater per day from a part of the city that is free of hospitals ("city"). In order to analyze the influence of the hospitals, the total daily loads of resistance genes in the two influents were calculated by multiply ing the average gene concentrations per ml with the average daily waste water volumes. Fig. 8 demonstrates that the daily load of bla_{VIM} genes carried by the clinically influenced wastewater to the WWTP ("clinic mixed with city") was about a hundredfold higher than that of the city wastewater ("city"). For more common genes ($bla_{ctx\text{-}M\text{-}15}$, *sul1*) the in crease in clinically influenced wastewater was about 5 10 fold and only twofold for *mcr* 1. In total, the "city" wastewater contributed only about 16% of all resistance genes to the daily load reaching the WWTP.

4. Discussion

In this study, the highest number of 4MDRO was detected in the med ical sector. A dissemination of carbapenemase producers by hospital wastewater has been described in many countries, e. g., Ireland (Cahill et al., 2019), Sweden (Khan et al., 2018), India (Bardhan et al., 2020), and China (Zhang et al., 2020). However, even after dilution with "city" wastewater, i. e., at the influent of the WWTP, the percentages of 4MDRO, 3MDRO_Pip/Taz and bacteria resistant to several antibiotics of last resort were still significantly increased in clinically influenced waste water compared to municipal wastewater. A similar study (Galvin et al., 2010) reached significance only for sulfamethoxazole resistance in the mixture of hospital and municipal wastewater.

In the clinical wastewater, the concentration of resistance genes was high: A study that measured bla_{VIM}/16S rRNA genes in Romanian hospi tal wastewater reached lower values (ranging from 1.85×10^{-6} to

