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Optimization and harmonization of microbiological and molecular detection and typing methods for carbapenemase-producing Enterobacteriaceae

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Vorgelegt von
Natalie Pauly
Biologin, M.Sc.
aus Aachen, Deutschland

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Dekan: Univ.-Prof. Dr. Uwe Rösler

Erster Gutachter: Univ.-Prof. Dr. Diana Meemken

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AMR	Antimicrobial resistance
BfR	Bundesinstitut für Risikobewertung
β	Beta
bla	β-lactamase gene
bp	base pair(s)
BPW	Buffered peptone water
CP	Carbapenemase-producing
CPE	Carbapenemase-producing Enterobacteriaceae
CR	Carbapenem-resistant
CTX	Cefotaxime
DNA	Deoxyribonucleic acid
ECDC	European Center for Disease Control
EFSA	European Food Safety Authority
EJP	European Joint Project
ESBL	Extended-spectrum β-lactamase
EU	European Union
Inc	Incompatibility
IMPART	Improving phenotypic Antimicrobial Resistance Testing by development of sensitive screening assays for emerging resistances and setting missing ECOFFs
IMP	Imipenemase
IS	Insertion sequence
ITR	Inverted tandem repeat
KPC	*Klebsiella-pneumoniae*-carbapenemase
MDR	Multidrug resistance
MEM	Meropenem
MIC	Minimal inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
NDM	New Delhi Metallo-β-lactamase
NC-IUBMB	Nomenclature Committee of the International Union of Biochemistry

OIE	World Organization for Animal Health
oriT	Origin of transfer
OXA	Oxacillinase
PBP	Penicillin-binding protein
PFGE	Pulsed-field gel electrophoresis
ST	Sequence type
TE	Transposable element
USA	United States of America
VIM	Verona Integron Metallo-β-lactamase
VRE	Vancomycin resistant enterococci
WGS	Whole genome sequencing
WHO	World Health Organization
WP	Work packages

1 Introduction

1.1 Antimicrobial agents

With the discovery of the first antibiotics in the 20th century, such as penicillins, a new way to treat infectious diseases became available (Fleming 2001). The medical world was revolutionized by their discovery and the development of more of such substances. Today, researchers and medics differentiate between antibiotics and antimicrobial agents. The term “antibiotics” describes natural substances with antibacterial effect, whereas “antimicrobial agents” includes natural and synthetic substances that are able to kill or to inhibit the growth of microorganisms (Waksman 2010).

Since antibiotics are natural substances, corresponding resistances are the result of the natural bacterial evolution (Bengtsson-Palme et al. 2018; Wright 2010). This is confirmed by studies, which illustrated the presence of resistance genes, encoding degrading enzymes for beta (β)-lactams, tetracyclines and glycopeptides, in samples of 30,000-year-old permafrost soil (D`Costa et al. 2011). The use of antibiotics resulted in an accelerated increase of the development and distribution of antimicrobial resistance (AMR) over the last decades (Wight 2010; Levy and Marshall 2004). Antimicrobial agents either inhibit the bacterial growth (bacteriostatic) or kill the cells (bactericidal). They target different physiological or biochemical mechanisms of the bacteria (Table 1) (Silver 2016; Davey et al. 2015). Based on these mechanisms, most common antimicrobial agents can be assigned to different classes (Silver 2016; Davey et al. 2015).

Table 1: Most common antimicrobial classes and their corresponding bacterial targets.

Drug class	Effect	Bacterial target
β-Lactams	bactericidal	Cell wall
Glycopeptides	bactericidal	
Quinolones	bactericidal	DNA synthesis
Nitroimidazoles	bactericidal	
Ansamycins	bactericidal	RNA synthesis
Aminoglycosides	bactericidal	Protein synthesis
Macrolides	bacteriostatic	
Phenicols	bacteriostatic	
Tetracyclines	bacteriostatic	
Oxazolidinones	bacteriostatic	
Lipopeptides	bactericidal	Membran and/or cell wall
Sulphonamides	bacteriostatic	Folate synthesis
Diaminopyrimidines	bacteriostatic	

According to the historical usage of antimicrobial agents in livestock, one of the first reports on AMR in animals was published in 1969 (Swann et al. 1969). The so-called “Swann Report” associated the usage of antimicrobial agents with the increase in resistances (Swann et al. 1969). Therefore, the authors concluded that the usage of antimicrobial agents in livestock led to AMR in enteric bacteria. They concluded a hazard not only for animal but also for human health (Swann et al. 1969). Three decades later, the relation between non-therapeutic use of a glycopeptides in pigs and poultry and the emergence of vancomycin-resistant enterococci (VRE) in animals and humans was reported (Bager et al. 1997; Aarestrup et al. 1996). Thus, the European Commission developed a strategy to stop the dissemination of AMR. One central point of this strategy was the European Union (EU)-wide refrain from further authorization of antimicrobial agents as growth promotors in animal feed, and the ban of avoparcin, with the Regulation (EC) No 1831/2003, which came into force on January 1 in 2006. The World Health Organization (WHO) also recognized the important concern for public and animal health. Together with the World Organization for Animal Health (OIE), they developed the framework for responsible and prudent use of antimicrobial agents (WHO 2017; OIE 2007). Both institutions set up ranking criteria for antimicrobial agents according to their importance for human resp. veterinary medicine (https://www.who.int/foodsafety/publications/antimicrobials-sixth/en/, December 21, 2020). The first criterion contains the availability of therapeutic options; the second criterion considers the non-human sources of bacteria or the potential acquirement of resistance genes. The highest class, called “Critically Important Antimicrobial Agents”, meets both criteria (WHO 2017; OIE 2007). Within this class, there is an additional prioritization based on three assumptions, the absolute numbers of potentially affected people, the overall frequency of use of the drugs in human medicine, and usage to treat pathogens with a potential transmission of bacteria or their genes from non-human sources to humans (WHO 2017; OIE 2007). An antimicrobial that meets all three criteria is categorized as “Highest Priority Critically Important Antimicrobial“ (WHO 2017).

1.1.1 β-lactam antimicrobials

One of the most important class of antimicrobial agents are β-lactams, which target the bacterial cell wall (Silver 2016; Kong et al., 2010). They inhibit the bacterial cell wall synthesis by covalent binding to the penicillin-binding proteins (PBPs). The inhibition results in stopping cell division or forming spherical cells. Another possibility is the arrest of cell division, resulting in filamentation. The type of inhibition corresponds to the type of PBP (Bush and Bradford 2016).

The β-lactams are the most commonly used antimicrobial agents to treat several bacterial infections in human and animals (Bush and Bradford 2016). This is due to their broad spectrum of activity and good tolerability (Bush and Bradford 2016; Fisher and Mobashery 2016; Tipper and Strominger 1965). They contain diverse groups of antibiotics as penicillins, cephalosporins, monobactams and carbapenems, which are described in more detail below. All of them carry the β-lactam ring in their structure (Figure 1) (Bush and Bradford 2016; Jeon et al. 2015; Nordmann et al. 2012).

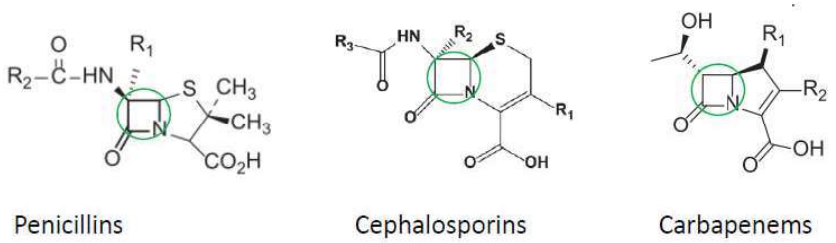

Figure 1: Chemical structures of β-lactam antibiotics according to Terico and Gallagher 2014. A: Core structure of penicillins. B: Core structure of cephalosporins. C: Core structure of carbapenems. R represents side chains that differ among members of the same class.

1.1.1.1 Penicillins

In 1941, benzylpenicillin (Penicillin G) was discovered. It was the first clinically effective antibiotic used to treat infections with Gram-positive bacteria (Bush and Bradford 2016; Davey et al. 2015). Beside its beneficial effects, Penicillin G has several disadvantages like the inactivation by gastric acid, fast elimination and hydrolysis by degrading enzymes and a severe allergic reaction in a small number of patients (Bush and Bradford 2016). Therefore, the research for more variants of penicillin was initiated. This research resulted i.e., in penicillin V for oral usage or ampicillin that also showed activity against Gram-negative bacteria (Bush and Bradford 2016; Davey et al. 2015; Tipper and Strominger 1965). Oxacillin is a penicillinase-stable penicillin and has a narrow spectrum of activity against Gram-positive bacteria (Wright 1999).

A five-membered thiazolidine ring is fused to the β-lactam ring (Figure 1) (Palzkill 2013). Resistances against penicillins are mostly due to degrading enzymes, so-called penicillinases, which attack the β-lactam ring. To ensure their activity, penicillins are often used in combination with β-lactamase inhibitors (i.e. clavulanic acid, sulbactam, tazobactam) or with aminoglycosides (Tooke et al. 2019; Bush and Bradford 2016). Latter combination is due to synergistic effects of both substances. Penicillins enable the entry of aminoglycosides into the bacterial cell, where these inhibit the protein synthesis. However, penicillins act on reproducing cells which is severely limited by paralyzed protein biosynthesis influenced by aminoglycosides (Winstanley and Hastings 1989).

1.1.1.2 Cephalosporins

Comparable to the described structure of penicillins, cephalosporins also contain this typical β-lactam ring, to which a six-membered dihydrothiazine ring is fused (Figure 1) (Palzkill 2013). Cephalosporins are one of the most prescribed group of antibiotics due to their relatively good tolerability and their broad spectrum of activity. They are divided into five generations, according to their spectrum against Gram-positive and Gram-negative bacteria and their discovery (Table 2). Due to their extra carbon atom in the dihydrothiazidin ring, more opportunities for modifications are given than in the five-membered ring of the penicillins (Bui and Preuss 2020; Das et al., 2019).

Table 2: Examples for cephalosporins of each generation (Das et al. 2019).

Generation	Examples	Description against Gram-positives	Description against Gram-negatives
First	Cefazolin, cefadroxil, cephalexin	Activity against penicillinase producing, methicillin-susceptible, staphylococci and streptococci	Moderate activity against *Proteus mirabilis*, *Escherichia coli*, and *Klebsiella pneumoniae*
Second	Cefaclor, cefuroxime, cefoxitin	Less activity than first-generation	Greater activity than first-generation
Third	Ceftiofur, cefoperazone, cefotaxime, cefdinir	Decreased activity	Broad spectrum of activity and more increased activity than previous generations
Fourth	Cefquinome, cefepime, cefpirome, cefozopran	Extended-spectrum activity as first-generation cephalosporins	Zwitterions can penetrate the outer membrane of Gram-negative bacteria. They also have a greater resistance to β-lactamases than the third generation cephalosporins
Fifth	Ceftobiprole, ceftaroline, ceftolozane	-	Strongly active against Pseudomonas sp. and appears to be less susceptible to resistance development

The first generation includes cefazolin and cephalexin (Chaudhry et al. 2019; Eljaaly et al. 2018). Their spectrum of action contains staphylococci, streptococci and some Gram-negative bacteria like *Proteus mirabilis*, *Escherichia* (*E.*) *coli*, and *Klebsiella* (*K.*) *pneumoniae* (Chaudhry et al. 2019; Eljaaly et al. 2018).

The second generation of cephalosporins is divided into the true second generation cephalosporins and the cephamycins. Cefuroxime and cefprozil can be assigned to the true second generation cephalosporins. The cephamycins include cefoxitin, cefotetan, and cefmetazole. This generation is often prescribed for treatment of respiratory infections such as bronchiolitis or pneumonia (Bui and Preuss 2020). Due to their broad spectrum, also cephalosporins of the third generation are widely used in the treatment of severe infections. This generation includes ceftriaxone, cefotaxime, ceftazidime, ceftazidime/avibactam, cefdinir, cefpodoxime, cefixime, but also cefoperazone and ceftiofur. It is the first generation that is considered as extended-spectrum cephalosporins and shows more stability to β-lactamases than the two previous (Arumugham and Cascella 2020; Chaudhry et al. 2019). The fourth generation cephalosporins includes cefepime and cefquinome, which has an additional quaternary ammonium group. This group allows a better penetration of the outer membrane of Gram-negative bacteria (Bui and Preuss 2020). Based on its broad spectrum, it is reserved to treat multi-resistant organisms.

Finally, the fifth generation cephalosporins, the so-called anti-methicillin-resistant *Staphylococcus* (*S.*) *aureus* (MRSA) cephalosporins, include ceftaroline and ceftibiprole (Zhanel et al. 2008). They exhibit a broad-spectrum activity against many important community-acquired Gram-positive and Gram-negative pathogens (including multidrug-resistant strains) (Duplessis and Crum-Cianflone 2011).

1.1.1.3 Monobactams

The structure of monobactams differs from that of the other β-lactams. They are characterized by a non-fused β-lactam nucleus, which means the β-lactam ring is not fused with additional rings as described in the other β-lactams (Palzkill 2013). The only monobactams, which gained the approval for the human therapeutic use is aztreonam (Bush and Bradford 2016; Sykes et al. 1982). It is used to treat human infections with aerobic enteric bacteria, *Pseudomonas* (*P.*) *aeruginosa*, *S. aureus*, *Streptococcus* (*S.*) *pneumoniae*, and *Enterococcus* (*E.*) *faecalis* (Sykes et al. 1982). However, aztreonam is not approved for use in veterinary medicine.

1.1.1.4 Carbapenems

The discovery of carbapenems was a result in the search for a potent antibiotic, initiated by increasing reports about resistances against the other β-lactams (Papp-Wallace et al. 2011; Zhanel et al. 2007). The first carbapenem, thienamycin, was limited in use due to its instability in liquid solutions and its sensitivity to mild base hydrolysis (Bush and Bradford 2016; Papp-Wallace et al. 2011). Nowadays, there are four commonly used carbapenems; imipenem, ertapenem, meropenem and doripenem (Nordmann et al. 2012; Papp-Wallace et al. 2011). In addition to the β-lactam ring, its five-membered ring contains a carbon in the C-1 position and a double bond between C-2 and C-3 (Figure 1) (Terico and Gallagher 2014; Palzkill 2013). These days, carbapenems are the β-lactams with the broadest spectrum of activity. This is mainly due to their stability against most β-lactamases and their ability to bind several types of PBPs (Hashizume et al. 1984). Carbapenems are categorized as Highest Priority Critically Important Antimicrobials by the WHO (WHO 2017). As a part of the earlier described Regulation (EC) No 1831/2003 to contain resistances, carbapenems are not approved for use in the veterinary food chain sector in the EU (OIE 2007). They may be used in non-food producing animals, such as dogs and cats, according to the cascade regulations. Nevertheless, cases of carbapenem-resistant (CR) bacteria in livestock and food animals, in wildlife animals, and in companion animals have been reported frequently in recent years (Köck et al. 2018).

1.2 Antimicrobial resistances

The discovery of antimicrobial agents was a milestone on the way to modern medicine. Therefore, it is not surprising that AMR is a threat to modern health care (Cassini e al. 2019; Eichenberger and Thaden 2019). Annually 33,000 deaths per year are a direct consequence of an infection with resistant bacteria (Cassini e al. 2019) and may increase to 10 million deaths per year by 2050 (De Kraker et al. 2016). Bacterial resistances can be either natural/intrinsic or acquired (Wright 2010). Intrinsic resistance is often caused by the absence or inaccessibility of a target for the antimicrobial agent in a bacterial species or genus. Bacteria with acquired resistance become resistant to a previously effective antibiotics through natural mutations or the acquisition of specific resistance genes through horizontal gene transfer (Antão et al. 2018). Nowadays, resistant bacteria are reported from the human and the veterinary sector as well as from environmental samples (Dolejska et al. 2019; Antão et al. 2018; Madec and Haenni 2018). Since AMR appears in such versatile environments, preventive, interdisciplinary approaches are needed (Taneja and Sharma 2019).

1.2.1 Resistance against β-lactam antimicrobials including carbapenems

There are different mechanisms that induce resistance to β-lactams (Davies and Davies 2010). A resistance could be based on an alteration of PBPs, the prevention of the entering of antimicrobial agents into the bacterial cell by altering the cell wall, an active efflux of antimicrobial agents and the production of degrading enzymes (Davies and Davies 2010). These so-called β-lactamases play an important role in the spread of β-lactam resistances. They are defined by the Nomenclature Committee of the International Union of Biochemistry, as enzymes hydrolyzing amides, amidines and other C-N bonds (Webb 1989). In this process, the enzymes inactivate β-lactams by hydrolyzing the β-lactam ring (Abraham and Chain 1988).

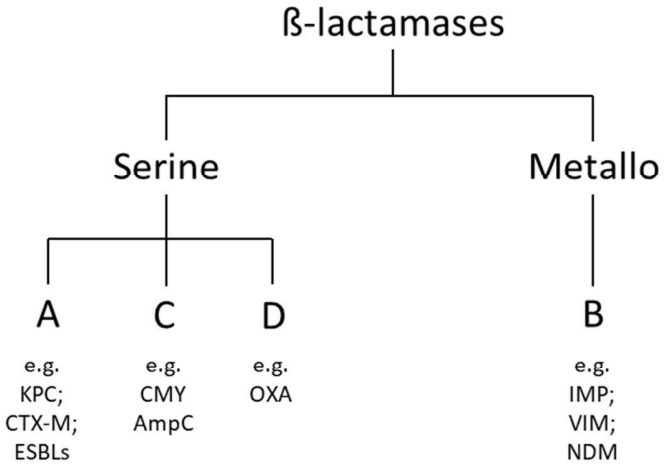

Figure 2: Classification of β-lactamases according to Ambler 1980.

Initially, the structural classification of β-lactamases was proposed by Ambler et al. in 1980. They classified the enzymes into molecular Ambler classes A, B, C and D (Ambler 1980). They are denoted with a "*bla*" (for "β-lactamase") followed by an enzyme-specific name as subscript, e.g. $bla_{\text{CTX-M-14}}$. Beside this Ambler classification, the combination of their molecular and biochemical classification resulted in 17 functional groups (Bush 2018). The molecular classes A, C and D use a serine-based mechanism for hydrolysis of β-lactams whereas the molecular class B is zinc-based and therefore classified as a Metallo-β-lactamase (Figure 2) (Kong et al. 2010).

1.2.1.1 Class A β-lactamases

The first of the three serine-based β-lactamase groups can be encoded chromosomally or by plasmidal resistance genes. The first plasmid-mediated class A β-lactamase was the TEM-1. It was detected in an *E. coli* isolate of clinical origin in Greece (Bush 2018). Another β-lactamase of class A is the SHV-1 (sulfhydryl variable) (Kliebe et al. 1985). Both encode resistance to penicillins and early-stage cephalosporins (Bush 2018). Since their first detection, several variants had developed, a lot of them showing an extended-spectrum activity against β-lactams (Bush 2018). Another group within the class A β-lactamases are the CTX-M β-lactamases (Bauernfeind et al. 1990). They are considered the most prevalent extended-spectrum β-lactamases (ESBL) in many European countries today (Livermore et al. 2007; Bauernfeind et al. 1990). The encoding genes are often associated with conjugative plasmids. For example, plasmids of the incompatibility (Inc) group. IncI1 seems to be an important factor of the spread of CTX-M-1 in European food products (Bevan et al. 2017; Irrgang et al. 2017a; Livermore et al. 2007).

In addition, some carbapenemases are classified as class A β-lactamases. An important representative, expressed by the carbapenemase gene *bla*$_{KPC-1}$, was first detected on a non-conjugative plasmid in a *K. pneumonia* isolate collected in an US American hospital (Yigit et al. 2001). Nowadays, *Klebsiella-pneumoniae*-carbapenemases (KPC) are harbored by different enterobacteria and found in samples of diverse origin (Taneja and Sharma 2019; Bonardi and Pitino 2019).

1.2.1.2 Class B β-lactamases

Class B β-lactamases are also known as Metallo-β-Lactamases (Figure 2). Their effect is based on zinc ions (Zn^{2+}) in the active center (Felici et al. 1993). These enzymes are able to inactivate most β-lactams (Palzkill 2013). Their broad spectrum includes an activity against some carbapenems. CR bacteria could be isolated from human and environmental samples, but also from samples of animal origin (Eichenberger and Thaden 2019; Köck et al. 2018; Albiger et al. 2015; Patel and Bonomo 2013).