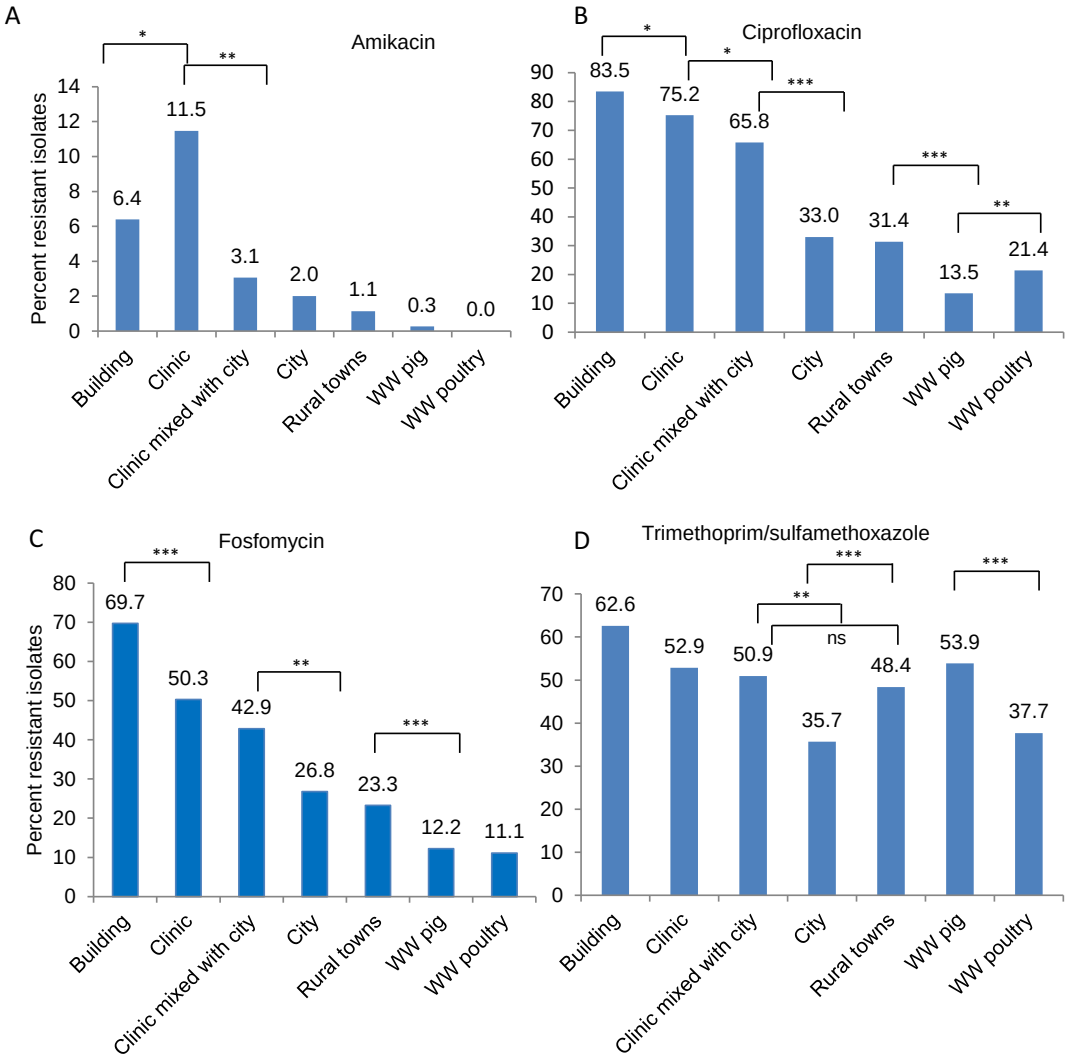

Fig. 4. Percentage of A) amikacin, B) ciprofloxacin, C) fosfomycin, and D) trimethoprim/sulfamethoxazole resistant isolates among strains with resistance to 3rd generation cephalosporins from different sampling sites.

5.84×10^{-5}) than shown here for the "ward" and "clinic" samples (Szekeres et al., 2017). The values obtained for bla_{NDM} at the sampling point "clinic" were lower or in the same range as those in earlier studies of hospital effluents in Tunisia (7.28×10^{-3}) (Nasri et al., 2017) and Spain (6.88×10^{-4} and 7.32×10^{-4}) (Subirats et al., 2017). In contrast, the values for the sampling point "ward" were also higher than these lit erature values.

When carbapenemase producers started to appear in hospitals, co listin was advertised as "re emerging antibiotic for multidrug resistant Gram negative bacterial infections" (Li et al., 2006). In the clinical strains, resistance to colistin was present in 20% of the isolates. This re sistance can either be mediated by chromosomal mutations that enable modification of lipid A or by a transferable gene of the *mcr* type (Liu et al., 2016). In the clinical isolates, transferable colistin resistance genes were not detected, indicating that the resistance depended on chromosomal mutations (Moffatt et al., 2019). In addition, the *mcr 1* genes detected in the wastewaters of the clinical and municipal system may have been present in bacteria that did not carry ESBL genes and, therefore, were not selected and analyzed in this study, as was also shown for a part of the slaughterhouse isolates. The rate of colistin resis tant isolates from slaughterhouse wastewater (4.4% (pigs) 7.8% (poul try)) was lower than that of the clinical isolates and not very different from that of isolates from the municipal wastewater (2.8% (rural towns) 6.5% (city)). However, the transferable colistin resistance gene *mcr* 1 was only detected in the strains from slaughterhouse wastewater. This gene is also present in municipal WWTPs in Germany (Hembach

Table 4
XDR strains isolated from clinically influenced wastewater. The table describes all strains that were susceptible or intermediately susceptible to only one antibiotic.

	Epidemic type	Carbapenemase	Susceptible to (MIC in mg/l)	Sampling point
P. aeruginosa cuww1402	ST273	VIM	Colistin (2)	Clinic
P. aeruginosa cuww1717	ST235	VIM	Colistin (2)	Building
P. aeruginosa cuww2103	ST235	VIM	Colistin (≤1)	Building
K. pneumoniae cuww1225	ST253	NDM, Oxa-48, Oxa48-like	Tigecycline (0.5)	Clinic
K. pneumoniae cuww1760	ST253	NDM, Oxa-48, Oxa48-like	Tigecycline (1)	Clinic mixed with city
K. pneumoniae cuww2208	ST16	NDM	Tigecycline (0.5)	Clinic mixed with city
K. pneumoniae cuww1896	ST15	NDM	Fosfomycin (32)	Clinic

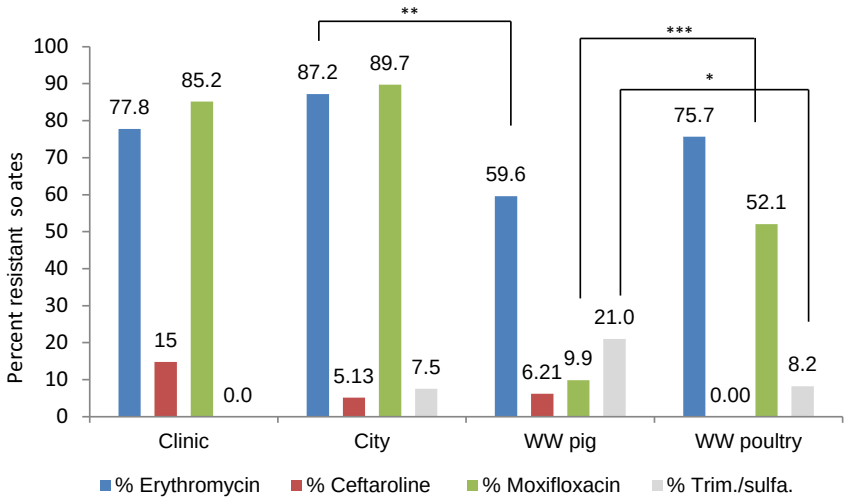

Fig. 5. Resistance of MRSA from different sampling sites (Trim/sulfa.: Trimethoprim/sulfamethoxazole). Since the number of isolates was low, the sampling site "clinical ww" comprises all isolates from the clinical wastewater (building + clinic + clinic mixed with city) and "municipal ww" all isolates from municipal wastewater (city + rural towns) as defined in Table 1.

et al., 2017) as well as in German surface waters (Falgenhauer et al., 2019) and the continued use of colistin in farming could favor its spread, in spite of a decrease in colistin consumption from 127.4 to 73.6 tons per year between 2011 and 2017 (AG Antibiotikaresistenz am BVL, 2018).

Unfortunately, the new antibiotic combination of ceftazidime/avibactam (Spiliopoulou et al., 2020) cannot inhibit the growth of strains producing metallo β carbapenemases, e. g., NDM, VIM, and GIM. Similarly ceftozolane/tazobactam does not target the growth of carbapenem resistant *Enterobacteriaceae* or strains harboring metallo β lactamases (Cho et al., 2015). Thus, the high concentration of these resistant strains in clinical wastewater mirrors the fact that the most frequently detected carbapenemase genes were metallo β lactamases. Also temocillin will not target these bacteria, since it is ineffective against strains producing OXA 48, VIM, and NDM (Cavaco et al., 2019).

Although we were not able to isolate carbapenemase producers from the wastewater and process water of slaughterhouses, several studies describe the isolation of VIM producing *Enterobacteriaceae* from pigs and poultry after subculturing in the absence of antibiotics and specific enrichment procedures (Irrgang et al., 2019; Roschanski et al., 2018; Roschanski et al., 2019). Our results indicate that these

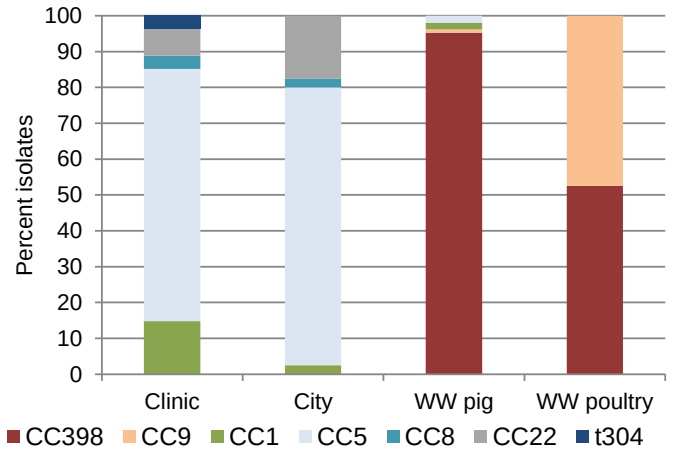

Fig. 6. Clonal complexes (CC) of MRSA isolates from different sampling sites. The high prevalence of the hospital associated MRSA CC5 is typical for this area. CC398 and CC9 strains are livestock-associated MRSA. Since the number of isolates was low, "clinical ww" subsumes all isolates from the clinical wastewater (building + clinic + clinic mixed with city) and "municipal ww" all isolates from municipal wastewater (city + rural towns) as defined in Table 1.

isolates are probably still rare in Germany, as carbapenems are not licensed for animal production. Therefore, there is a lack of selection pressure that might otherwise favor the spread of these genes in farming animals. In conclusion, the resistance phenotypes detected in this study reflect the usages of antibiotics and hence selection pressures in the different areas of clinical medicine and food production. Penicillins, cephalosporins, colistin, and enrofloxacin in poultry and pig production (AG Antibiotikaresistenz am BVL, 2018) select for 3MDRO_Pip. The Gram negative bacteria (Fig. 4B and D) and MRSA (Fig. 6) from pig slaughterhouses showed higher resistance to sulfonamides and lower resistance to quinolones than the isolates from poultry, indicating a different consumption of these antibiotics by different species. In contrast, carbapenems, piperacillin/tazobactam, and other new substances are only employed in hospitals. The resulting selection pressure forms a biotope with high abundance of 4MDRO with additional resistances and 3MDRO_Pip/Taz. Especially the quinolone ciprofloxacin seems to exert a strong selection pressure, since ciprofloxacin residues in wastewater or in sanitary installations have been shown to correlate with the presence of resistant bacteria (Voigt et al., 2020). Extensive genome comparisons of recent and historic strains (isolated in the nineteen eighties, when quinolones were introduced into the clinics) have demonstrated that the ciprofloxacin selection pressure has led to the rise of the most problematic multidrug resistant pandemic strains. Among them were the ESBL producing ST131 *E. coli* (Ben Zakour et al., 2016), the carbapenemase producing ST235 *P. aeruginosa* (Treepong et al., 2017), and the epidemic MRSA 15 (ST22) (Holden et al., 2013).