The Metallo-β-Lactamase imipenemase (IMP) is an important representative of this group. The genes are located in class 1 integrons carried by plasmids and therefore able to spread horizontally among different species (Bonardi and Pitino 2019). Moreover, the New Delhi Metallo-β-Lactamases (NDM) and the Verona Integron Metallo-β-Lactamase (VIM) are the most common Metallo-β-Lactamases that are also able to hydrolyze carbapenems (Bonardi and Pitino 2019). *bla*$_{NDM}$ genes were reported from species like *E. coli*, *Acinetobacter* (*A.*) spp. and *Pseudomonas* spp. independent of the sample origin (Pitout et al. 2015; Mushtaq et al. 2011; Walsh et al. 2011). They can be located on plasmids and within the chromosome. Therefore, the dissemination occurs by horizontal as well as by vertical gene transfer (Poirel et al. 2011a). Today, the NDM variants NDM-1 to 31 are known (National Center for Biotechnology Information 2020). Lauretti et al. described the first discovery of a VIM carbapenemase in 1999. The carbapenemase-producing (CP) *P. aeruginosa* was isolated in Verona, Italy from a clinical sample (Lauretti et al. 1999). Since this report, at least 73 VIM variants were described(National Center for Biotechnology Information 2020). According to the variants, VIM carbapenemases were detected in Enterobacteriaceae, in *Acinetobacter* and *Pseudomonas* species (Bonardi and Pitino 2019). *bla*$_{VIM}$ genes are often associated with class 1 integrons. The VIM-1 enzyme was the first carbapenemase detected in European livestock in 2012 (Fischer et al. 2012a). The *E. coli* and *Salmonella* spp. isolates were recovered from German pig and poultry farms and harbored an IncHI2 plasmid (Fischer et al. 2013a). Since that first reports, the *bla*$_{VIM-1}$ gene is the most reported carbapenemase gene in German livestock.

1.2.1.3 Class C β-lactamases

The first report about penicillin-hydrolyzing enzymes was a chromosomal AmpC β-lactamase of *E. coli* in 1940. The name AmpC correspond to the *ampC* gene, which is chromosomally located in almost all Enterobacteriaceae and in *P. aeruginosa* (Jacoby 2009). Historically, it was the third mutation in an *ampA* strain and resulted in reduced resistance. *AmpC* was classified as the structural gene for the enzyme due to their little production of any β-lactamase (Burman et al. 1973). Such enzymes are inducible (Lindström et al. 1970). By mutations, they can be expressed at high levels which confers to phenotypical resistance to broad-spectrum cephalosporins.

Since the late 1980s, plasmid-encoded AmpC-type enzymes were reported on high-copy-number plasmids (Philippon et al. 2002; Walther-Rasmussen and Høiby 2002). The first plasmid encoded AmpC beta-lactamase CMY-1 was described by Bauernfeind in 1989 (Bauernfeind et al. 1989). It may have arisen by mobilization of chromosomal c*AmpC* genes from various Enterobacteriaceae (i.e. *Citrobacter* (*C.*) *freundii* and *Enterobacter* (*E.*) *cloacae*) (Philippon et al. 2002). In the meantime, both chromosomally and plasmidal located enzymes are widely distributed in several Enterobacteriaceae around the world (Jacoby 2009). Other examples for plasmid-mediated AmpC β-lactamases are varieties of FOX, ACC, DHA and others (Jacoby 2009).

Moreover, the spectrum can arise by mutations related to outer membrane porins (reduced influx) (Van Boxtel et al. 2016) or to efflux pumps (enhanced efflux) (Quale et al. 2006; Lindström et al. 1970). Meanwhile, there are reports of AmpC producers in clinical, environmental and animal samples (Dorado-Garcia et al. 2018).

1.2.1.4 Class D β-lactamases

Class D β-lactamases, or oxacillinases (OXA), are able to hydrolyze different types of β-lactams, e.g. the name-giving oxacillin. One example, the variant OXA-48 expresses resistances to penicillins and partially to carbapenems (Naas and Nordmann 1999). The first OXA-48 producer was detected in 2001 in Turkey from a human clinical sample (Poirel et al. 2004). Meanwhile, these enzymes have spread globally (Ceccarelli et al. 2017; Bush and Bradford 2016; Nordmann et al. 2012 ; Poirel et al. 2004).

Despite their potential to cause resistance to penicillins and carbapenems, most OXA β-lactamases are susceptible to cephalosporins (Codjoe and Donkor 2017). This susceptibility complicates the detection and isolation of OXA-producers. These difficulties are due to the classical isolation methods for ESBL- or CP bacteria which are based on selective media containing cefotaxime (Bortolaia and Hendriksen 2018). In addition, these genes are often weakly expressed, resulting in only slightly increased minimal inhibitory concentrations (MICs) in the usual phenotypic tests. Therefore, an underestimation of their prevalence is assumed (Nordmann et al. 2012). Moreover, the mutation potential of OXA β-lactamases can lead to an extension of their enzymatic activity (Codjoe and Donkor 2017). One example is OXA-163, which hydrolyses expanded-spectrum cephalosporins, and weakly carbapenems (Abdelaziz et al. 2012; Poirel et al. 2011b).

1.3 Transfer of antimicrobial resistances

1.3.1 Transfer of mobile genetic elements

Mobile genetic elements (MGEs), e.g. gene cassettes, and transposons, are defined by their capability of changing their position within and between different genomes (Siguier et al. 2014). The movement of DNA is based on enzymes which enable a ´cut and paste´ or a ´copy and paste´ mechanism, i.e. recombinases, transposases, integrases, and resolvases. Another group of MGEs are plasmids.

Gene cassettes are small MGEs which often consist only of a single gene and a recombination site. They can exist in a circular form for a short time or incorporate into larger genetic structures. Their movement often depends on their association with self-transmissible genetic elements, but they can also be components of non-conjugative transposons or corresponding plasmids. Therefore, they often integrate by site-specific recombination (Norman et al. 2009). An integrase recognizes a recognition site *attC* and the gene cassette incorporates into an *attI* specific site next to the integrase encoding genes of the integron (Norman et al. 2009). Integrons are genetic elements that can contain one or more gene cassettes. Therefore, they contribute to the genomic complexity of bacteria. Integrons self-possess an integron-associated promotor which leads to the expression of the 5´ adjacent incorporated gene cassettes (Hall 2012). There are different classes of integrons, each of which code for a class specific integrase (Norman et al. 2009).

Transposable elements (TEs) or in short transposons are DNA sequences, which are able to change the position in a genome (Bourque et al. 2018). Therefore, a movement within a DNA sequence (chromosome or plasmid) and even between sequences (i.e. from a plasmid to another) is possible. Insertion sequences (ISs) are the most simple form of transposons. They carry transposase genes for their transposition (Siguier et al. 2014) and are commonly flanked by inverted tandem repeats (ITR). These ITR are essential for the transposition mechanism. A transposition can be non-replicative (the target DNA is dislocated) or replicative (a copy of a genetic element is generated) (Siguier et al. 2014). Composite transposons are flanked by such IS elements which code for the transposition functions (Bennett 2008).

Plasmids are circular, double-stranded, extrachromosomal, autonomously replicating DNA elements (Lerminaux and Cameron 2019; Norman et al. 2009). They contain core genes and evolutionary acquired accessory genes (Norman et al. 2009). Moreover, they can carry ISs, conjugative or non-conjugative transposons and a multitude of additional genes, i.e. genes encoding resistance to antimicrobial agents, heavy metals, or biocides (Carattoli 2013).

Therefore, the size of plasmids is very variable and ranges from several kilobases (kb) to several hundred kb. They can be conjugative, non-conjugative or mobilizable by so-called helper plasmids. Due to this property, they are associated with the spread of i.e. resistance or virulence genes (Carattoli 2013).

Plasmids can be classified into so-called incompatibility (Inc) groups. Their incompatibility depends on the similar replication and partition systems. In general, bacteria can harbor more than one plasmid. In that case, the plasmids must have various replication systems. Otherwise, they cannot occur or be replicated and are therefore incompatible with each other (Dionisio et al. 2019). Nowadays, 27 different Inc groups are described in Enterobacteriaceae. Some of them are often associated with AMR genes, i.e. IncF, IncI, IncA/C, IncH, IncL/M and IncN (Shintani et al. 2015).

1.3.2 Horizontal gene transfer

While the vertical transmission describes the passing of the genetic information by cell division to the next generation, the horizontal gene transfer means the spread of genetic elements between bacteria of the same or different species or even genera (Lerminaux and Cameron 2019). The horizontal gene transfer between bacteria can occur by either of the three mechanisms: transformation, transduction and conjugation/mobilization (Lerminaux and Cameron 2019).

Transformation is the uptake of free DNA which binds to the DNA-binding protein on the surface of competent cells. The DNA is taken into the cell and bound on a second protein that protects the DNA against degradation. The DNA will be incorporated by recombination (Juhas 2015).

Transduction describes a mechanism of DNA transfer by bacteriophages (Juhas 2015). Bacteriophages need to inject their genetic material into bacterial host cell for their replication. Based on their life cycle, bacteriophages are divided into virulent and temperate ones. Temperate bacteriophages are initially in the lysogenic mode. In this inactive mode, they are referred to as prophages. After external induction (i.e. by UV-light), the lytic mode is started, and the entire cell metabolism is switched to the formation of phage particles. The replication of the phage DNA and the production of the corresponding proteins is followed by the assembly of phage particles, which – when released – cause the death of the bacterial cell (Juhas 2015; Balcazar 2014). Bacterial DNA, including plasmids, can be mistakenly packed into the phage capsid. The spread of foreign DNA by phages is limited by the amount of DNA that can be incorporated into the phage capsids. Moreover, the receptor specificity limits the host range of the phage (Balcazar 2014).

The main way of transfer of large DNA molecules like plasmids, is conjugation (figure 3). Conjugation is an important mechanism for the spread of resistance genes between bacteria not only of the same species but also of different species (Cabezón et al 2017). AMR genes are often located on large, conjugative plasmids which control their own copy number (Norman et al. 2009; Nordström 2006). Plasmids that are not able to conjugate themselves (non-conjugative plasmids), can be transferred by so-called helper plasmids (Partridge et al. 2018). In both cases, a close contact between the donor cell and the recipient is required to transfer the single-stranded DNA. This close contact is enabled by the formation of a complex, known as mating pair formation, in which a conjugation pilus is generated. This pilus extends from the donor to the recipient cell and enable the exchange of the plasmid. For this purpose, the conjugative double-stranded plasmid is cleaved by an endonuclease at the origin of transfer (*oriT*). Thereafter, the so-called relaxosome is formed. It contains the single-stranded plasmid DNA and proteins needed for the DNA transfer, including relaxase. A coupling protein mediates the further uptake in the recipient cell. Finally, the relaxosome enables the formation of the second strand of plasmid DNA in both the donor and recipient cell (Cabezón et al. 2017; Getino and De la Cruz 2017). Non-conjugative plasmids that are in the same host cell can use the transfer machinery of conjugative plasmids and are called mobilizable plasmids (Getino and De la Cruz 2017).

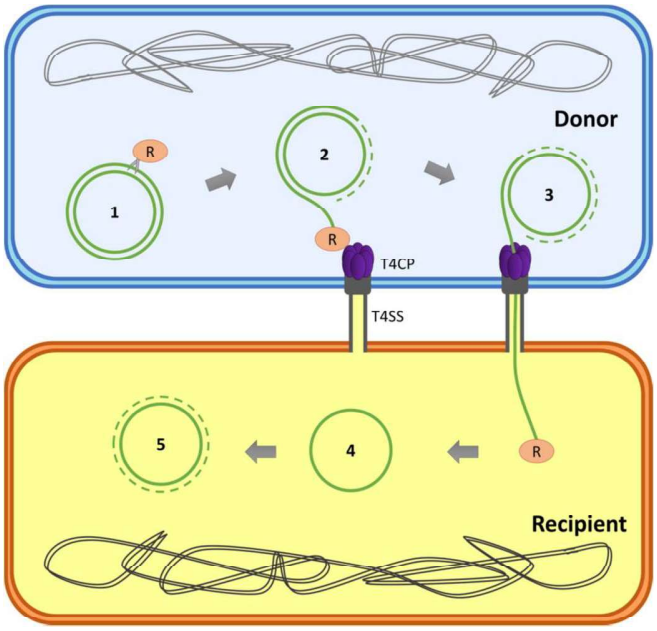

Figure 3: Transfer of plasmids during bacterial conjugation by Getino and De la Cruz 2017. R; Relaxase

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1.3.3 The One Health principle

The One Health principle describes the approach of close connections between humans, animals and the environment (Antão et al. 2018). Infectious agents, including resistant bacteria, may cross species and environmental barriers to move between these compartments. Therefore, these three systems are closely linked by various vectors (figure 4).

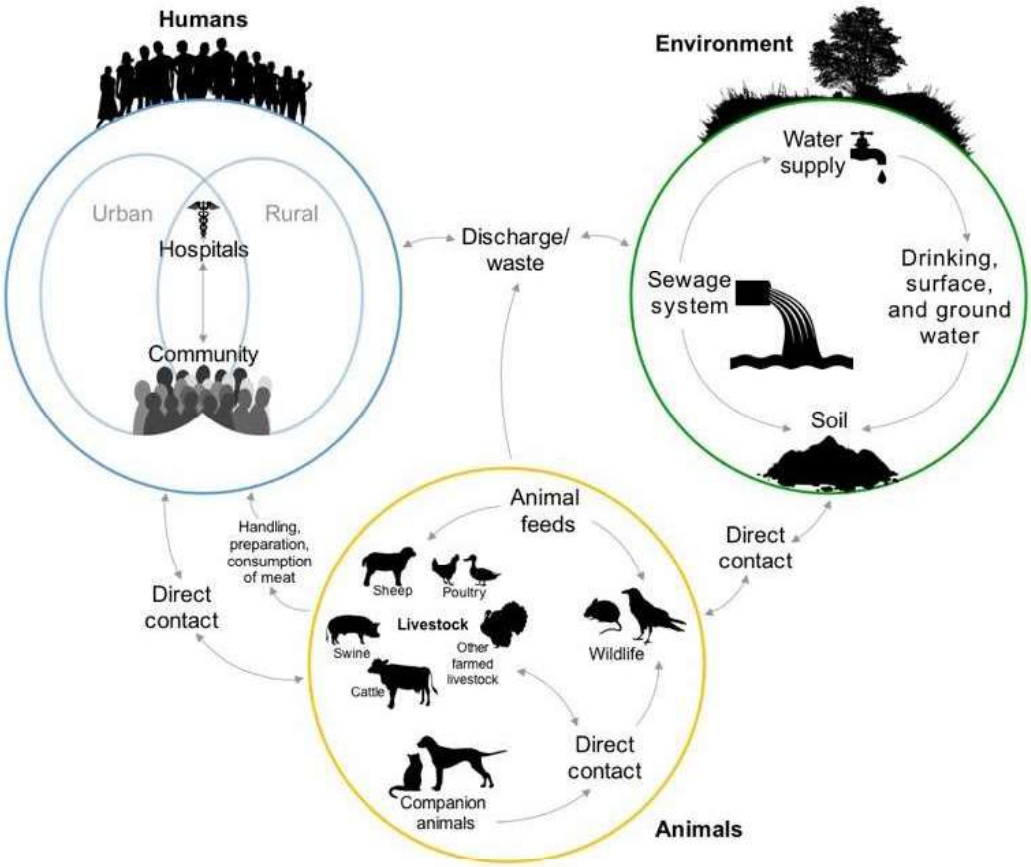

Figure 4: Graphical representation of the One Health Principle adapted from https://www.ucdavis.edu/one-health-in-action-amr/ (last access at 18.01.2021).

None of these compartments can be considered on their own. This approach can be applied to the problem of antibiotic resistance. The spread of resistant bacteria is hard to control. On one hand, that is because of the transmission of bacteria between areas. One illustrative example is the dissemination by water. Not just the movement of waters, i.e. rivers, but also the proximity of wildlife to rivers and the wastewater disposals are described as potential increasing factors of the dissemination of CPE and their corresponding genes (Proia et al. 2018).

On the other hand, the dissemination via MGEs plays an important role beyond all compartments. Despite the interconnected compartments, there are bacteria that are adapted to a specific niche. There are clonal lineages that are adapted to a specific reservoir or host. In this sense, the importance of reservoirs or possible transmission pathways should be addressed. In particular, the transmission from animals to humans via the food chain or via direct contact between animals and humans are of interest. An illustrative example for the transmission of zoonotic pathogens and resistance genes via the food chain was the enteroaggregative Shiga-toxin producing *E. coli* O104:H4-B1-ST678-$bla_{CTX\text{-}M\text{-}15}$ outbreak in 2011 in Germany (Beutin and Martin 2012). Starting from fenugreek seeds and sprouts, more than 3,800 patients became ill. The parallel transmission between humans, and humans and food complicated epidemiological investigations. Regarding raw food preparation in private and public kitchens, this example underlined the possible contaminations and disseminations. In addition, various studies discussed the possible transmission of pathogens and resistances via pets or food-producing animals and human (Köck et al. 2018; Stolle et al. 2013; Fischer et al. 2013b. Furthermore, the possible transmission between wild animals and humans was discussed. In Austrian rats (*Rattus norvegicus*) (Desvars-Larrive et al. 2016) and Spanish yellow legged gulls (*Larus michahellis*) (Vergara et al. 2017), carbapenemases were described and the authors discussed the potential transmission way through waste or wastewater.

1.4 Dissemination of carbapenemase genes

The first detected carbapenemase was the Metallo-β-lactamase IMP-1 in 1991 in Japan (Watanabe et al. 1991). Six years later, the VIM-1 was discovered in Italy (Lauretti et al. 1999). In 2002, *bla*$_{SMP}$ was found in Brazil (Queenan and Bush 2007) and the *bla*$_{NDM}$ gene was initially reported in 2008 in India (Yong et al. 2009). Since the first documentation, more and more carbapenemases were discovered and characterized. The acquirement of such carbapenemase genes is one of the most important reason for resistances against carbapenems of clinically important bacteria (Meletis 2016). Additionally, these genes are often co-located with further resistance determinants, which results in multidrug resistance (Meletis 2016). Nowadays, carbapenemase genes were reported from samples of human and veterinary origin as well as from environmental samples around the world. Some carbapenemase genes, like *bla*$_{NDM}$, are globally distributed, whereas other genes (i.e. *bla*$_{GIM}$, *bla*$_{SIM}$ or *bla*$_{POM}$) are only sporadically reported. As example, an overview of the prevalence of carbapenemase genes in human samples in Europe is shown in Figure 5.

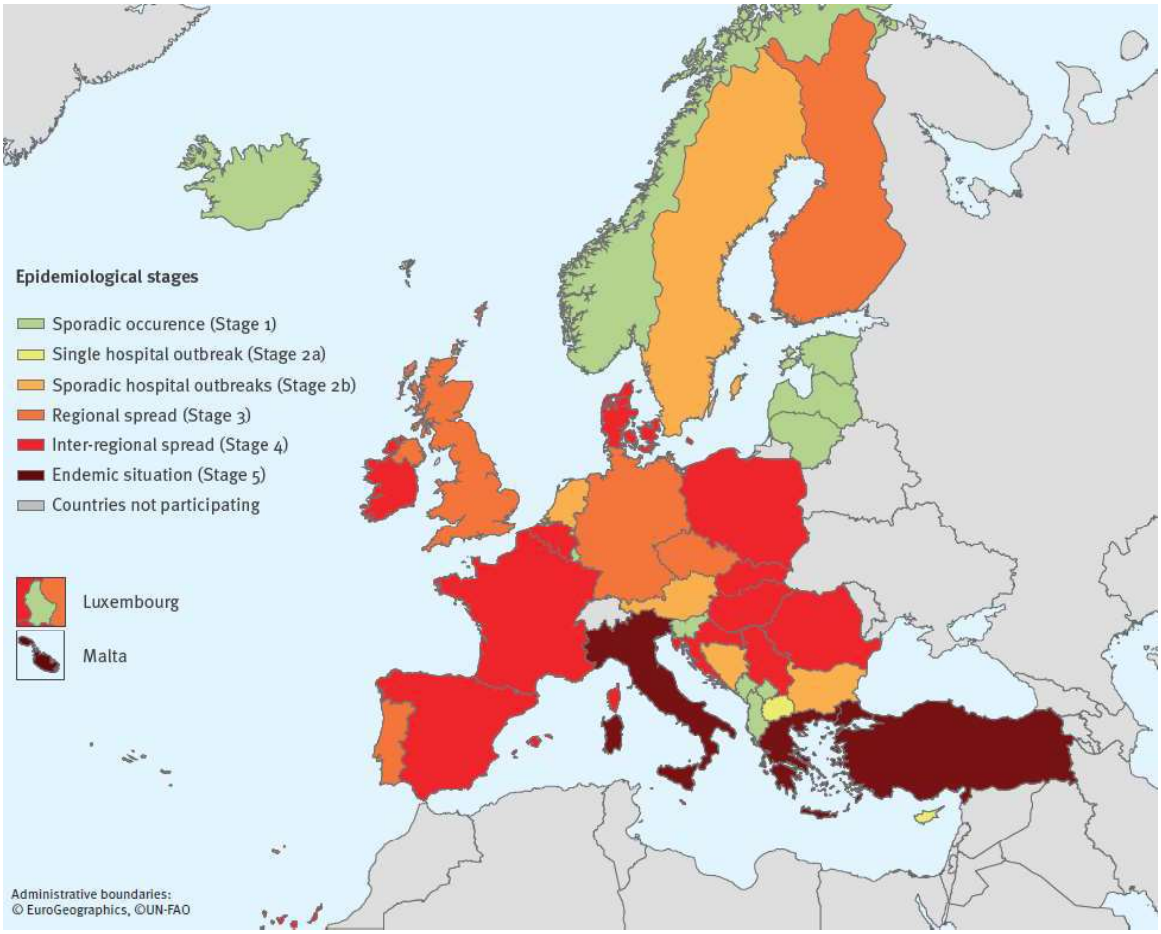

Figure 5: The spread of carbapenemase-producing Enterobacteriaceae in Europe in 2018 by Brolund et al. 2019 .

NDM variants are reported around the globe, but China, India and the central Balkans seem to be probable epicenters (Meletis 2016; Khan and Nordmann 2012). Predominant in *K. pneumonia* and *E. coli*, but in *A. baumannii* and *P. aeruginosa* as well, bla_{NDM} genes were detected in isolates from hospital equipment, environmental and animal samples from farms or humans (Hammoudi Halat and Ayoub Moubareck 2020). Another rapid dissemination was reported for the bla_{KPC} genes. After the first description in the late 1990s in the United States of America (USA) (Yigit et al. 2001), carbapenemases have spread to Latin America, Europe and China (Meletis 2016). Today, KPC producers became global and include different genera of Enterobacteriaceae (Bush and Bradford 2016). The bla_{KPC} genes are located on conjugative plasmids, which is a reason for its fast dissemination, and are associated to a single transposon, Tn*4401* (Hammoudi Halat and Ayoub Moubareck 2020). Next to clinical reports, KPC producers were isolated from environmental and animal samples worldwide (Hammoudi Halat and Ayoub Moubareck 2020; Köck et al. 2018).

The OXA-48 carbapenemases were mostly isolated in the Middle East and North Africa, but also detections in Europe, Turkey, India and other countries were reported (Meletis 2016), indicating a global spread. In Germany, this carbapenemase was the most frequently detected in clinical samples in 2018 (Pfennigwerth 2018). Typically, the bla_{OXA-48} gene is located on a highly conserved plasmid-prototype that carries an IncL/M replicon (Manageiro et al. 2015). Such plasmids were detected in isolates, i.e. isolated from *K. pneumoniae*, *E. coli* and *E. cloacae*, which have caused hospital outbreaks in many countries worldwide (Nordmann and Poirel 2014). Moreover, OXA-48 producing bacteria were isolated from livestock and food, companion animals and wildlife around the world (Köck et al. 2018).

Guiana extended spectrum (GES) enzymes are encoded by genes on integrons or plasmids. Based on point mutations, the ESBL phenotype can lead to increased hydrolyzing activity and carbapenem resistance. Therefore, only some lead to reduced sensitivity to carbapenems, such as GES-2, GES-5 and GES-20 (Hammoudi Halat and Ayoub Moubareck 2020). The corresponding bla_{GES} genes were described in various bacteria (i.e. *A. baumannii, Morganella* (*M.*) *morganii, E. coli* and *P. aeruginosa*) isolated from different sources (i.e. clinical samples, livestock) around the world (i.e. Brazil, Belgium, Canada, South Africa) (Hammoudi Halat and Ayoub Moubareck 2020).

The *bla*$_{VIM}$ genes are carried on a gene cassette inserted into a class 1 integron (Lauretti et al. 1999). These integrons can be incorporated in plasmids and chromosomes (Hammoudi Halat and Ayoub Moubareck 2020). After the discovery of VIM-1 in Italy, at least 77 additional variants were described around the globe (https://www.ncbi.nlm.nih.gov/pathogens/refgene/#gene_family:bla%20Vim, August 02, 2021). VIM-producers, i.e. Enterobacteriaceae and pseudomonads, were isolated from different sources in United Arab Emirates, North America, various states of the EU, among others. These examples illustrate the worldwide problem of increasing diversification of resistance genes and their dissemination in different bacteria.

1.4.1 Actual situation in veterinary medicine in Europe

Several European countries, i.e. Germany, Italy, France, and Greece, reported the sporadic isolation of CPE from livestock and food over the last years. The examined samples originated from various animal species (poultry, pigs, dairy cows, and sheep), animal products (meat and milk) and settings (farms, aquaculture farms, slaughterhouses). The most described carbapenemase gene in European food chain was *bla*$_{VIM-1}$ (Garcia-Graells et al. 2020; Irrgang et al. 2019; Borowiak et al. 2017; Irrgang et al. 2017b; Roschanski et al. 2017), which was mostly located on various plasmids (IncY, IncA/C$_2$, IncX3, IncHI2). Moreover, the plasmidal encoded OXA variants *bla*$_{OXA-181}$ (Pulss et al. 2017) and *bla*$_{OXA-55}$ (Zago et al. 2020) were detected. These genes were described in different Enterobacteriaceae (i.e. *E. coli*, *Salmonella* spp., *Klebsiella* spp., *Enterobacter* spp. and *Citrobacter* spp.).

By focusing on companion animals in Europe, most CPE were isolated from dogs and cats (Nigg et al. 2019; Boehmer et al. 2018; Pulss et al. 2018; Melo et al. 2017; Gonzalez-Torralba et al. 2016; Schmiedel et al. 2014; Stolle et al. 2013). Both usually are in closer contact to humans than other companion animals, or animals of livestock or wildlife. Different studies described the detection of *bla*$_{VIM-1}$, *bla*$_{NDM-5}$, *bla*$_{OXA-48}$, or *bla*$_{OXA-181}$-harbouring plasmids in Enterobacteriaceae or other species like *M. morganii, P. aeruginosa* or *P. mirabilis* (Köck et al. 2018). An interesting similarity was the location on small (up to ~60 kb), conjugative plasmids of different Inc groups (IncFII, IncX3, IncL/M-1 and IncL). One study described a chromosomally located gene *bla*$_{NDM-5}$ in *E. coli* isolated from a dog (Reynolds et al. 2019). The author reported an integrated 100 kb IncF plasmid.

In recent years, various studies focussed on the screening and characterization of CPE originating from wildlife. In different countries, species like birds, rats, foxes and wolves, seals and more were investigated, but just four studies described successful isolation of CPE (Duff et al. 2020; Desvars-Larrive et al. 2019; Fischer 2013b). The described CPE included two *E. xianfangensis* (NDM-1, rats) (Desvars-Larrive et al. 2019), one *K. pneumoniae* (OXA-48; seals) (Duff et al. 2020), one *S. enterica* subsp. *enterica* serovar Corvallis (NDM-1; wild bird) (Fischer 2013b) and two *E. coli* isolates (VIM-1, KPC-2; Gulls) (Vergara et al. 2017).

1.4.2 European monitoring on antimicrobial resistances

In 2012, a VIM-1 producing *E. coli* was isolated from a pig farm (Fischer et al. 2012a). That was the first report of a livestock-associated CPE worldwide. In order to avoid missing the spread of CPE through livestock, the European Reference Laboratory for Antimicrobial Resistances (EURL-AR) elaborated an isolation method for CP *E. coli* from meat and caecum samples. This method is based on a 1:10 enrichment in buffered peptone water without any selective pressure. After an incubation under aerobic conditions at 37 °C for 16-18 h, the enrichment should be inoculated on a selective agar (https://www.eurl-ar.eu/protocols.aspx). This procedure was implemented into the voluntary CP *E. coli* monitoring for the EU member states (Schrijver et al. 2018) and became mandatory in the monitoring programmes from 2021 (EU/2020/1729).

Since the voluntary monitoring in 2014, only six isolates with a resistant phenotype against carbapenems were detected. These isolates could be confirmed as four VIM-1 producing *E. coli*, one OXA-48 producing and one bla_{GES-5} producing *E. coli* (EFSA and ECDC 20207; EFSA and ECDC 2017). After publication of the initial isolations, reports about CPE in animals, especially in food and livestock, became more frequent in Europe, in the USA, in Asia and in Africa (Köck et al. 2018). Experts raised concern that animals may represent an unsuspected reservoir of CPE. Nevertheless, still little is known about carriage and dynamics of CPE in the livestock. Since the transmission from humans to animals and food, or vice versa could play an important role, a systematic monitoring was and is needed to protect consumers (Schrijver et al. 2018). In 2017 and 2018, a total of 20 countries participated in the European monitoring and analyzed 30,698 livestock associated samples. In Germany, one *E. coli* was confirmed as VIM-producer (EFSA and ECDC 2020). Interestingly, a second CP *E. coli* was isolated within the ESBL/AmpC producing *E. coli* monitoring, while the same sample specifically investigated for CPE was evaluated as CPE negative. All CP *E. coli* isolates from the German part of this monitoring from 2017-2020 were characterized in detail for the present work.

The low number of isolated CP *E. coli* from the monitoring can be interpreted differently. On one hand, this suggests an extremely low prevalence of CPE in the livestock in Europe. On the other hand, it could conclude to a lack of sensitivity of the isolation method. Due to the increasing number of reports of CPE in different veterinary sectors found in the literature, an optimization of the method seems rational. In addition, a modified and harmonized method would be supportive in terms of consumer protection and comparability of research data. Therefore, such a modification was one aim of the European Joint Project (EJP) "Improving phenotypic Antimicrobial Resistance Testing by development of sensitive screening assays for emerging resistances and setting missing ECOFFs" (IMPART).

1.5 The IMPART project

With the beginning of 2018, the European joint research project IMPART was launched (https://onehealthejp.eu/jrp-impart/) as a part of the One Health EJP Programme. The initiation of this project was one result of the EU Action Plan Against the rising threats from AMR: Road Map (EU 2015) (EFSA and ECDC 2020). One topic of this EU Action Plan was reinforced joint cross-border research to address AMR, e.g. the development of diagnostic tools for the detection of bacteria and resistance markers and harmonization of surveillance and monitoring (EFSA and ECDC 2020).

The IMPART project included four work packages (WP) related to the development and harmonization of phenotypic methods for detection of AMR, which were worked on by partners across Europe; Animal & Plant Health Agency (APHA), United Kingdom; DTU National Food Institute, Denmark; French Agency for Food, Environmental and Occupational Health & Safety (ANSES), France; The German Federal Institute for Risk Assessment (BfR), Germany; National Institute for Public Health and the Environment (RIVM), United Kingdom; Norwegian Veterinary Institute (NVI), Norway; National Veterinary Institute (SVA), Sweden; National Veterinary Institute - National Research Institute (PIWET), Poland; Netherlands Centre for One Health, Netherlands; Public Health England (PHE), United Kingdom; Statens Serum Institute (SSI), Denmark.

WP1 dealt with a selective isolation and detection of colistin-resistant Enterobacteriaceae, whereas WP2 dealt with carbapenemase-producing Enterobacteriaceae. The development of a disk diffusion method for antimicrobial susceptibility testing of *Clostridium difficile* was the topic of WP3. In WP4, the setting of epidemiological cut-off value (ECOFF) for specific bacterial pathogen/antimicrobial agent combinations was focussed. The BfR was a partner institution, active in all four WP and leader in WP3.

This doctoral thesis was conducted within WP2. Due to earlier detections of CPE in the German food chain, the aim of a sensitive and specific detection and isolation method is essential to determine the prevalence and the distribution of CPE in animals. Therefore, the isolation method provided by the EU for monitoring purposes was selected as the basis of this thesis.

1.6 Objectives of the PhD thesis

Over the last years, the detection of CPE in livestock increased. These detections led to the discussion on the importance of livestock as a reservoir for CPE and the resulting risk of transmission along the food chain. To detect the dynamics and occurrence of CPE, the EU has established a carbapenem resistance monitoring for *E. coli* isolates from food and livestock (EFSA and ECDC 2020). In Germany, we recognized that this procedure lacks efficacy for the recovery of VIM producing *E. coli* from caecum samples. Samples were negative in CPE monitoring, while CPE were detected in the same sample via ESBL monitoring. This lack of sensitivity justified one long-term goal of the IMPART project to optimize the microbiological and molecular detection and typing methods for CPE in this area. To detect the occurrence of CPE at an early stage in the future, a selective and sensitive cultivation method for the detection and isolation of low CPE concentrations is indispensable. The detailed characterization of recently isolated CPE from animal origin is a second aim of the project. Comparing such isolates was intended to provide insights into resistance mechanisms and dissemination processes, among others.

Therefore, as a part of this doctoral thesis, following objectives have been identified:

- The optimization of the commonly used isolation method for CPE by variation of microbiological parameters.
- The optimization of the detection of carbapenemase genes by validation of molecular methods.
- The validation of the modified method.
- The detailed characterization of new isolates as well as the comprehensive investigation of corresponding farms.

2 Published articles

The contribution of Natalie Pauly to the following articles was evaluated according to the following scheme:

A. Has contributed (0-33%)

B. Has contributed significantly (34-66%)

C. Has worked essentially independent (67-100%)

2.1 Publication 1

Pauly N, Hammerl J A, Schwarz S, Grobbel M, Meemken D, Malorny B, Tenhagen B-A, Käsbohrer A, Irrgang A.

Co-occurrence of the *bla*$_{VIM-1}$ and *bla*$_{SHV-12}$ genes on an IncHI2 plasmid of an *Escherichia coli* isolate recovered from German livestock

doi: https://doi.org/10.1093/jac/dkaa436

J Antimicrob Chemother 2021: 19; 76: 531-533.

The contribution of Natalie Pauly:

1. Design of the project including the design of the individual experiments: B

2. Conduct of the experimental part of the study: C

3. Analysis of the experimental results: B

4. Writing – original draft of the manuscript including data visualization: C

5. Writing – review and editing of the manuscript: B

Journal of Antimicrobial Chemotherapy

J Antimicrob Chemother
doi:10.1093/jac/dkaa436

Co-occurrence of the bla_{VIM-1} and bla_{SHV-12} genes on an IncHI2 plasmid of an *Escherichia coli* isolate recovered from German livestock

N. Pauly[1]*, J. A. Hammerl [1], S. Schwarz[2], M. Grobbel[1], D. Meemken[3], B. Malorny[1], B.-A. Tenhagen[1], A. Käsbohrer[1,4] and A. Irrgang [1]

[1]*Department Biological Safety, German Federal Institute for Risk Assessment, Berlin, Germany;* [2]*Institute of Microbiology and Epizootics, Freie Universität Berlin, Berlin, Germany;* [3]*Institute of Food Safety and Food Hygiene, Freie Universität Berlin, Berlin, Germany;* [4]*Unit for Veterinary Public Health and Epidemiology, University of Veterinary Medicine, Vienna, Austria*

*Corresponding author. E-mail: natalie_pauly@hotmail.com

Sir,

The dissemination of carbapenemase-producing Enterobacterales (CPE) is an important public health issue. The number of human CPE isolates has been steadily increasing during recent years, worldwide. Despite the fact that carbapenems are not licensed for use in veterinary medicine, increasing numbers of CPE from the veterinary sector have been reported.[1] The transmission of CPE between pets/livestock and exposed humans as well as via food has been demonstrated.[2] In this study, a detailed characterization of a carbapenem-resistant porcine *Escherichia coli* co-harbouring bla_{VIM-1}, bla_{SHV-12} and bla_{ACC-1} genes, along with other resistance genes, is provided.

Within the German annual monitoring of ESBL/AmpC β-lactamase-producing *E. coli* from animals and food in 2017–18, the isolate 17-AB02384 was recovered from the caecal content of a fattening pig at slaughter. Based on EUCAST epidemiological cut-off values (https://www.eucast.org/mic_distributions_and_ecoffs/), the isolate showed a non-WT phenotype to carbapenems and other antimicrobials [Table S1 (Table S1 is available as Supplementary data at *JAC* Online)]. Molecular analysis revealed that *E. coli* 17-AB02384 belonged to the phylogenetic group A and the multilocus sequence type (MLST) 7593.[3] To our knowledge, this ST has not yet been described in *E. coli* from German livestock or food chain. However, ST7593 was reported for some NDM-5-producing isolates of retail meat samples in China.[4]

Initial S1-PFGE plasmid profiling and subsequent DNA–DNA hybridization[3] indicated that the bla_{VIM-1} gene was located on an approximately 300 kb IncHI2 plasmid, designated pEC17-AB02384, which was transferable into the *E. coli* J53 by conjugation at a transfer rate of 2×10^{-4}. For a detailed characterization, the whole-genome sequence of *E. coli* 17-AB02384 was determined by Illumina (CA, USA) short-read and PacBio (Menlo Park, USA) long-read sequencing, according to the manufacturers' recommendations. Hybrid assembly of the plasmid sequence was carried out using unicycler v.0.44. The resulting sequence of the plasmid pEC17-AB02384 was deposited at GenBank (NCBI) under the accession number MT163739. MLST, resistance and virulence genes were determined using online tools that were provided by the Danish Technical University (http://www.genomicepidemiology.org) . The annotation was carried out by RAST2 provided by PATRIC (www.patricbrc.org). Characteristics of the isolate and its plasmid are summarized in Table S2.

Besides bla_{VIM-1}, this plasmid harboured the ESBL gene bla_{SHV-12} and the AmpC β-lactamase gene bla_{ACC-1}. Overall, pEC17-AB02384 showed similarity to the VIM-1-encoding plasmids pSE15-SA01028 (90% identity, CP026661.1) and pRH-R178 (93% identity, HG530658.1) from *Salmonella enterica* subsp. *enterica* and *E. coli*, respectively, which were both recovered from German pigs (Figure 1a).[5] In contrast to these plasmids, pEC17-AB02384 harboured three additional resistance gene-carrying segments. The 9773 bp segment 1 comprised the resistance genes *sul1* and *qnrA1* and was flanked by 124 bp inverted repeats. It was inserted into the region upstream of the bla_{VIM-1}-carrying class 1 multiresistance integron in pEC17-AB02384 (Figure 1b). Downstream of the integrase gene *intI1* of the bla_{VIM-1}-carrying integron, the segment 2 was integrated. It carried the macrolide resistance operon *mph*(A)-*mrx*-*mphR*, the trimethoprim resistance gene *dfrA14* and another *intI1* gene. Immediately downstream of both *intI1* genes, 202 bp direct repeats were found (Figure 1b). It appears possible that a translocatable unit comprising the entire segment 2 was inserted into plasmid pEC17-AB02384 by recombination with the *intI1* gene of the bla_{VIM-1}-carrying integron and its adjacent repeat region. The 4979 bp segment 3 was inserted into segment 2 between the Δ*rec* and the *mph*(A) gene. It carried the ESBL gene bla_{SHV-12} and was flanked by IS*26* elements in opposite orientation. However, no direct repeats were detected at the immediate boundaries of segment 3, suggesting that the IS*26*-bounded segment 3 does not function as a transposon.