Animals that are in contact with 4MDRO in wastewater may also be colonized, as shown for rats colonized with ESBL and NDM 1 producing *Enterobacter xiangfangensis* in Vienna (Desvars Larrive et al., 2019) and gulls (Atterby et al., 2017; Vittecoq et al., 2017). We have demonstrated that 4MDRO are still present after wastewater treatment and are released into surface waters (H. Müller et al., 2018). There is still a debate about how and where resistant bacteria might be transmitted from surface water back to humans, especially as long as they are not present in drinking water or food. However, after a drowning accident, a patient developed an infection with IMI 2 producing *Enterobacteriaceae* present in the river (Laurens et al., 2018), and a higher colonization with ESBL producing bacteria was also shown for surfers (6.5%) compared to non surfers (1.5%) in England (Leonard et al., 2018). In addition, resistant bacteria may find their way into the food chain, since ESBL producing bacteria have recently been isolated from fresh products in Germany (Blau et al., 2018) and hyper virulent ESBL producing EHEC (enterohemorrhagic *E. coli*) O104:H4 were transferred by fenugreek sprouts in the German outbreak in 2011 (Fruth et al.,

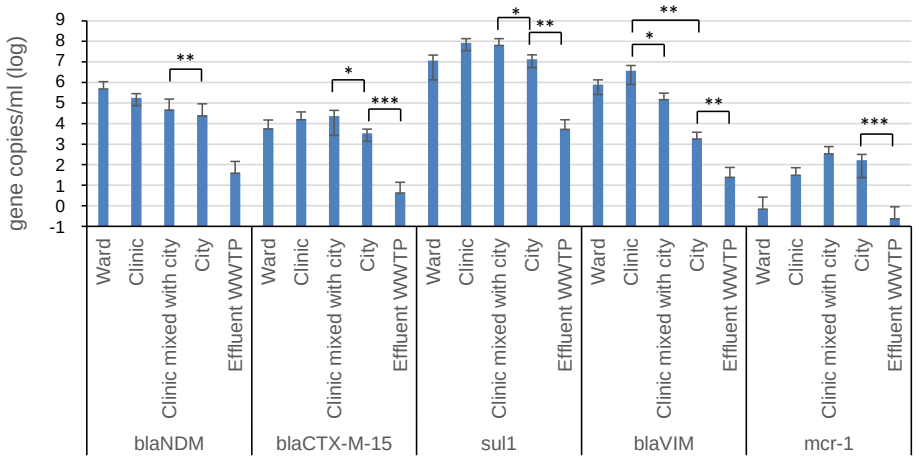

Fig. 7. Average concentrations of gene copies per ml water as detected by qPCR in nine samples. Carbapenemase genes (bla_{NDM}, bla_{VIM}) compared to frequent genes (*sul1*), ESBL genes (bla_{ctxM15}) and *mcr*-1. In this graph, "ward" designates wastewater from the building housing the hemato-oncological clinic. The absence of values in some samples (effluent of WWTP and ward) indicates that the genes were below the detection level of the qPCR in these samples. The effluent of the WWTP consists of a mixture of treated wastewater from both influents.

2015). On the other hand, there are numerous reports stating that colonization of the sanitary installations in hospitals with 4MRDO may lead to colonization of patients in the affected rooms, leading to long lasting, low level outbreaks (Clarivet et al., 2016; Wendel et al., 2015). It was also shown that the colonization with 4MDRO in the sanitary system is promoted by the presence of antibiotic residues that are excreted by the patients and which may be pres ent in selective concentrations in the toilets and siphons (Sib et al., 2019).

The burden of multidrug resistance in bacteria released by waste and process water of the slaughterhouses was lower than that of clinical or often even municipal wastewater. However, in contrast to clinical and municipal wastewater, the application of manure from animal hus bandry as a fertilizer on fields might lead to a spread of such bacteria into the environment in high concentrations and without any passage through a WWTP. Therefore, it might be wise to abolish use of colistin and quinolones in animal production. Both antibiotics are categorized as critically important with the highest priority by the WHO (WHO, 2019b). For quinolones a ban from animal production is already practiced in Australia and has kept resistance levels low (Cheng et al., 2012). In the US, the use of quinolones was banned in poultry produc tion in 2005 (Price et al., 2007).