To our knowledge, the co-occurrence of bla_{VIM-1} and bla_{SHV-12} has not yet been described in plasmids from isolates of animal origin. Co-location of these genes was reported only for a single human clinical *Aeromonas caviae* isolate (KR869764) in 2014.[6] The corresponding plasmid belonged to the replicon type IncA/C and showed no substantial similarities to pEC17-AB02384. SHV-12 is an extended-spectrum β-lactamase that is commonly detected in isolates from poultry, but rarely from pigs.[7] Alonso *et al.*[7] provided further data on bla_{SHV-12}-carrying plasmids from human, animal and food sources. Among them, the *E. coli* plasmid pCAZ590 (LT669764) exhibits a similar SHV-12 region as the one identified in pEC17-AB02384. In general, bla_{SHV-12} seems to be associated with

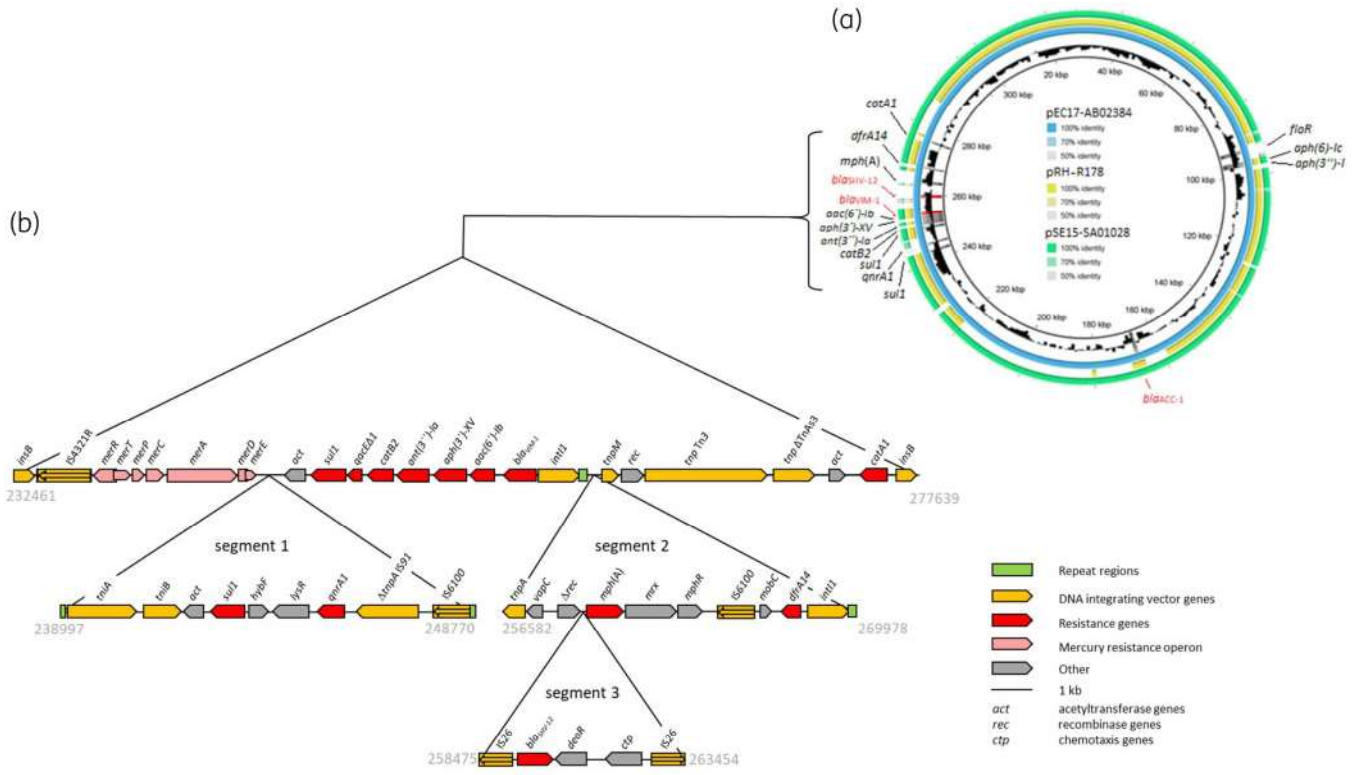

Figure 1. (a) Schematic illustrations of plasmids pRH-R178 and pSE15-SA01028 in comparison with plasmid pEC17-AB02384 described in this sudy. (b) Schematic illustration of the multidrug resistance region of plasmid pEC17-AB02384. The reading frames are displayed as arrows with the arrowhead showing the dirction of transcription. The numbers refer to the whole plasmid sequence of pEC17-AB02384, which is deposited in the GenBank database under accession no. MT163739. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

IS*26* elements, which might support its mobility. The ability of IS*26* to mobilize neighbouring genes might play an important role in the persistence of antimicrobial resistances.[8]

A comparison of the few known bla_{VIM-1}-carrying plasmids from German livestock revealed close relationships. This might indicate that a prototype-plasmid has adapted to bacteria in different animal populations and persists in an unknown reservoir. The characterization of CPE isolates and their plasmids will contribute to the further understanding of reservoirs, potential transmission pathways and persistence factors for plasmid maintenance in bacteria from animal husbandry.

Acknowledgements

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Transparency declarations

We declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online.

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2.2 Publication 2

Pauly N, Hammerl J A, Grobbel M, Käsbohrer A, Tenhagen B-A, Malorny B, Schwarz S, Meemken D, Irrgang A.

Identification of a *bla*$_{VIM-1}$-Carrying IncA/C2 Multiresistance Plasmid in an *Escherichia coli* Isolate Recovered from the German Food Chain

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Identification of a *bla*$_{VIM-1}$-Carrying IncA/C_2 Multiresistance Plasmid in an *Escherichia coli* Isolate Recovered from the German Food Chain

Natalie Pauly [1,*], Jens Andre Hammerl [1], Mirjam Grobbel [1], Annemarie Käsbohrer [1,2], Bernd-Alois Tenhagen [1], Burkhard Malorny [1], Stefan Schwarz [3], Diana Meemken [4] and Alexandra Irrgang [1,*]

1 Department Biological Safety, German Federal Institute for Risk Assessment, 10589 Berlin, Germany; Jens-Andre.Hammerl@bfr.bund.de (J.A.H.); mirjam.grobbel@bfr.bund.de (M.G.); annemarie.kaesbohrer@bfr.bund.de (A.K.); Bernd-Alois.Tenhagen@bfr.bund.de (B.-A.T.); Burkhard.Malorny@bfr.bund.de (B.M.)
2 Institute for Veterinary Public Health, University of Veterinary Medicine, 1210 Vienna, Austria
3 Institute of Microbiology and Epizootics, Freie Universität Berlin, 14163 Berlin, Germany; stefan.schwarz@fu-berlin.de
4 Institute of Food Safety and Food Hygiene, Freie Universität Berlin, 14163 Berlin, Germany; Diana.Meemken@fu-berlin.de
* Correspondence: natalie_pauly@hotmail.com (N.P.); alexandra.irrgang@bfr.bund.de (A.I.)

Abstract: Within the German national monitoring of zoonotic agents, antimicrobial resistance determination also targets carbapenemase-producing (CP) *Escherichia coli* by selective isolation from food and livestock. In this monitoring in 2019, the CP *E. coli* 19-AB01133 was recovered from pork shoulder. The isolate was assigned to the phylogenetic group B1 and exhibited the multi-locus sequence-type ST5869. Molecular investigations, including whole genome sequencing, of 19-AB01133 revealed that the isolate carried the resistance genes *bla*$_{VIM-1}$, *bla*$_{SHV-5}$ and *bla*$_{CMY-13}$ on a self-transmissible IncA/C_2 plasmid. The plasmid was closely related to the previously described VIM-1-encoding plasmid S15FP06257_p from *E. coli* of pork origin in Belgium. Our results indicate an occasional spread of the *bla*$_{VIM-1}$ gene in Enterobacteriaceae of the European pig population. Moreover, the *bla*$_{VIM-1}$ located on an IncA/C_2 plasmid supports the presumption of a new, probably human source of carbapenemase-producing Enterobacteriaceae (CPE) entering the livestock and food chain sector.

Keywords: carbapenem; metallo-β-lactamase; IncA/C_2; WGS; *bla*$_{VIM-1}$

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1. Introduction

Carbapenems are last resort antimicrobial agents against several infections caused by multidrug-resistant bacteria in human medicine. The World Health Organization defines them as "Highest Priority Critically Important Antimicrobials". Nevertheless, the reports of carbapenem resistant bacteria are not limited to human medicine [1]. The repeated detection of carbapenemase-producing (CP) Enterobacteriaceae (CPE) in the German food-production chain is of considerable concern [1,2]. In general, CPE have been isolated sporadically from non-human sources (i.e., livestock, pets, wildlife) although carbapenems are not approved for veterinary application [2]. One of the main mechanisms of carbapenem resistance is the production of degrading enzymes, so called carbapenemases, which can hydrolyze almost all available β-lactams, including carbapenems [3]. The corresponding genes are often located on mobile genetic elements, in particular plasmids, which often carry additional antimicrobial, biocide, or heavy metal resistance genes [4]. These plasmids can be horizontally spread to bacteria under the selective pressure also imposed by non-β-lactam agents [5,6]. Since 2011, CPE isolates, which harbored VIM-1 encoding IncHI2 plasmids, were detected in livestock and food [7–12]. The first report described *Salmonella* Infantis and

E. coli isolates detected in 2011 from fattening pigs in Germany [10]. They could persist in the animal host and closely related isolates could be detected in fattening pigs and minced meat in the following years [7,8]. Another VIM-1 report described the detection of this gene on an IncY plasmids in *E. coli* originated from seafood samples in Germany [13]. Besides the increasing reports of VIM-1 producing Enterobacteriaceae in the German food chain, the variants of carbapenemase genes increased as well. In 2019 within the annual resistance monitoring in the food chain in Germany, three CP *E. coli* were isolated. These include an OXA-48 producing *E. coli* [14] and a GES-5 producing *E. coli* [15], both isolated from pig feces, and another VIM-1 producing *E. coli* from a pork sample. This $bla_{\text{VIM-1}}$ harboring *E. coli* isolate 19-AB01133 and its comprehensive molecular characteristics are described in this study. It shows great similarities to an *E. coli* isolate described by Garcia-Graells et al. (2020) [16] that was isolated from a pork sample in Belgium. Both isolates harbored almost identical IncA/C_2 plasmids, which encode the $bla_{\text{VIM-1}}$ gene. Furthermore, the genome data hint to a specific human entry source.

2. Materials and Methods

2.1. Isolate Origin and Antimicrobial Susceptibility Testing

The *E. coli* isolate 19-AB01133 was obtained in 2019 in the framework of the annual resistance monitoring in the food chain in Germany. The selective isolation of the CP *E. coli* was performed at a Federal State Laboratory, following Commission Implementing Decision (CID) 2013/652/EC and protocols provided by the European Union Reference Laboratory for Antimicrobial Resistance (EURL-AR) (https://www.eurl-ar.eu/protocols.aspx). The *E. coli* isolate 19-AB01133 was recovered from a shoulder meat sample of a pig raised in Germany. The pig was fattened and slaughtered in Brandenburg. The isolate was sent to the German Federal Institute for Risk Assessment for further confirmation and phenotypic and genotypic characterization. Antimicrobial susceptibility testing according to the EN ISO20776-1:2006 was conducted by broth microdilution using commercial plates (Thermo Fisher Scientific, Schwerte, Germany) with antimicrobial substances and concentrations defined by CID 2013/652/EC. The minimum inhibitory concentration (MIC) values were interpreted based on epidemiological cut-off values defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org) and fixed in CID 2013/652/EU.

2.2. Phenotypic and Genotypic Characterization

Species confirmation of the isolate was conducted by MALDI-TOF Microflex LT/SH (Bruker Daltonics, Bremen, Germany) according to the manufacturer's recommendations. Therefore, α-Cyano-4-hydroxycinnamic acid (HCCA, Bruker, MA, USA) was used as the matrix. Molecular determination of the carbapenem-resistance genes and initial phylogenetic typing were performed as previously described [14]. The *E. coli* 19-AB01133 was subjected to S1-nuclease pulsed-field gel electrophoresis (S1-PFGE) [17] (https://www.cdc.gov/pulsenet/pathogens/protocols.html) followed by Southern blot hybridization [18] against a digoxigenin-labelled $bla_{\text{VIM-1}}$ probe while using DIG EasyHyb and DIG Wash and Block Buffer Set (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendations. The transmissibility of the plasmid was investigated by in vitro filter-mating studies using *E. coli* K12 J53 as the recipient. Moreover, the plasmid pEC19-AB01133 was extracted by using the CosMCPrep Plasmid Purification Kit (Beckman Coulter, Krefeld, Germany) according to the manufacturer's recommendations. The plasmids were transformed into highly competent *E. coli* DH10B (ElectroMAXTM DH10BTM Cells; InvitrogenTM, Thermo Fisher Scientific, Schwerte, Germany) by electroporation using disposable cuvettes with 1 mm gap and, 1.8 kV (E = 18 kV/cm) in a Bio-Rad MicroPulser (Bio-Rad Laboratories, Feldkirchen, Germany). Potential transconjugants and transformants were analyzed for their plasmid content and resistance phenotype. The *E. coli* isolate 19-AB01133 was subjected to short-read (MiSeq, Illumina, CA, USA) and long-read whole genome sequencing (MinIon; Oxford Nanopore, Oxford, UK) followed by a hybrid

assembly of the obtained sequences using Unicycler v0.4.4 under default parameters [19]. Based on this assembly, the multi-locus sequence-type (MLST) as well as resistance and virulence genes were determined using online tools that were provided by the Danish Technical University (http://www.genomicepidemiology.org). The annotation was carried out by RAST2 provided by PATRIC (www.patricbrc.org). The assembly of the plasmid pEC19-AB01133 was deposited in GenBank (NCBI) under the accession number MT682138.

2.3. Farm Investigation

Three months after detecting the isolate 19-AB01133, the farm and the corresponding slaughterhouse were re-investigated. Therefore, composite fecal samples (n = 8) were taken from all pens. Moreover, ten environmental samples were taken in the farm. These samples include cobwebs, dust, water trough, wet residues and sock swabs. In the slaughterhouse, additional 17 samples were taken, including minced and sausage meat, fat, a smear of work surfaces and drains, curing water, hand washing water and more. These samples were analyzed according to the EURL method combined with a second enrichment step in lysogeny broth (LB) supplemented with 1 mg/L cefotaxime (CTX) and in LB supplemented with 0.125 mg/L meropenem (MEM). Afterwards, 10 µL of all enrichments (BPW, LB + CTX and LB + MEM) were streaked out on self-made selective agar (McConkey agar (McC) supplemented with 0.125 mg/L MEM and McC supplemented with 0.125 mg/L MEM and 1 mg/L CTX) and on chromID® CARBA SMART (bioMérieux, Nürtingen, Germany). Plates were incubated for 16–18 h at 37 ± 2 °C. Up to ten colonies with different morphologies were picked and further analyzed by real-time PCR to confirm the presence or absence of bla_{GES}, bla_{KPC}, bla_{NDM}, $bla_{\text{OXA-48}}$ and bla_{VIM} [14] and by MALDI-ToF MS for species confirmation of presumptive CPE.

3. Results and Discussion

The phenotypic analysis of *E. coli* 19-AB01133 indicated a non-wildtype phenotype for the tested β-lactam antimicrobial agents, including penicillins (ampicillin MIC > 64 mg/L), cephalosporins (cefotaxime MIC 64 mg/L, ceftazidime MIC > 128 mg/L, cefepime MIC > 32 mg/L, cefoxitin MIC > 64 mg/L, ceftazidime MIC > 8 mg/L) and carbapenems (imipenem MIC 8 mg/L, ertapenem MIC > 2 mg/L and meropenem MIC 8 mg/L), and also for (fluoro)quinolones (ciprofloxacin MIC > 8 mg/L, nalidixic acid MIC > 128 mg/L) and aminoglycosides (gentamicin MIC > 32 mg/L) (Table 1). Further typing assigned the isolate to the phylogenetic group B1. This group is often associated with enhanced antimicrobial resistance but low virulence potential [20,21]. The virulence potential of the *E. coli* 19-AB01133 is composed of the genes *iss*, *ifpA* and *gad*, which code for an increased serum survival, for long polar fimbriae and for a glutamate decarboxylase, respectively. The S1-PFGE with subsequent Southern blot hybridization and in vitro filter-mating and transformation studies identified a single, conjugative ~190 kb plasmid carrying the $bla_{\text{VIM-1}}$ gene.

The bioinformatic analysis revealed assigned *E. coli* 19-AB01133 to the sequence type ST5869, which has not been described before from livestock and food in Germany. The plasmid pEC19-AB01133 (190,205 bp) exhibited an IncA/C_2 replicon type. The characteristics of the presented isolate 19-AB01133 and its plasmid pEC19-AB01133 are summarized in the Supplemental Material Table S1. The encoded $bla_{\text{VIM-1}}$ gene is highly likely to be responsible for the resistance to the tested carbapenems (imipenem, ertapenem and meropenem) in the *E. coli* isolate 19-AB01133. To the best of our knowledge, $bla_{\text{VIM-1}}$-carrying IncA/C_2 plasmids were detected sporadically in human samples [22,23], but so far only once in livestock and food in Europe [16]. Recently, Garcia-Grealls and her colleagues (2020) described an almost identical VIM-1 encoding plasmid (99.99% identical over the entire plasmid nucleotide sequence, Acc. No. PRJNA564835) of *E. coli* (Acc. No. MN477204.1) isolated from minced pork in Belgium in 2015 [16]. The corresponding isolate was assigned to the same sequence type, which might indicate a clonal relationship. This observation leads to the question of repeated contamination of pigs and pork with this strain, or to a persistence

of this strain in Central European pig production. The *E. coli* isolate 19-AB01133 further showed a close relationship to the *E. coli* isolate TZ 116, which is used by the EURL-AR as second (backup) strain for the validation of selective plates for the detection of CP *E. coli* (https://www.eurl-ar.eu/protocols.aspx). This backup strain was isolated from a human sample. According to personal communication, the strain was not used in the isolating laboratory at that time or in our lab and thus, a cross-contamination can be excluded.

Apart from this control strain, some more $bla_{\text{VIM-1}}$ harboring IncA/C_2 plasmids of isolates from human origin showed a close relationship to the described plasmid pEC19-AB01133. For example, Drieux et al. (2012) [23] described a plasmid (Acc. No. JQ824049) from a Greek *Providencia stuartii* multiresistant strain. By comparing this plasmid with the plasmid pEC19-AB01133, a query coverage of 87% was observed. Further, plasmids from Enterobacteriaceae of clinical and environmental samples from Canada showed also high similarities [24] to the described plasmid pEC17-AB01133. The plasmid pIncAC-KP4898 (Acc. No. KY882285.1) and the plasmid pRIVM0001_VIM-1 (Acc. No. MH220284.1) had a query coverage of 70% and the plasmid pKPC_CAV1344 (Acc. No. CP011622.1) a query coverage of 81%. Taken together, the close relationship and the repeated detection of the strain in food chain samples hints to a spill-over of the strain from humans to food.

The plasmid pEC19-AB01133 harbored a variety of antimicrobial resistance genes, which are located within a ~60 kb multiresistance region (Figure 1). The $bla_{\text{VIM-1}}$ gene is located on a class 1 integron. In addition, the displayed region exhibited three further interesting segments. The first segment comprised two mercury resistance operons (Figure 1), which confer narrow-spectrum resistance to inorganic mercury [25]. Two *tnpA* genes encompass the 5768 bp segment 2. This DNA-segment, originally identified in *Citrobacter freundii* (Acc. No. AY339625.2), encodes the small multidrug resistance efflux transporter SugE and the AmpC β-lactamase CMY-13 [26]. To date, the occurrence of $bla_{\text{CMY-13}}$ has only sporadically been reported [26]. Another neighboring class 1 integron conferred resistance to aminoglycosides [*aac(6′)-Il*, *aac(3)-l*, *ant(3″)-Ia*] and to sulfonamides (*sul1*). Downstream of this class 1 integron, the third segment was integrated. This segment carried the ESBL β-lactamase gene $bla_{\text{SHV-5}}$. The $bla_{\text{SHV-5}}$ gene has been previously reported from isolates of *E. coli*, *Klebsiella pneumoniae* and *Providencia stuartii* from humans, livestock and wildlife [27], but not yet in animals or food in Germany.

The farm and the corresponding slaughter facility were re-investigated three months later. Thereby, 35 samples from feces, farm and slaughter facility environment and different meat products were taken. No CPE were detected in these samples. This suggests that the strain was unable to persist on the farm and spread of the VIM-1 plasmid did not occur. Nevertheless, the detection of an isolate with such high similarities to the Belgian isolates might hint to the ability for it to persist within the pork production chain [15]. A re-introduction by human, i.e., by farmers or workers, is also possible as there was no obvious link between the two fattening pig farms.

The detection of multiresistance IncA/C_2 plasmids identified in *E. coli* isolates from food chain is a threat to public health. Previous studies from Germany described the location of $bla_{\text{VIM-1}}$ only on IncHI2 or IncY plasmids. However, for IncA/C_2 plasmids, a high impact on the dissemination of antimicrobial resistance genes has been reported [20]. The detection of new plasmid types and the recurrence of carbapenemase-producing isolates in the German pork production chain emphasizes the importance of the CPE monitoring [4,8,9]. The occurrence of the $bla_{\text{VIM-1}}$ located on an IncA/C_2 plasmid in an *E. coli* isolate from pork suggests a new, probably human source of CPE entering the food chain.

Table 1. Minimal inhibitory concentrations (MIC) of following antimicrobial agents (mg/L) to 19-AB01133 and its transkonjugant TK_19AB01133. MIC values classified as non-wildtype are colored in red. Ampicillin (AMP); Azithromycin (AZI); Cefepime (FEP); Chloramphenicol (CHL); Ciprofloxacin (CIP); Colistin (COL); Ertapenem (ERP); Cefotaxime (FOT); Cefoxitin (FOX); Gentamicin (GEN); Imipenem (IMI); Kanamycin (KAN); Meropenem (MERO); Nalidixic acid (NAL); Sulfamethoxazole (SMX); Ceftazidime (TAZ); Tetracycline (TET); Tigecycline (TGC); Trimethophrim (TMP).

Isolate	AMP	AZI	CHL	CIP	COL	ERP	FEP	FOT	FOX	GEN	IMI	MERO	NAL	SMX	TAZ	TET	TGC	TMP
19-AB01133	>64	16	16	>8	≤1	>2	>32	>64	>64	32	8	8	>128	>1024	>8	4	≤0.25	>32
TK_19-AB01133	>64	8	8	≤0.015	≤1	>2	>32	>64	>64	4	8	4	≤4	>1024	>8	4	≤0.25	>32
K12 J53	4	8	≤8	≤0.015	≤1	≤0.015	≤0.06	≤0.25	4	2	0.25	≤0.03	≤4	≤8	≤0.5	4	0.5	≤0.25

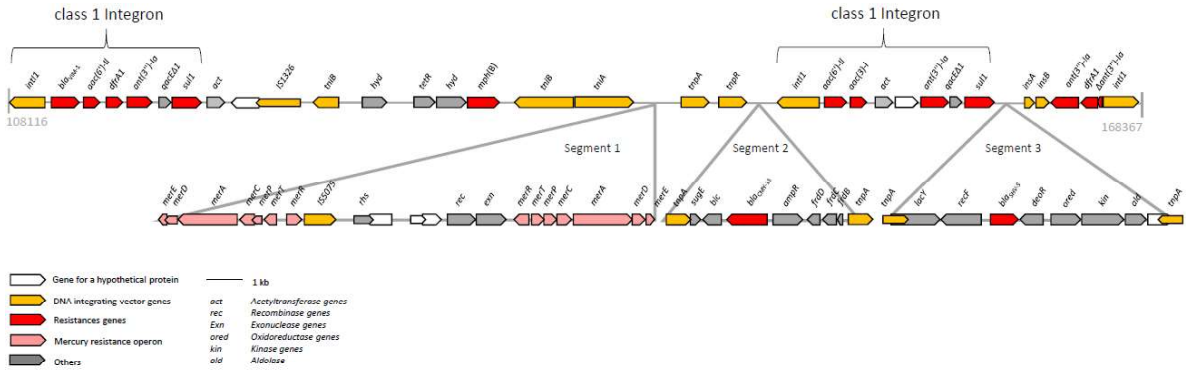

Figure 1. Schematic illustration of the multidrug-resistance region of plasmid pEC19-AB01133. The reading frames are displayed as arrows with the arrowhead showing the direction of transcription. The numbers refer to the whole plasmid sequence of pEC19-AB01133, which is deposited in the GenBank database under accession no. MT682138.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-2607/9/1/29/s1. Table S1: General information about the isolate 19-AB01133 and its plasmid pEC19-AB01133.

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Data Availability Statement: Data is contained within the article and supplementary material. Moreover, the plasmid sequence was deposited in GenBank (NCBI) under the accession number MT682138.

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Conflicts of Interest: The authors declare no conflict of interest.

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2.3 Publication 3

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METHODS
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ChromID® CARBA Agar Fails to Detect Carbapenem-Resistant *Enterobacteriaceae* With Slightly Reduced Susceptibility to Carbapenems

Natalie Pauly[1], Jens A. Hammerl[1], Mirjam Grobbel[1], Bernd-Alois Tenhagen[1], Annemarie Käsbohrer[1,2], Sandra Bisenius[3], Jannika Fuchs[4], Sabine Horlacher[5], Holger Lingstädt[6], Ute Mauermann[7], Silke Mitro[8], Margit Müller[9], Stefan Rohrmann[10], Arthur P. Schiffmann[11], Birgit Stührenberg[12], Pia Zimmermann[13], Stefan Schwarz[14], Diana Meemken[15] and Alexandra Irrgang[1]**

[1] German Federal Institute for Risk Assessment, Berlin, Germany, [2] Unit for Veterinary Public Health and Epidemiology, University of Veterinary Medicine, Vienna, Austria, [3] Institute for Fish and Fishery Products (LAVES), Cuxhaven, Germany, [4] Chemical and Veterinary Investigation Office, Karlsruhe, Germany, [5] Chemical and Veterinary Investigation Office, Fellbach, Germany, [6] State Office for Consumer Protection Saxony-Anhalt, Stendal, Germany, [7] Food and Veterinary Institute, Oldenburg, Germany, [8] State Investigation Institute for Health and Veterinary Services, Chemnitz, Germany, [9] Chemical and Veterinary Investigation Office Rhein-Ruhr-Wupper, Krefeld, Germany, [10] Chemical and Veterinary Investigation Office, Arnsberg, Germany, [11] Hessian State Laboratory, Gießen, Germany, [12] Chemical and Veterinary Investigation Office, Detmold, Germany, [13] Bavarian Health and Food Safety Authority, Oberschleißheim, Germany, [14] Institute of Microbiology and Epizootics, Freie Universität Berlin, Berlin, Germany, [15] Institute of Food Safety and Food Hygiene, Freie Universität Berlin, Berlin, Germany

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Edited by:
Shaolin Wang,
China Agricultural University, China

Reviewed by:
Rong Zhang,
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Ruichao Li,
Yangzhou University, China

***Correspondence:**
Natalie Pauly
natalie.pauly@bfr.bund.de
Alexandra Irrgang
alexandra.irrgang@bfr.bund.de;
alex.irrgang@gmail.com

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After first detections of carbapenemase-producing Enterobacteriaceae (CPE) in animals, the European Union Reference Laboratory for Antimicrobial Resistance has provided a protocol for the isolation of carbapenemase-producing *Escherichia* (*E.*) *coli* from cecum content and meat. Up to now, only few isolates were recovered using this procedure. In our experience, the choice of the selective agar is important for the efficacy of the method. Currently, the use of the prevailing method fails to detect CPE that exhibit a low resistance against carbapenems. Thus, this study aims to evaluate the suitability of selective media with antibiotic supplements and commercial ChromID® CARBA agar for a reliable CPE detection. For comparative investigations, detection of freeze-dried carbapenemase-resistant bacteria was studied on different batches of the ChromID® CARBA agar as well as on MacConkey agar supplemented with 1 mg/L cefotaxime and 0.125 mg/L meropenem (McC+CTX+MEM). The suitability of the different media was assessed within a time of 25 weeks, starting at least six weeks before expiration of the media. Carbapenem-resistant isolates exhibiting a serine-based hydrolytic resistance mechanism (e.g., bla_{KPC} genes) were consistently detected over 25 weeks on the different media. In contrast, carbapenemase producers with only slightly reduced susceptibility and exhibiting a zinc-catalyzed activity (e.g., bla_{VIM}, bla_{NDM}, and bla_{IMP}) could only be cultivated on long-time expired ChromID® CARBA, but within the whole test period on McC+CTX+MEM. Thus, ChromID® CARBA agar appears to be not suitable for the detection of CPE with slightly increased minimum inhibitory concentrations (MIC) against carbapenems, which have been detected in German livestock and thus, are of main interest in the national monitoring programs.

Our data are in concordance with the results of eleven state laboratories that had participated in this study with their ChromID® CARBA batches routinely used for the German CPE monitoring. Based on the determined CPE detection rate, we recommend the use of McC+CTX+MEM for monitoring purposes. This study indicates that the use of ChromID® CARBA agar might lead to an underestimation of the current CPE occurrence in food and livestock samples.

Keywords: carbapenemase, isolation, media, specificity, sensitivity

INTRODUCTION

Carbapenems are important antimicrobial substances with broad activity against almost all β-lactams. They are essential to treat severe human infections with multidrug-resistant Gram-negative bacteria. Due to their high impact in human medicine, dissemination of carbapenem-resistant bacteria should be avoided (Sharland et al., 2018; European Medicines Agency [EMA], 2019). Actually, more than 2,000 variants of carbapenemase resistance genes have been described that were allocated to the β-lactamases of Ambler classes A, B, and D (Codjoe and Donkor, 2017; Grundmann et al., 2017). *Klebsiella pneumoniae* carbapenemases (KPC) and defined OXA enzymes are common Ambler class A and D carbapenemases, respectively, with a serine residue in their active sites (Codjoe and Donkor, 2017). New Delhi metallo-β-lactamases (NDM) and Verona integron-encoded metallo-β-lactamases (VIM) are predominant Ambler class B enzymes. Their resistance mechanism depends on the interaction of the carbapenems with zinc ions in the active site of the enzymes (Codjoe and Donkor, 2017). Carbapenemase genes are often located on mobile genetic elements (i.e., transposons/integrons, plasmids, phages), which support horizontal transmission of the genes between different bacteria (Carattoli, 2009; Miriagou et al., 2010; Tato et al., 2010). The genes are often co-located with genes mediating resistance to other antimicrobial classes. This leads to an important reduction of therapeutic options in human medicine (Woodford et al., 2014). In addition, various chromosomal mechanisms are also involved in resistance development against carbapenems, i.e., changes in membrane permeability due to the loss of specific outer membrane porins (Miriagou et al., 2010).

For a long time, carbapenemase-producing Enterobacteriaceae (CPE) were almost exclusively associated with human medicine. However, the number of reported isolates from wildlife, companion animals, livestock, and food increases worldwide (Köck et al., 2018). To get deeper insights into the dynamics of occurring CPE, the European Union (EU) set up a carbapenem-resistance monitoring for *Escherichia coli* isolates from food products and livestock (Schrijver et al., 2018). Aside from clinical settings, the carbapenemase enzymes OXA, VIM, NDM, and KPC possess the highest impact in wildlife, pets, and the food chain (Grundmann et al., 2017; Köck et al., 2018). In Germany, France, Italy, Spain, and the Netherlands, reports have been published about carbapenem-resistant Enterobacteriaceae (CRE) in companion animals (i.e., dogs, cats, horses) (Stolle et al., 2013; Schmiedel et al., 2014; Gonzalez-Torralba et al., 2016; Melo et al., 2017; Vittecoq et al., 2017; Pulss et al., 2018), seafood (i.e., shrimps, blue mussels, cockles) (Roschanski et al., 2016; Ceccarelli et al., 2017), wild animals (i.e., yellow-legged gulls, black kite) (Fischer et al., 2013b; Vergara et al., 2017; Vittecoq et al., 2017), and food of livestock animals (i.e., beef, chicken meat, pork) (Schwaiger et al., 2008; Leverstein-van Hall et al., 2011; Poirel et al., 2012; Zurfluh et al., 2016; Ceccarelli et al., 2017; Fischer et al., 2017; Pulss et al., 2017; Randall et al., 2017; Roschanski et al., 2017, 2018). In 2011, VIM-1-producing Enterobacteriaceae (*Salmonella* Infantis and *E. coli*) were found in several chicken and pig farms in Germany (Borowiak et al., 2017; Falgenhauer et al., 2017; Fischer et al., 2017; Irrgang et al., 2017, 2019; Roschanski et al., 2018). The European Union Reference Laboratory for Antimicrobial Resistance (EURL-AR) together with national experts on antimicrobial resistances developed a culture-dependent procedure for the isolation of carbapenemase-producing *E. coli* from meat and cecum content samples[1]. As no validated method for isolates from food exists, this method is currently used in the annual monitoring on carbapenemase-producing *E. coli* from livestock and food in Germany and other European member states. In Germany, we recognized on several occasions within the monitoring and during additional studies that the method failed to detect CPE that were identified in the same sample using a different method. One example was an isolate in 2017, which was detected in the monitoring on ESBL/AmpC β-lactamases but could not be detected within the German CPE monitoring. It was a VIM-1-producing *E. coli* from the cecum content of a fattening pig at slaughter that exhibited minimum inhibitory concentrations (MIC) ranging around the current cutoff values for the tested carbapenems. These cutoff values are considerably lower than the MIC of CPE of human clinical origin (European Food Safety Authority [EFSA], and European Centre for Disease Prevention and Control [ECDC],, 2017, 2019). Thus, there was the necessity to improve the isolation method for CPE with low carbapenem MIC values from food and livestock samples.

Due to the prevailing deficit in reliable detection of CPE exhibiting low MIC values, this study aims to determine the sensitivity of the selective cultivation after enrichment of the samples for the laboratory routine in detecting CPE. Thus, the suitability of different selective media for the recovery of CPE was assessed. Beside the frequently used ChromID® CARBA agar (bioMérieux, Nürtingen, Germany) (Nordmann et al., 2012; Girlich et al., 2013; Papadimitriou-Olivgeris et al., 2014),

[1]https://www.eurl-ar.eu/protocols.aspx

which is also predominantly used by the German federal state laboratories, we also determined the sensitivity of MacConkey agar supplemented with cefotaxime and meropenem over a time interval of 25 weeks.

MATERIALS AND EQUIPMENT

Bacterial Strains

Five carbapenem-resistant isolates were used for the evaluation of the different selective media (**Table 1**). We chose three CPE (*E. coli*: CP-3 and CP-9; *Salmonella* Corvallis: CP-5), which had previously been recovered from German food and livestock samples (Fischer et al., 2013b; Irrgang et al., 2017), one carbapenemase-producing *Vibrio parahaemolyticus* (CP-8) from seafood and one carbapenem-resistant *K. pneumoniae* isolate from a pig slaughterhouse that lacks carbapenemase production (CR-1). As references for quality assurance concerning the selectivity of the media, the ESBL-producing *E. coli* isolate AR-1 (Wilkinson et al., 2012) and *E. coli* ATCC 25922 were used.

Further information on the bacterial isolates used in this study is summarized in **Table 1**. Detailed information on the MIC values of the isolates for 14 antimicrobials (ampicillin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, colistin, florfenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim) is provided in the **Supplementary Table S1**.

Media and Supplements

If not stated otherwise, all bacteria were cultivated in lysogeny broth (LB) at 37°C for 16–20 h under shaking conditions (120–160 rpm). Solid media agars containing 0.8% (w/v) Bacto agar (Oxoid, Dassel, Germany) were used. If necessary, antimicrobial substances were supplemented to the media as indicated. All media (listed in **Table 2**) of this study were stored at 4–6°C in a refrigerator and only exposed to light during processing of the experiments.

METHODS

Antimicrobial Susceptibility Testing

The susceptibility to antimicrobials of the isolates was determined by broth microdilution using defined antimicrobial substances and concentrations from the harmonized EU panel [plates EUVSEC and EUVSEC2; TREK Diagnostic Systems (Thermo Fisher Scientific, Schwerte, Germany)]. Resistance testing was conducted according to the EN ISO20776-1:2006 (European Food Safety Authority [EFSA], and European Centre for Disease Prevention and Control [ECDC],, 2018), and MIC values were interpreted based on EUCAST definitions[2] using epidemiological cutoff values. The testing was repeated three times (**Supplementary Table S1**). As quality control strain, *E. coli* ATCC 25922, was used.

[2]www.eucast.org

Preparation of Freeze-Dried Bacteria

To ensure that equal amounts of bacterial cells were used throughout the whole study, a single batch of freeze-dried isolates was prepared using an Epsilon 2-10D LSC lyophilization instrument (Christ, Osterode am Harz, Germany) with the following procedure. Bacterial enrichments were prepared by inoculating 2 mL LB broth, supplemented with 1 mg/L cefotaxime (CTX), with a single colony of the carbapenem-resistant or ESBL-producing isolates, while cultivation of *E. coli* strain ATCC 25922 was conducted in LB without antimicrobials. The inoculated broths were cultured for 16–20 h at 37 ± 2°C on a rotational shaker (180 rpm). Following incubation, 150 µL of the respective cultures was transferred into 2 mL of fresh LB for further incubation at 37 ± 2°C until an optical density ($OD_{600\ nm}$) between 0.5 and 0.6 was reached. Thereafter, the culture was diluted to 1:10,000 (10^3 to 10^4 CFU/mL) and centrifuged at 4,000 g for 10 min. The bacterial cell pellet was resuspended in lyophilization medium [glutamic acid 10.0 g/L; NaOH (8%) 30.0 mL/L; Bacto tryptone 25.0 g/L; sucrose 50.0 g/L; thiourea (CH_4N_2S) 2.0 g/L] and was applied in aliquots of 1 mL to the borosilicate glasses. Lyophilization was conducted according to the standardized and validated procedure of the German Federal Institute for Risk Assessment (BfR) (freezing; condenser cooling to −50°C; vacuuming the drying chamber; main drying and post-drying). After lyophilization, the bacterial concentrations (CFU/mL) of the freeze-dried bacteria were determined under selective and non-selective conditions. The bacterial concentration after resuspension, plating on LB agar without any supplement and incubation overnight at 37°C accounted between 7.5 and 45 × 10^3 CFU/mL.

Enumeration of Bacteria on Different Media

To evaluate the performance of different agar types and the impact of increasing storage time of ChromID® CARBA plates, bacterial growth on different media was determined weekly over a period of 25 weeks as described below. Therefore, the respective lyophilisates of the bacteria were resuspended in 1 mL double-distilled water and incubated for 30–60 min at room temperature for adaption of the bacteria. To exclude potential effects of the commercial agar of the growth performance of the bacteria, five different ChromID® CARBA batches were tested. Aliquots (50 µL) of the lyophilisate suspensions were plated on ChromID® CARBA plates. For comparative analysis of the commercial agar to other media, the same lyophilisate suspensions were also applied to LB agar and to MacConkey agar supplemented with 1 mg/L CTX and 0.125 mg/L meropenem (MEM) (McC+CTX+MEM). Both LB agar and McC+CTX+MEM were produced *in-house* at the BfR. After an incubation period of 16–18 h at 37 ± 2°C, the bacterial concentration (CFU/mL) was determined. For comparability, the storage time the bacterial growth on the five ChromID® CARBA batches was calculated according to the expiry date.

Freeze-dried bacteria were also provided to 11 participating federal state laboratories for comparative investigation. The laboratories of Baden-Württemberg (Fellbach, Karlsruhe),

Bavaria (Oberschleißheim), Hesse (Gießen), Lower Saxony (Cuxhaven, Oldenburg), North Rhine-Westphalia (Arnsberg, Detmold, Krefeld), Saxony (Chemnitz), and Saxony-Anhalt (Stendal) conducted growth investigation according to the specifications of the BfR. Overall, the selectivity of the ChromID® CARBA agar was tested on seven different batches. In contrast to the long-term procedure of the BfR, the federal state laboratories investigated the growth performance of the different isolates on different selective media at three defined time points: (i) at the expiry date, (ii) 2 weeks before, and (iii) 5 weeks after the expiry date. The systematic procedure of all steps is shown in **Figure 1**.

RESULTS

Commercial selective medium is not suitable for routine monitoring purposes on isolates from livestock and food that exhibit low carbapenem MIC values.

Therefore, the commercial ChromID® CARBA agar was tested out of date because of previous observations and experiences. Freeze-dried bacteria, which were produced within this study, showed growth between 5×10^2 CFU/mL and 4.5×10^4 CFU/mL (**Figure 2**). Over the 25-week period, the bacterial titer of the isolates did not change significantly under non-selective conditions (LB). However, we observed a slight decrease in the bacterial titer for the isolates AR-1, CR-1, and CP-8 over the first time points of enumeration (**Figure 2**). After 25 weeks, all isolates were still reliably detectable under non-selective conditions (**Figure 2**).

The results of the comparative analysis are summarized in **Supplementary Table S2**. Results of comparative testing of the different federal state laboratories were very similar. They

TABLE 1 | Strains and species used in this study with their resistance determinants (threefold determined MIC values, variations are indicated *with a "/" between the values*).

Isolate	Bacterium	Origin	Carbapenemase genes	Carbapenemase production	ERP (mg/L)	IMI (mg/L)	MEM (mg/L)	References
ATCC 25922	*E. coli*	DSMZ	None	–	≤0.015	0.25	≤0.03	DSMZ
AR-1	*E. coli*	Bovine cecum	None	–	0.25	0.5	0.6	This work
CP-3	*E. coli*	Colon content of a slaughter pig	bla_{VIM-1}	+	0.12	4	1	Irrgang et al., 2017
CP-5	*Salmonella* Corvallis	Wild bird	bla_{NDM-1}	+	>2	4	8	Fischer et al., 2013b
CP-8	*V. parahaemolyticus*	Seafood	bla_{NDM-1}	+	0.25	1/2/4	0.25	This work
CP-9	*E. coli*	Human	bla_{KPC-2}	+	>2	4	8	This work
CR-1	*K. pneumoniae*	Porcine cecum	None	–	2	0.5/1	0.5	This work

ERP, ertapenem; IMI, imipenem; MEM, meropenem. DSMZ, German collection of microorganisms and cell cultures. Moreover CP, carbapenemase-producing; CR, carbapenem-resistant; and AR, antibiotic resistant.

TABLE 2 | List of media, reagents, materials, and equipment.