5. Conclusion

In conclusion, it is tempting to speculate that an introduction of waste water treatment in hospitals or of other measures that inhibit the coloni zation of the hospital sanitary system would reduce the high levels of multidrug resistant bacteria and resistance genes detected in the clinical samples to the lower levels in the municipal wastewater. Also an intro duction of an ultrafiltration as last step of wastewater treatment would inhibit dissemination of these bacteria into the environment, as there was no final disinfection of wastewater in the analyzed WWTPs. Such measures might be costly, but they would avoid dissemination of bacteria that cause difficult to treat infections and help to prevent the post antibiotic era that was predicted by the WHO (WHO, 2015).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.140894.

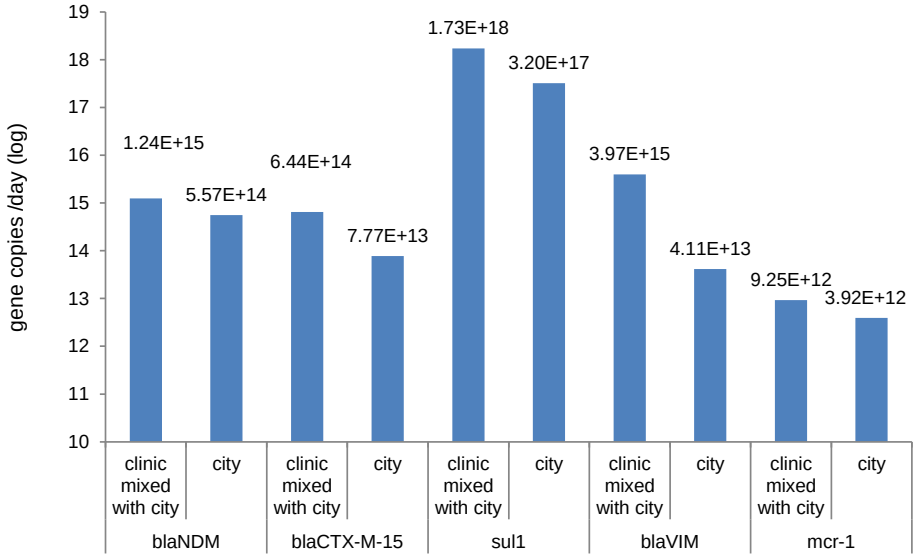

Fig. 8. Daily loads of antimicrobial resistance genes transported by clinically influenced wastewater in influent 1 and municipal wastewater in influent 2 to the WWTP (calculated from the average concentration of resistance genes/ml multiplied with corresponding average wastewater volumes per day). bla_{VIM} and bla_{NDM} encode carbapenemases, whereas bla_{ctxM15} encodes an ESBL enzyme. *sul1* confers resistance to sulfamethoxazole and *mcr-1* encodes transferable colistin resistance. The average daily volume of the clinically influenced wastewater influent 1 is a higher (27,498 m^3) than that of the municipal wastewater (23,389 m^3) and therefore clinically influenced wastewater delivered 90% of all carbapenemase and 84% of all resistance genes measured here.

CRediT authorship contribution statement

Esther Sib: Validation, Investigation, Data curation, Writing review & editing. **Franziska Lenz-Plet:** Investigation. **Vanessa Barabasch:** Investigation. **Ursula Klanke:** Investigation. **Mykhailo Savin:** Investigation, Resources, Writing review & editing. **Norman Hembach:** Investigation, Writing review & editing. **Anna Schallenberg:** Investigation. **Katja Kehl:** Investigation. **Cathrin Albert:** Investigation. **Mike Gajdiss:** Investigation. **Nicole Zacharias:** Resources. **Heike Müller:** Resources. **Ricarda Maria Schmithausen:** Project administration. **Martin Exner:** Conceptualization, Funding acquisition. **Judith Kreyenschmidt:** Conceptualization, Supervision, Writing review & editing, Funding acquisition. **Christiane Schreiber:** Conceptualization, Supervision, Resources, Writing review & editing, Funding acquisition. **Thomas Schwartz:** Conceptualization, Methodology, Writing review & editing, Supervision, Funding acquisition. **Marijo Parčina:** Conceptualization, Investigation, Writing review & editing, Funding acquisition. **Gabriele Bierbaum:** Conceptualization, Supervision, Validation, Data curation, Writing original draft, Writing review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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