Material	Produced by	Further information	Country
Lysogeny broth	*In-house*	Yeast extract 5 g/L; peptone from casein 10 g/L; NaCl 10 g/L	
Lysogeny agar	*In-house*	Yeast extract 5 g/L; peptone from casein 10 g/L; NaCl 10 g/L; agar 12 g/L	
Bacto agar	Oxoid		Dassel, Germany
Lyophilization medium	*In-house*	Glutamic acid 10.0 g/L; NaOH (8%) 30.0 mL/L; Bacto tryptone 25.0 g/L; sucrose 50.0 g/L; thiourea (CH_4N_2S) 2.0 g/L	
MacConkey agar	*In-house*	Peptone from casein 17 g/L; peptone from meat 3 g/L; NaCl 5 g/L; lactose 10 g/L; bile salt mixture 1.5 g/L, neutral red 0.03 g/L; crystal violet 0.001 g/L, agar 13.5 g/L	
ChromID® CARBA	bioMérieux		Nürtingen, Germany
Cefotaxime	Sigma-Aldrich		Taufkirchen, Germany
Meropenem	Sigma-Aldrich		Taufkirchen, Germany
Plates EUVSEC and EUVSEC2	Thermo Fisher Scientific		Schwerte, Germany
Epsilon 2-10D LSC lyophilization instrument	Christ		Osterode am Harz, Germany
Sensititre AIM™ Automated Inoculation Delivery System	TREK Diagnostic		East Grinstead, United Kingdom
Sensititre™ SWIN™ Software System 3.3	Thermo Fisher Scientific		Schwerte, Germany

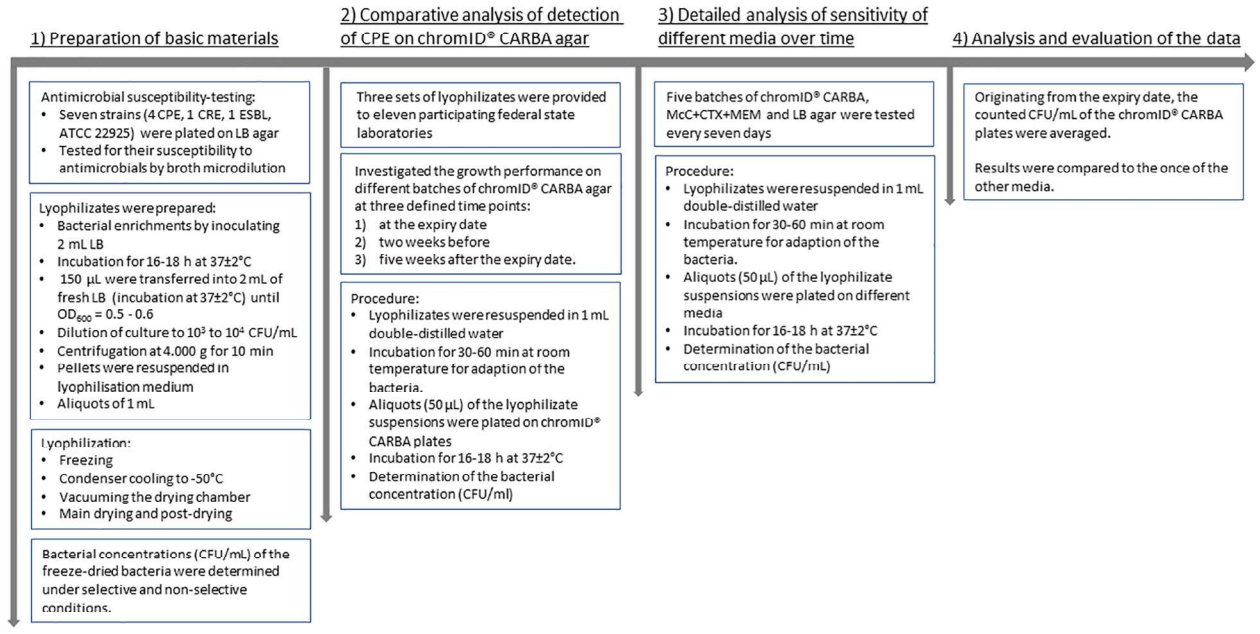

FIGURE 1 | Flow diagram of the systematic procedure.

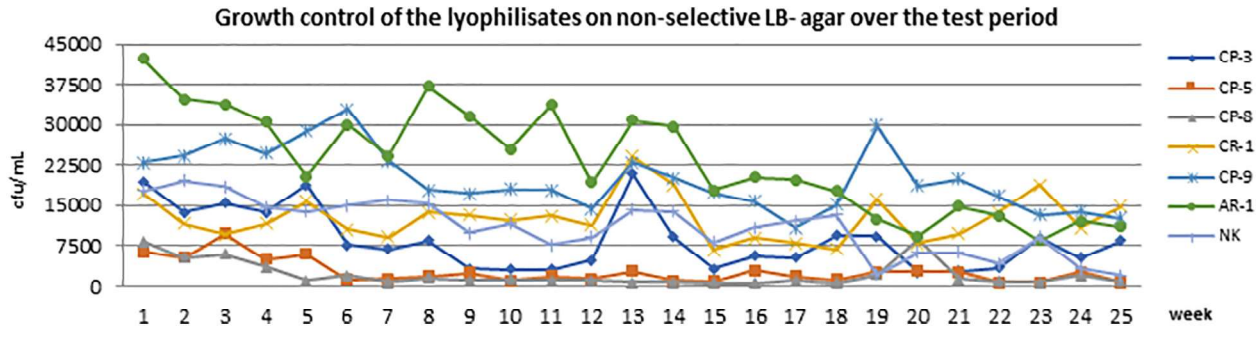

FIGURE 2 | The growth of all lyophilisates on non-selective LB agar over the entire test period of 25 weeks. The *x*-axis (abscissa) shows the time in weeks, and the *y*-axis (ordinate) shows CFU/mL.

indicated that only one isolate (CP-9) could be detected on the used agar batches at all three time points. This respective isolate, a human *E. coli* which carried the gene bla_{KPC-2}, exhibited a high MIC values against the tested carbapenems (**Table 1**). None of the other isolates showed bacterial growth at the selected time points.

To investigate the suitability of the ChromID® CARBA agar for the detection of CPE exhibiting low carbapenem MIC values, the growth ability of the aforementioned isolates was investigated at the NRL-AR over a time period of 25 weeks using five different batches of the agar. The diagrams 2A–2E show that at the beginning of the test, bacterial growth could only be detected for isolate CP-9, exhibiting high MIC values for the carbapenems ertapenem, imipenem, and meropenem (**Table 1** and **Supplementary Table S1**). Slight bacterial growth could be detected for the isolates CP-3, CP-5, and CP-8 2 weeks before expiry date (week 7) (**Figures 3A–C**). This detection increased over time and also after the expiry date. The carbapenemase-producing isolate CP-9 showed a highly reliable average growth rate between 5.0×10^3 and 2.5×10^4 CFU/mL at all points of investigations over the 25 weeks (**Figure 3D**). At the end of the trial, four carbapenem-resistant isolates (CP-3; CP-5; CP-8; and CR-1) showed increased growth rates (average: CP-3 with bla_{VIM-1}: 4 to 32 CFU/mL; CP-5 with bla_{NDM-1}: 4 to 368 CFU/mL; and CP-8 with bla_{VIM-1}: 4 to 24 CFU/mL). The CR-1 isolate that exhibits no carbapenemase-production showed growth rates between 20 and 3920 CFU/mL and was first detected 1 week before the expiry date of the media (**Figure 3E**). The quality control isolate AR-1 (**Table 1** and **Supplementary Table S1**) and the control strain ATCC 25922 did not grow on ChromID® CARBA plates during the investigated period.

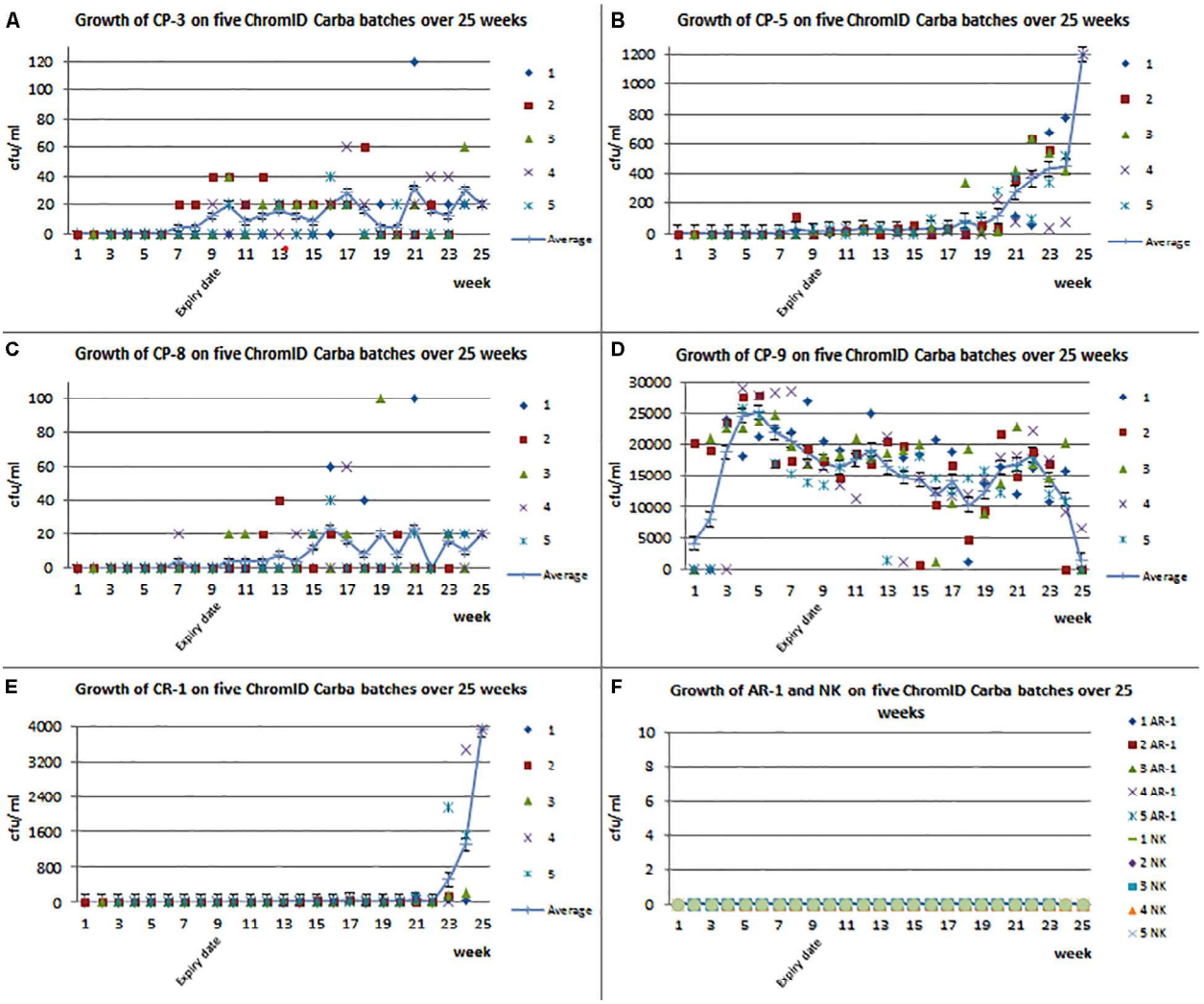

FIGURE 3 | The *x*-axis shows the time in weeks where "9" the expiry date is. The *y*-axis shows CFU/mL. **(A)** Growth of CP-3 on five tested batches of ChromID® CARBA agar. Moreover, the average of all batches is shown. **(B)** Growth of CP-5 on five tested batches of ChromID® CARBA agar. Moreover, the average of all batches is shown. **(C)** Growth of CP-8 on five tested batches of ChromID® CARBA agar. Moreover, the average of all batches is shown. **(D)** Growth of CP-9 on five tested batches of ChromID® CARBA agar. Moreover, the average of all batches is shown. **(E)** Growth of CR-1 on five tested batches of ChromID® CARBA agar. Moreover, the average of all batches is shown. **(F)** Growth of the both control strains NK and AR-1 on five tested batches of ChromID® CARBA agar.

Supplementing 1 mg/L CTX and 0.125 mg/L MEM to the agar basis of the MacConkey medium can overcome growth inhibition during cultivation of low-carbapenem MIC-exhibiting isolates.

The growth performance of the abovementioned isolates was also determined on McC+CTX+MEM agar. In contrast to ChromID® CARBA agar, all carbapenem-resistant isolates were reliably detected at all time points within the 25-week period of investigation (**Table 3**). The growth of the CP-9 isolate was similar on McC+CTX+MEM, ChromID® CARBA agar as well as under non-selective conditions (LB agar) ranging between 2.4×10^3 and 3.3×10^4 CFU/mL. With the focus on the isolates (AR-1 and NK) used as quality controls, McC+CTX+MEM has a reliable specificity of up to 22 weeks, as within this period no bacterial growth was observed.

DISCUSSION

Previous experiments in our laboratory showed that the ChromID® CARBA agar becomes more sensitive with increasing storage time. Based on the observations and experiences within the laboratory routine (International Organization for Standardization, 2006; Fischer et al., 2013a,b, 2017; Irrgang et al., 2017, 2019), detection of carbapenemase-producing bacteria is challenging using the EURL-AR method in combination with ChromID® CARBA agar. We assume that the concentration of different ingredients and thus the selectivity will decrease with time or after the expiry date. This justifies the choice of times to determine a possible decrease in antimicrobial concentration after the shelf life of ChromID® CARBA agar.

TABLE 3 | Growth of the seven lyophilisates tested once a week over 25 weeks on MacConkey agar supplemented with 0.125 mg/L meropenem and 1 mg/L cefotaxime.

Week	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
AR-1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
CP-3	+	+	+	+	+	+	+	+	+	+	+	+	+	++	+	++	++	++	++	+	+	++	++	++	++
CP-5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CP-8	+	–	–	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	–	+	+	+	+
CP-9	+++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
CR-1	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
NK	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

"–" means no growth; "+" means 1–100 cfu/mL; "++" means 100–1000 cfu/mL; "+++" means ≥1000 cfu/mL.

Within this study, we observed strong differences in the growth performance of the used carbapenem-resistant isolates on the selective media. These different growth performances exceed the observed slight decrease of AR-1, CR-1, and CP-8 during the first time points of enumeration, which seems to represent a natural adaption of the specific isolates to the prevailing cultivation conditions (**Figure 2**).

As previously reported (Fischer et al., 2012; Köck et al., 2018), carbapenem-resistant isolates from food products or livestock exhibit significantly lower MIC values for the respective carbapenems than clinical isolates. There may be different reasons for this observation. It could be due to molecular conditions, such as a variable number of gene copies, a different expression of the genes, or a different genetic localization (plasmid versus chromosomal DNA). It could also be due to the different antimicrobial selection pressures to which the strains are exposed. As carbapenems are not licensed for use in food animals, previous exposure of the strains with a livestock origin is unlikely. The chosen isolates for the evaluation of the selective cultivation step of the detection procedure represent different bacterial genera and carbapenem resistance genes as well as resistance mechanisms (Viau et al., 2012; Köck et al., 2018). The *E. coli* isolate CP-9 carries a bla_{KPC-2} gene and exhibited high MIC values against all tested carbapenems. CP-9 represents typical MIC values for carbapenems of human isolates (**Supplementary Table S1**). Other target isolates, except CP-5 (the NDM-producing *Salmonella*) exhibited carbapenem resistance but showed only MICs ranging around the current cutoff values (**Supplementary Table S1**). As the used isolates exhibited very low MIC values, we suppose that the remaining concentration of the supplemented antimicrobials is still high enough to prevent a growth of these isolates.

The KPC enzymes belong to Amber class A carbapenemases and use a serine-based hydrolytic mechanism to inactivate carbapenems. In contrast, the VIM-1 and NDM-1 enzymes belong to Ambler class B carbapenemases and convey the resistances by a zinc-catalyzing active site (Codjoe and Donkor, 2017). Viau et al. (2016) reported that most of their ten tested media performed reasonably well with different screening methods for the detection of class A enzymes. Performance with class B and D enzymes was more variable. However, Viau and co-workers used up to 180 different CRE originating from human clinical sources. They determined a good performance of the ChromID® CARBA medium if tested with rectal/perirectal swabs (Jeon et al., 2015). The differences between CPE from human samples and CPE from non-clinical animal samples have not been investigated systematically so far as the number of available non-clinical animal isolates is still limited. As the ingredients of the ChromID® CARBA agar are unknown and have not been provided by the manufacturers on request, it is difficult to assess the selective additives of the medium and to expect their influence on the cultivation of CPE with low MIC. It might be that an additive supports the serine-based hydrolytic resistance mechanism in a better way than the zinc-based mechanism.

Within our study, CP-9 was the only isolate that was consistently detected in all participating laboratories and with all selective media. The determined amount of the adjusted, freeze-dried bacteria ranged between 5×10^2 and 4.5×10^4 CFU/mL between the laboratories. The other carbapenem-resistant isolates (CP-3; CP-5; CP-8; and CR-1) showed no detectable growth on the ChromID® CARBA plates within the different federal state laboratories up to 5 weeks after expiration date of the plates (**Supplementary Table S2**). At this time point, the agar will not be used by accredited laboratories as the agar will be handled as expired according to the recommendations of the manufacturers. However, each of these isolates was reliably detected on McC+CTX+MEM (**Table 3**). The quality control isolate AR-1, a CTX-M-1-producing *E. coli* with a low phenotypical resistance against ertapenem (**Table 1** and **Supplementary Table S1**), and the quality control strain ATCC 25922 did not grow on ChromID® CARBA plates at any time (**Figure 3F**). As they grew at the last three time points on McC+CTX+MEM, it can be assumed that the specificity of the plates did not decrease considerably within a period of 20 weeks after production (**Table 3**). In contrast, the number of CFU of the isolates CP-3; CP-5; CP-8; and CR-1 increased at the end of the investigated time interval. This indicates that the sensitivity of the commercial agar increased over time.

In contrast to our study, other groups found that ChromID® CARBA agar was one of the most sensitive and specific chromogenic medium for the detection of CPE (Vrioni et al., 2012; Papadimitriou-Olivgeris et al., 2014; Simner et al., 2015; Viau et al., 2016). However, in these studies, the MIC values of the CPE are in general much higher than the MIC values of the isolates tested here. In comparison, Vrioni et al. (2012) used clinical isolates with very high MIC values of up to over 32 mg/L to MEM, but they also used some strains with MIC values below the resistance breakpoint. The detection of the isolates with these

high MIC values might correlate much more with the detection of CP-9 and is not comparable with the detection of isolates exhibiting low MICs (CP-3, CP-8, and CR-1).

Limited detection of CPE might be also attributable to a loss of the resistance-harboring plasmids during lyophilization (Wagman and Weneck, 1963; Berman et al., 1968; Wein et al., 2019). We can exclude this as an important factor in our study because we observed similar bacterial titers on selective and non-selective *in-house* media (data not shown). We had also confirmed the presence of the plasmid in some representative colonies from selective and non-selective media.

Overall, we have detected more CFU/mL by using the conventional MacConkey agar supplemented with antibiotics over the entire period for almost all isolates of this study. Due to the lacking resistance against carbapenems, the quality control strain ATCC 25922 could not be detected on McC+CTX+MEM, indicating the appropriate specificity of the medium. Some spontaneous growth of the *E. coli* isolate AR_1 was observed 22, 23, and 24 weeks after agar production, indicating that the prepared agar worked with a high specificity for up to 22 weeks. In contrast to this observation, the AR_1 isolate could not be detected on the ChromID® CARBA agar. The concentration of meropenem (0.125 mg/L) in the supplemented agar is lower than the current ECOFFs (0.25 mg/L) (Giske et al., 2013). Based on this concentration, CPE with low MIC values are also able to grow successfully and will further be detected within the monitoring programs. Viau et al. (2012) reported about "silent" dissemination of *K. pneumoniae*, because some isolates were not identified as carbapenem-resistant based on their MIC, even though they carried a carbapenemase gene and showed slightly increased MIC values against ertapenem and meropenem (Fischer et al., 2012).

Berthoin et al. (2010) reported that the stability of meropenem in solutions depends on time, temperature, and concentration (Berthoin et al., 2010). These observations were supported by Keel et al. (2011). A potential effect of light on the stability of meropenem can be excluded, as the agar plates were only exposed to light during plating and visual inspection. Therefore, it was assumed that the stability of meropenem in the media might be limited. In our study, the McC+CTX+MEM agar was stably usable for up to 22 weeks without a loss of specificity when stored at 4 ± 2°C. The supplementation with cefotaxime supported the selective effect, but for OXA-48 producers this antibiotic should be omitted. Changes in sensitivity and specificity of MacConkey agar supplemented with meropenem only over time were not tested in our study. Adler et al. (2011) used MacConkey agar supplemented with imipenem at 1 mg/mL (McC+IMI) and compared this medium with a commercial selective medium (CHROMagar KPC, CHROMagar Company, Paris, France). They described a similar performance of both media during clinical evaluation. Moreover, they argued with the lower costs of McC+IMI to prefer it for detection of CRE in their study (Adler et al., 2011). We decided to use meropenem as supplement because it is more stable than imipenem (Zhanel et al., 2007). Based on our data, we recommend using McC+CTX+MEM for selective cultivation of carbapenem-resistant isolates from food products and livestock samples.

Another important difference between the different agars is that the ingredients of the *in-house* prepared agar are known. In McC+CTX+MEM agar, no further substances were supplemented for inhibition of accompanying bacteria. It is possible that the commercial selective medium comprises unspecified substances (e.g., zinc that is used to inhibit non-carbapenemase-producing bacteria), which may also suppress the growth of some CPE, e.g., with a low MIC. Although less selective substances are used, no increased difficulties were found with the accompanying flora in other experiments so far.

A limitation of this study is that only one batch of McC+CTX+MEM plates was used throughout the experiment. This was due to its function as a control medium, and it was not the primary aim to compare both media.

CONCLUSION

The results of our study indicate that the ChromID® CARBA agar is not suitable for the cultural detection of CPE with low MIC to carbapenems, while clinical isolates with higher MIC values can be detected reliably. Overall, the use of the ChromID® CARBA agar in monitoring programs may lead to an underestimation of the CPE occurrence in livestock and food as the method lacks sensitivity.

We further provide data, that MacConkey agar with 1 mg/L cefotaxime and 0.125 mg/L meropenem provides a better sensitivity for the detection of such isolates and may be more suitable for the carbapenemase monitoring in livestock and food.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

This study uses strains obtained from humans (*E. coli* CP-9), livestock (*E. coli* AR-1 and CP-3, *Klebsiella pneumoniae* CR-1), wildlife (*Salmonella* Corvallis CP-5) and seafood (*Vibrio parahaemolyticus* CP-8). All isolates were part of the strain collection of the German NRL AR and had been submitted to the NRL in the framework of its routine tasks in monitoring and surveillance programs or during previous scientific collaborations. None of the isolates was collected specifically for the current study. Therefore we did not consider it necessary to seek for ethical approval for using the isolates.

AUTHOR CONTRIBUTIONS

JH, NP, and AI designed the study and developed the draft of the manuscript. NP performed the preparation of adjusted, freeze-dried bacteria, and the determination of the growth performance of the isolates at the NRL-AR. MG, AI, and JH initially characterized the carbapenem-resistant isolates. SB, JF, SH, HL, UM, MM, SR, AS, SS, BS, and PZ performed the individual growth investigations on the commercial agar at the different federal state laboratories. NP, AI, JH, MG, AK, B-AT, SS, and DM performed the data analysis. All authors supported

the finalization of the manuscript and supported editing of the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01678/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.4 Publication 4

Pauly N, Klaar Y, Skladnikiewicz-Ziemer T, Juraschek K, Grobbel M, Hammerl J A, Hemmers L, Käsbohrer A, Schwarz S, Meemken D, Tenhagen B-A, Irrgang A.

Isolation Procedure for CP *E. coli* from Caeca Samples under Review towards an Increased Sensitivity

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Isolation Procedure for CP *E. coli* from Caeca Samples under Review towards an Increased Sensitivity

Natalie Pauly [1], Yvonne Klaar [1], Tanja Skladnikiewicz-Ziemer [1], Katharina Juraschek [1], Mirjam Grobbel [1], Jens André Hammerl [1], Lukas Hemmers [2], Annemarie Käsbohrer [1,3], Stefan Schwarz [4,5], Diana Meemken [6], Bernd-Alois Tenhagen [1] and Alexandra Irrgang [1,*]

1 German Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung), D-10589 Berlin, Germany; natalie_pauly@hotmail.com (N.P.); Yvonne.Klaar@bfr.bund.de (Y.K.); Tanja.Skladnikiewicz-Ziemer@bfr.bund.de (T.S.-Z.); Katharina.juraschek@bfr.bund.de (K.J.); mirjam.grobbel@bfr.bund.de (M.G.); Jens-Andre.Hammerl@bfr.bund.de (J.A.H.); annemarie.kaesbohrer@bfr.bund.de (A.K.); bernd-alois.tenhagen@bfr.bund.de (B.-A.T.)
2 State Office for Health and Social Affairs Berlin, D-10639 Berlin, Germany; lukas.hemmers@lageso.berline.de
3 Unit for Veterinary Public Health and Epidemiology, University of Veterinary Medicine, AT-1210 Vienna, Austria
4 Institute of Microbiology and Epizootics, Freie Universität Berlin, D-14163 Berlin, Germany; Stefan.Schwarz@fu-berlin.de
5 FAO Reference Centre for Antimicrobial Resistance, Freie Universität Berlin, D-14163 Berlin, Germany
6 Institute of Food Safety and Food Hygiene, Freie Universität Berlin, D-14163 Berlin, Germany; diana.meemken@fu-berlin.de
* Correspondence: Alexandra.irrgang@bfr.bund.de

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Abstract: Due to the increasing reports of carbapenemase-producing Enterobacteriaceae (CPE) from livestock in recent years, the European Reference Laboratory for Antimicrobial Resistances (EURL-AR) provided a protocol for their recovery from caecum and meat samples. This procedure exhibited limitations for the detection of CPE with low carbapenem MIC values. Therefore, it was modified by a second, selective enrichment in lysogeny broth with cefotaxime (CTX 1 mg/L) and with meropenem (MEM 0.125 mg/L) at 37 °C under microaerophilic conditions. By Real-time PCR, these enrichments are pre-screened for the most common carbapenemase genes. Another adaptation was the use of in-house prepared MacConkey agar with MEM and MEM+CTX instead of commercial selective agar. According to the EURL-method, we achieved 100% sensitivity and specificity using the in-house media instead of commercial agar, which decreased the sensitivity to ~75%. Comparing the method with and without the second enrichment, no substantial influence on sensitivity and specificity was detected. Nevertheless, this enrichment has simplified the CPE-isolation regarding the accompanying microbiota and the separation of putative colonies. In conclusion, the sensitivity of the method can be increased with slight modifications.

Keywords: Isolation; carbapenemase; CPE detection; selective media; specificity; sensitivity

1. Introduction

The World Health Organization defines carbapenems as 'High Priority Critically Important Antimicrobials' for human medicine [1]. This is due to their role in the treatment of severe human infections with multidrug-resistant bacteria. Up to now, in European countries carbapenems are not licensed for use in veterinary medicine. Nevertheless, carbapenems may be used in specific circumstances in companion animals [2,3]. In recent years, an increasing number of carbapenem resistant enterobacteria, including carbapenemase-producers, have been reported [4]. Interestingly, the reports are not only limited to human medicine [4–6] as carbapenem-resistant bacteria (i.e., *E. coli*, *Salmonella* enterica subsp. enterica) have also been detected repeatedly in environmental or animal samples [4,5].

Besides various chromosomally encoded mechanisms (i.e., changes in membrane permeability, efflux pumps), resistance to carbapenems is often mediated by degrading enzymes called carbapenemases that can hydrolyze carbapenems and most other β-lactams [7]. Typically, the corresponding carbapenemase genes are located on mobile (integrative and conjugative) genetic elements (i.e., plasmids) [8,9]. They exhibit different mechanisms of action (i.e., serine-based (i.e., KPC, OXA) and zinc-catalysed carbapenemases (i.e., NDM, VIM)) [10,11]. Carbapenemases usually show a broad activity against most β-lactams and are typically associated with resistance determinants against other antimicrobial classes often leading to a drastic reduction of therapeutic options in case of infections [12,13]. In the European Union (EU), OXA, VIM, NDM, and KPC carbapenemases are most frequently detected in human clinical settings [5,14,15]. In 2012, the first carbapenemase-producing (CP) *E. coli* was detected in livestock [16], and voluntary monitoring of CP *E. coli* in the EU member states along with the obligatory monitoring on extended spectrum β-lactamase (ESBL)/AmpC β-lactamases producing *E. coli* (CID 2013/652/EU) [17] was introduced. In 2021, through new legislation, the specific monitoring for carbapenem resistant *E. coli* became mandatory (CID 2020/1729/EU). For selective CP *E. coli* detection, the isolation method provided by the European Reference Laboratory for Antimicrobial Resistances (EURL-AR) (https://www.eurl-ar.eu/protocols.aspx, accessed on December 2019) is recommended. The method is based on a 1:10 non-selective enrichment of the samples in buffered peptone water (BPW), which needs to be inoculated onto suitable selective agars (i.e., the commercial chromID® CARBA agar). Several studies described the chromID® CARBA agar as one of the most sensitive and specific chromogenic media for the detection of CP Enterobacteriaceae (CPE) in clinical samples in human medicine [18–21]. In Germany, this agar represents one of the most commonly used selective media for CPE monitoring purposes [22]. By using a highly sensitive molecular pre-screening of enrichment cultures (i.e., by real-time PCR), laborious microbiological investigations might be avoided, and further efforts can focus on the recovery of CPE from presumptive-positive samples.

Unfortunately, for some CPE detected in German livestock the EURL-AR detection method failed in recent years [23,24]. For example, the isolate 17-AB02384 was not detected within the monitoring of CP *E. coli* but was recovered in the monitoring on ESBL/AmpC β-lactamase producing *E. coli* from the same sample [24]. Thus, we identified the necessity to improve the isolation method for CP *E. coli* from livestock samples, especially from caecum content. Difficulties in the microbiological processing of caeca samples are the growth of an accompanying microbiota, the low CPE concentrations, and the considerably lower MIC values of CPE from animal origin in comparison to human isolates [22]. Here, we present comparative analyses on the performance of the EURL-method with a number of alterations potentially leading to an improvement of the CPE detection and recovery rate. The focus of this study was on the recovery of CP *E. coli* from pig caecum, as the majority of the recently described CP *E. coli* from German livestock originate from this matrix. Therefore, samples were spiked and blinded to calculate the sensitivity and specificity of the approaches, which included different selective agars.

2. Materials and Methods

2.1. Pre-Studies

Initial experiments were carried out to determine the growth and the survival of CPE in feces. Therefore, the survival rate (colony forming units (cfu)/g pig feces) of seven different CPE (four VIM-1 producing *E. coli*, two VIM-1 producing *Salmonella* (*S.*) Infantis and one NDM-1 producing *S. Corvallis*) was investigated over a period of 10 days. For the alternative method to be tested, spiked samples were prepared. For this, the seven CPE were inoculated each in lysogeny broth (LB) supplemented with 1 mg/L cefotaxime (CTX) (LB + CTX) and were incubated at 37 °C for 16–18 h. After incubation, 10 µL of the suspension was transferred into LB and was additionally incubated at 37 °C with 180 rpm until an optical density ($OD_{600\,nm}$) of 0.5 was reached. The suspensions were centrifuged for 10 min at 4000 rpm and resuspended in 0.9 % saline solution (*w/v*). Thereafter, 5 mL of the

solution (~10^8 cfu/mL) was applied to 45 g feces and homogenized for 1 min in a BagMixer 400SW (Interscience, Wiesbaden, Germany). The prepared samples were stored at 4–6 °C. Every 24 h, 1 g of the spiked samples was diluted in 0.9% saline solution to 10^{-6}. Aliquots of 100 µL of the 10^{-5} and the 10^{-6} dilutions were plated out onto selective MacConkey agar (McC) supplemented with 0.125 mg/L meropenem (MEM) (McC+MEM) and 1 mg/L CTX and MEM (McC + CTX + MEM), and the cfu were counted after incubation for 20–24 h at 37 °C.

To extend the time until sample processing in the laboratory, a potential improvement of the CPE survival in fecal samples was tested by adding stabilizing substances. Trehalose [25,26], glycerol [27,28], and sodium chloride [29,30] were tested as potential additives for the improvement of the recovery rate of the seven CPE in pig feces. Previous studies reported on the protective characteristics or the enhancive influence of these substances for the recovery and growth of Enterobacteriaceae [25–30]. Therefore, the described previous experiments were repeated by adding 1 mL of the supplement (trehalose, glycerol, and sodium acid) to 9 g of the caeca sample. The samples were thoroughly mixed for 1 min and a decimal dilution series up to 10^{-6} in 0.9% saline solution was prepared. Afterwards, 100 µL of the 10^{-5} and the 10^{-6} dilution were applied onto selective McC + CTX + MEM, incubated overnight at 37 °C, and cfu/g were determined.

The influence of different liquid media (Mossel medium [31] and LB) on CPE growth was tested to determine the most appropriate culture medium for the second enrichment step. Furthermore, possible influences of a sublethal concentration of MEM (0.008 µg/mL) [32] and the addition of $ZnSO_4$ (70 µg/mL) [33] to the two liquid media were tested. Gullberg et al. (2014) [34] reported that sublethal antimicrobial concentrations are able to induce bacteria to retain plasmids and express corresponding genes.

Moreover, the growth of a CP *E. coli* and a CP *Salmonella* in LB + CTX and LB + MEM under aerobic and microaerobic conditions were compared. For spiking the samples, each CPE was inoculated in 4 mL BPW and incubated at 37 °C overnight. Thereafter, 50 µL of the culture was transferred to BPW and incubated at 37 °C and 180 rpm until an optical density ($OD_{600\,nm}$) of 1.0 was reached. From this suspension, 1 mL was added to 9 g fresh caeca sample. Thereafter, each 1 mL of this mix was transferred to 9 mL LB + CTX and incubated at 37 °C either under aerobic or under microaerobic conditions for 18 h. After incubation, 100 µL of the sample were plated four times onto McC + CTX + MEM and cfu were counted after 20–24 h of incubation at 37 °C. Potential differences induced by the two incubation conditions were assessed through a two-sided Wilcoxon signed rank test for independent samples.

2.2. Method Comparison

2.2.1. Bacterial Strains

In total, twelve CP *E. coli* were used for the evaluation of both isolation methods and the comparison of the different selective media. These isolates cover a wide range of carbapenemase genes (bla_{VIM-1}; bla_{GES-5}; bla_{KPC-2}; bla_{NDM-1} and bla_{OXA-48}) (Supplemental Material Table S1). As reference for quality assurance concerning the selectivity for *E. coli* of the media, the *E. coli* ATCC 25922 was used. In all three approaches, six caeca samples were CPE-negative and inoculated with the *E. coli* ATCC 25922. In each of the other twelve caeca samples, one of the twelve CP *E. coli* was inoculated with a concentration of 100 cfu/mL each.

2.2.2. Sample Preparation for the Method Comparison

All twelve CP *E. coli* and the *E. coli* control strain ATCC 25922 were inoculated in 4 mL BPW and incubated at 37 °C overnight. After incubation, 50 µL of the overnight culture was transferred to fresh BPW and incubated at 37 °C at 180 rpm until an optical density (OD_{600} nm) of 1 was reached. For each CP *E. coli*, 9 g fresh caeca sample was artificially contaminated with 1 mL of the 10^{-5} diluted bacterial suspension. For the control strain, six caeca samples were spiked. All spiked caeca samples were homogenized by

thorough mixing for 1 min. After sample preparation, the samples were blinded by a non-participating person. All 18 samples were analyzed by two methods (Figure 1) as described below. First, 1 g of each spiked sample was added to 9 mL BPW and incubated at 37 ± 2 °C for 16–18 h. Thereafter, the samples were separately subjected to investigations using the two different isolation procedures. For reliable interpretation, the 18 blinded samples were investigated with both methods in three independent approaches.

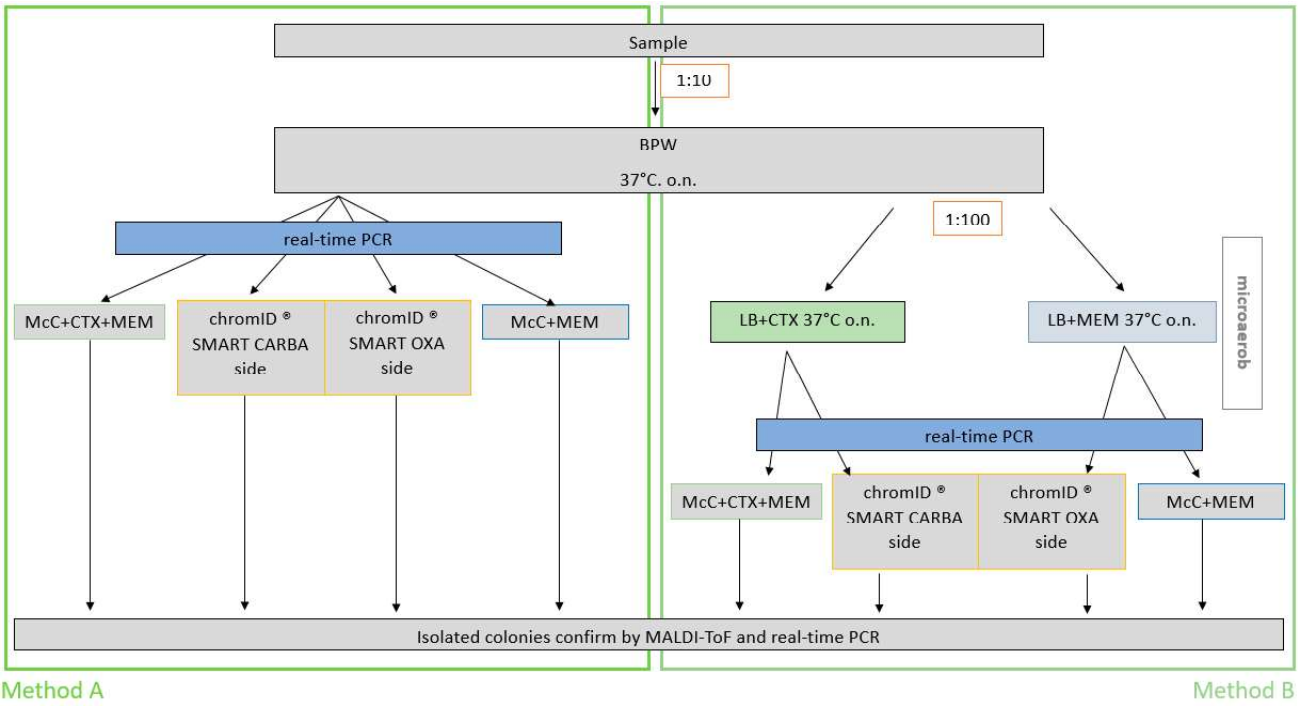

Figure 1. The whole procedure of method A and method B to compare both and to validate the agar plates. BPW = Buffered Peptone Water; McC + CTX + MEM = MacConkey Agar supplemented with 1 mg/L cefotaxim and 0.125 mg/L meropenem; McC + MEM = MacConkey Agar supplemented with 0.125 mg/L meropenem; LB + CTX = lysogeny broth supplemented with 1 mg/L cefotaxim; LB+MEM = lysogeny broth supplemented with 0.125 mg/L meropenem.

2.2.3. Isolation and Detection Methods

Method A represents the EURL-protocol for isolation of ESBL, AmpC and CP *E. coli* from caeca, (https://www.eurl-ar.eu/protocols.aspx, accessed in December 2019). For the method comparison, the commercial chromID® CARBA SMART agar (bioMérieux, Nürtingen, Germany) was used. This agar is composed of the CARBA agar and the OXA agar in equal parts. Therefore, the EURL-protocol was adapted by applying 5 µL on a half agar plate instead of 10 µL on a full agar plate. This adaption allowed the agar validation as part of the method comparison (5 µL per half chromID® CARBA SMART agar plate, 10 µL per a whole McC agar plate) (Figure 2). Additionally, a Real-Time PCR step was added after the enrichment in BPW (described below).

Method B represents the modified version of the isolation procedure. Therein, non-selective enrichment in BPW has been maintained, followed by a second selective enrichment step. For that purpose, 10 µL of the BPW pre-enrichment has been applied to 10 mL LB + CTX and 10 mL LB + MEM. Both enrichments were incubated at 37 ± 2 °C for 16–18 h under microaerophilic conditions. Thereafter, 5 µL of the LB + CTX enrichment was inoculated on the CARBA side, and 5 µL of the LB + MEM enrichment on the OXA side of the chromID® CARBA SMART plate (Figure 1). In addition, 10 µL of LB + CTX or LB + MEM were inoculated on in-house selective agars McC + CTX + MEM or McC + MEM, respectively. Each sample was stroked on each agar in duplicate (six plates per sample). Plates were incubated for 16–18 h at 37 ± 2 °C. Subsequently, one colony with

E. coli typical colony morphology was picked from each plate and enriched in 4 mL LB + MEM. The species of each colony was confirmed by PCR and MALDI-ToF. Confirmed colonies were incubated for 16–18 h at 37 ± 2 °C in LB and 500 µL aliquots were stored in 800 µL of 80% glycerol for further analysis. Samples spiked with OXA-producers were assessed as positive if growth was observed on the chromID® OXA side, likewise on McC + MEM. For the other CP *E. coli*, the sample was regarded positive if growth was observed at least on chromID® CARBA side or on McC + CTX + MEM.

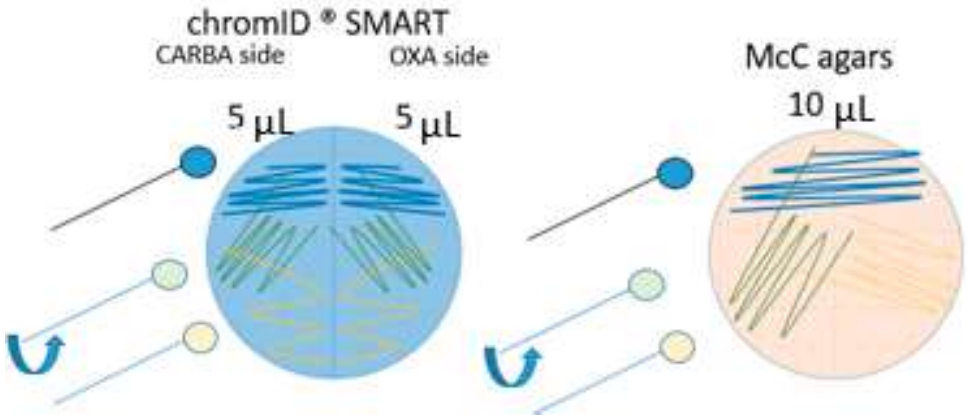

Figure 2. Spreading the sample on the plate. The first spread (blue, first third of the plate) was performed by using a 10 µL-loop. A second spread (green, second third of the plate) was performed by another 10 µL-loop, which was turned the last third of the spread (yellow).

To confirm the validity of the results, the whole experiment was independently repeated three times. Moreover, to ensure that personal handling had no influence, both methods were carried out by two different people per approach. Moreover, each agar type was used in duplicate and deviations (i.e., colony morphology) were noted. If at least one colony could be isolated and confirmed from one of the duplicates, the sample was rated positive for the corresponding method and agar type.

2.2.4. Typing

Antimicrobial Susceptibility-Testing (AST)

The antimicrobial susceptibility of the isolates (Supplemental Material Table S1) was determined by broth microdilution using defined antimicrobial substances and concentrations from the harmonized EU panel following Commission Implementing Decision (CID) 2013/652/EU [plates EUVSEC & EUVSEC2; TREK Diagnostic Systems (Thermo Fisher Scientific, Schwerte, Germany)]. AST was conducted according to the EN ISO20776-1:2006 [15] and MIC values were interpreted based on EUCAST definitions (www.eucast.org, November 2013) using epidemiological cut-off values fixed in CID 2013/652/2013. The strain ATCC 25922 was used for quality control. All isolates were tested twice. First, before using them for artificial contamination and second, after their successful recovery from the samples to assess possible changes in their antimicrobial resistance patterns.

MALDI-ToF MS

One putative colony of CP *E. coli* of each agar plate (six per sample) was chosen for species confirmation by MALDI-ToF MS. As matrix α-Cyano-4-hydroxycinnamic acid (HCCA, Bruker, USA) was used, and analyses were performed by MALDI Microflex Biotyper (Bruker Daltonics, Bremen, Germany) as recommended by the manufacturers.

Molecular Typing of CP *E. coli*

Molecular detection of the carbapenemase genes was carried out to confirm the presence/absence of bla_{GES}, bla_{KPC}, bla_{NDM}, bla_{OXA-48} and bla_{VIM} in enrichment cultures and in presumptive colonies. Both sample enrichments and presumptive colonies were

used for extraction of template DNA by heat treatment of bacterial suspensions. For this extraction, 800 µL were centrifuged by 10,000× *g* for 5 min. The cell pellet was resuspended in 300 µL double distilled water and boiled for 10 min at 99 °C. Thereafter, the samples were cooled on ice and centrifuged for 2 min at 16,000× *g*. Finally, 255 µL of the supernatant was mixed with 45 µL trehalose for stable storage at −20 °C. Real-time PCR was performed on a Bio-Rad CFX system. Primers and probes were adapted from Swayne et al. (2011) [35] and van der Zee et al. (2014) [36]. Amplification was conducted using Biozym qPCR Mastermix (Biozym Scientific, Oldendorf, Germany) and the following conditions: Initially 2 min at 95 °C, 30 cycles with 5 s at 95 °C and 60 s at 60 °C for product amplification.

2.2.5. Statistics

Results of both detection methods were expressed in percent (positive samples divided by all tested samples). The EURL-method and the modified method were compared in terms of their diagnostic accuracy indicated by sensitivity, specificity, false discovery and omission rates, and overall diagnostic accuracy. At this point, the blinding of the samples was dissolved. If the detected CP *E. coli* were confirmed using MALDI-ToF MS and real-time PCR, a sample was classified as positive. The sensitivity was expressed as the proportion of the positive samples correctly identified. The specificity was calculated as the proportion of the samples correctly identified as negative among all negative samples. False discovery rates were calculated as the proportion of all false positive samples among all positive samples. Likewise, false omission rates were calculated as the proportion of all false negative samples among all negative samples. Overall diagnostic accuracy was calculated as the proportion of all tests that gave a correct result. All estimates as well as their corresponding exact 95% confidence intervals (Clopper-Pearson 95% confidence intervals as precision measures honoring small sample sizes) were estimated using the epiR package in R [37].

3. Results

3.1. Modification of the EURL-Protocol for Isolation of CP E. coli from Caeca Samples

In order to determine the number of bacteria that survived the prevailing condition in the pig feces, the recovery rate of the bacteria in feces was determined after storage at 6 °C. Based on the cfu counts, a reduction of 2 log units within 24 h was detected in pig feces samples stored at 6 °C. The addition of glycerin, sodium chloride, and trehalose to the feces samples did not result in a positive effect on the CPE survival rate (Figure 3).

In a first step, the suitability of two liquid media (LB- or Mossel-medium) as basis of this second enrichment were compared. The Mossel-medium was developed especially for Enterobacteriaceae [31], whereas LB-medium is a nutrient-rich microbial broth used for the cultivation of E. coli. No differences in the recovery rate were observed in the direct comparison of LB- or Mossel-medium as second enrichment (data not shown). As LB is a common medium used in many laboratories, it was used for further investigations.

The addition of sublethal conditions of $ZnSO_4$ and MEM was intended to activate potentially present resistance mechanisms in the liquid culture so that the corresponding strains may have a growth advantage when subsequently plated onto the selective solid media. However, the slight selection pressure did not result in a better recovery rate. Therefore, we used the second enrichment in LB + CTX as previously described [23]. To isolate the OXA-48 producers as well, we tested another second enrichment in LB + MEM. Enterobacteriaceae are described as facultative anaerobes [38]. Therefore, the target species can survive in both aerobic and anaerobic environments, in contrast to some representatives of the accompanying microbiota. The microaerophilic incubation led to a median increase in isolated CP E. coli from 7 cfu/mL with aerobic incubation to 26.5 cfu/mL (Wilcoxon test, W = 0, $p = < 0.05$, $n = 8$). Likewise, the median number of CP Salmonella increased from 81 cfu/mL with aerobic incubation to 144.5 cfu/mL (Wilcoxon test, W = 0, $p = < 0.05$, $n = 8$).

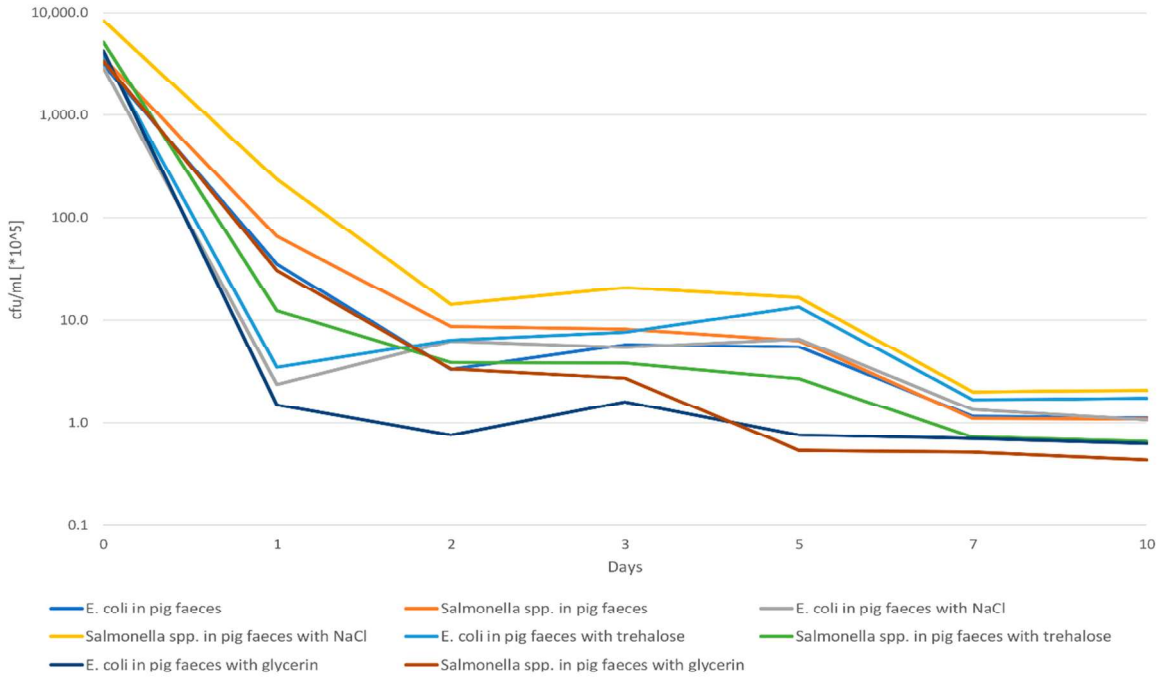

Figure 3. The decreasing number of CPE (*E. coli* and *Salmonella* each in average) in pig faeces with and without some additives over ten days.

Based on the above-mentioned observations, the procedure of the method was modified to promote the growth of CPE and to reduce the amount of accompanying microbiota (Figure 4). The modified procedure includes a first, non-selective enrichment in BPW, followed by two parallel selective enrichments (LB + CTX and LB + MEM) incubated under microaerophilic conditions. Aliquots (10 µL) of the second enrichment were applied onto McC agar supplemented either with MEM or CTX + MEM. In the following chapter, this modified method was compared to the EURL-method.

3.2. The Comparison of the Modified Method with the EURL-Method for Isolation of the CP E. coli from Caeca Samples

To determine the performance of the modified method (method B), its diagnostic accuracy was compared to the reference method of the EURL-AR, the official protocol for isolation of ESBL, AmpC and CP E. coli from caeca samples (method A). The complete procedure of the comparison and validation is schematically illustrated in Figure 1. In the following, the results of the three repetitions were considered together (sample size n = 54) for each method and agar type (Table 1).

Table 1. Summarized results on the repeated isolation of CPE from the spiked fecal samples (calculation by combining the OXA and CARBA- site of the ChromID® SMART CARBA agars and of the McC + CTX + MEM and the McC + MEM plate of the in-house agar). Method B is the modified method. Corresponding exact 95% confidence intervals are provided in brackets.

	Method A + ChromID® SMART CARBA agars	**Method A + *in-house* agars**	**Method B + *in-house* agars**	**Method B + ChromID® SMART CARBA agars**
Sensitivity	75 (57.8–87.9)	100 (90.2–100)	86.1 (70.5–95.3)	66.7 (49–81.4)
Specificity	100 (81.5–100)	100 (81.5–100)	100 (81.5–100)	100 (81.5–100)
False discovery rate	0 (0–12.8)	0 (0–9.7)	0 (0–11.2)	0 (0–14.2)
False omission rate	33.3 (16.5–54)	0 (0–18.5)	21.7 (7.5–43.7)	40 (22.7–59.4)
Accuracy	83.3 (70.7–92.1)	100 (93.4–100)	90.7 (79.7–96.9)	77.8 (64.4–88)

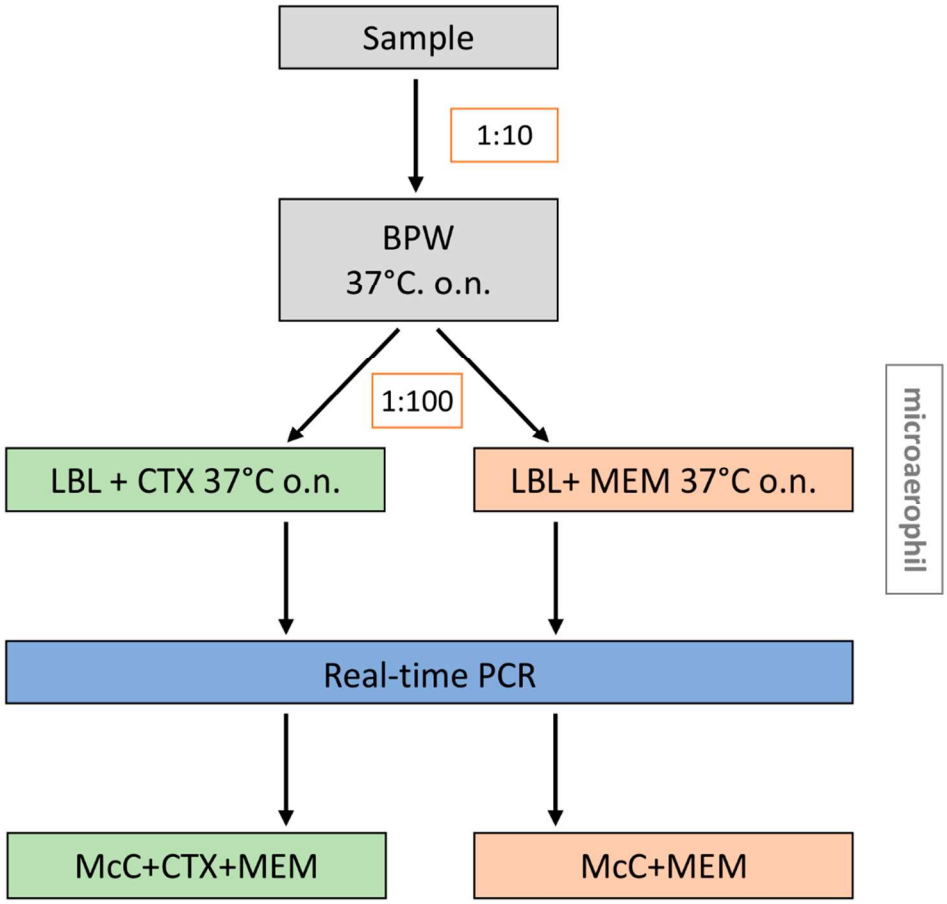

Figure 4. The final step-by-step guidance of the modified method (method B). BPW = Buffered Peptone Water; McC + CTX + MEM = MacConkey Agar supplemented with 1 mg/L cefotaxim and 0.125 mg/L meropenem; McC + MEM = MacConkey Agar supplemented with 0.125 mg/L meropenem; LB + CTX = lysogeny broth supplemented with 1 mg/L cefotaxim; LB + MEM = lysogeny broth supplemented with 0.125 mg/L meropenem.

Comparison of diagnostic accuracy measures show that the use of method A in combination with the in-house agar led to a significantly higher sensitivity, relative to the use of method A with the chromID® SMART CARBA agar. In addition to this overall evaluation, the results for the individual approaches are given in Supplemental Material Table S2. The best CPE detection was achieved by using the original EURL-protocol in combination with in-house prepared McC + MEM and McC + CTX + MEM agar plates. All negative samples (*n*/N) were correctly recognized, and CP E. coli were successfully recovered from each of the artificially contaminated samples (*n*/N). This corresponds to a 100% sensitivity and specificity. The sensitivity decreased to 75% when the in-house agar plates were replaced by the commercial chromID® CARBA agar. However, specificity remained at 100%. The modified method (B) reached a sensitivity of 86.1% when using the in-house agar and a sensitivity of 66.7% when using the commercial agar. However, all negative samples (*n*/N) were also correctly identified leading to a specificity of 100%. The added value of this modified method is the simplification of the isolation of putative colonies that are difficult to isolate. This could be shown by counting the agar plates (108 per agar type and method) with and without accompanying microbiota (i.e., Pseudomonas (P.) putida, P. monteilli, P. aeruginosa, Proteus mirabilis) (Table 2). Using the EURL-procedure in combination with the chromID® CARBA agar, on 44.5% (48/108) of the agar plates growth of accompanying microbiota was observed. That was reduced to 39.8% (43/108) using the modified method with the chromID® CARBA agar. In addition, for the chromID® OXA agar, a reduction of accompanying microbiota from 23.2% (25/108) of the plates by

using the EURL-procedure to 15.7% (17/108) using the modified method was observed. Using the in-house agars and the EURL-procedure, accompanying microbiota was observed on 37.0% (40/108) of the McC + MEM plates and on 33.4% (36/108) of the McC + CTX + MEM plates. By changing to the modified method, we detected accompanying microbiota on 29.6% (32/108) of the McC + MEM plates and on 36.1% (39/108) of the McC + CTX + MEM plates.

Table 2. Validation of the selective agars by counting the number of plates with growing of accompanying microbiota for each group per used method. McC + CTX + MEM = MacConkey Agar supplemented with 1 mg/L cefotaxim and 0.125 mg/L meropenem; McC + MEM = MacConkey Agar supplemented with 0.125 mg/L meropenem.

	ChromID® SMART CARBA Side	ChromID® SMART OXA Side	McC + MEM	McC + CTX + MEM
Method A	48/108 (44.45%)	25/108 (23.15%)	40/108 (37.04%)	36/108 (33.36%)
Method B	43/108 (39.82%)	17/108 (15.74%)	32/108 (29.63%)	39/108 (36.14%)

3.3. Molecular Detection of the Carbapenemase Genes

As previously described, the multiplex real-time PCR was used to pre-screen the enrichments (BPW, LB + CTX, and LB + MEM). Discrepancies were detected between the PCR and the results of the culture-based isolation. Comparing the PCR results with the true status of the samples, a sensitivity of 61.1% and a specificity of 94.4% was calculated for the molecular screening of the heat-treated BPW enrichments for carbapenemase genes. Fourteen out of thirty-six positive samples were not correctly identified. One of the 18 negative samples was incorrectly identified as positive for the bla_{GES}.

When considering the screening of both second enrichments (results listed in Table 3), just two of the false negatives remained false negative, and the false positive sample remained as well. Therefore, the PCR achieved a sensitivity and a specificity of 94.4% by using both second enrichments as DNA template. Moreover, the real-time PCR showed erroneous simultaneous detection of bla_{NDM} and bla_{VIM} in various samples (9.2%) that were only bla_{VIM}-positive. However, a high cp-valuefor bla_{NDM} was detected, suggesting low amplification. The results for the weekly approaches are given in Supplemental Material Table S3.

Table 3. Accumulated results on the weekly detection of carbapenemase genes from the spiked fecal samples. BPW was used for the first enrichment, LB + CTX and LB + MEM were used parallel as second enrichments. Corresponding exact 95% confidence intervals are provided in brackets.

	BPW	LB + CTX	LB + MEM	Both LB Enrichments
Sensitivity	61.1 (43.5–76.9)	83.3 (67.2–93.6)	58.3 (40.8–74.4)	94.4 (81.3–99.3)
Specifity	94.4 (72.7–99.6)	94.4 (72.7–99.6)	100 (81.5–100)	94.4 (72.7–99.9)
False discovery rate	4.3 (0.1–21.9)	3.2 (0.1–16.7)	0 (0–16.1)	2.9 (0.1–14.9)
False omission rate	45.2 (27.3–64)	26.1 (10.2–48.4)	45.4 (28.1–63.6)	10.5 (1.3–33.1)
Accuracy	72.2 (58.4–83.5)	87.0 (75.1–94.7)	72.2 (58.4–83.5)	94.4 (84.6–98.8)

4. Discussion

4.1. Modification Steps of the Official Isolation Protocol

All modifications were tested to reduce the amount of accompanying microbiota or to increase the recovery rate of the CPE. First, the recovery rate of the target bacteria in pig feces was determined at 6 °C and based on the bacterial growth calculated by the development of the cfu/g every day. To increase the survival of bacteria for better isolation, several additives to the pig feces were tested. In previous studies, the addition of sodium

chloride has been reported to support growth under difficult conditions like low pH and high lactate concentrations [29]. Another study evaluated trehalose for the protection of *E. coli* against carbon stress [25]. The absence of a positive effect of these additives in our study may be due to the lack of a clear definition of the individual influencing factors of each fecal sample, i.e., the microbiota and other factors, which could have an influence on the nature of the samples. It should be noted here that the detection of CPE was increasingly challenging over the investigated period. One major challenge was the differentiation of CPE from increasing amounts of accompanying flora. Therefore, we recommend a timely processing of samples, preferably within 24 h after collection as this was an important factor for reliable detection of CP *E. coli* in feces and food matrices.

After estimating bacterial survival, other options for improved detection of the target bacteria were tested. To account for the low MIC values of CPE isolated from samples of animal origin [22], we considered triggering gene expression by using a sublethal concentration of MEM. Moreover, the usage of $ZnSO_4$ to push the metallo-β-lactamases was tested. Both approaches did not improve the recovery rate. Previous experiments achieved a beneficial effect by using a second selective enrichment step. This enrichment had been conducted in LB+CTX [23]. As OXA-48 producers are not reliably able to survive the presence of other β-lactams, like CTX, we considered another parallel second enrichment using LB+MEM [39,40]. The usefulness of the combination of both enrichments was also proven by the sensitivity of 94.4% of the Real-Time PCR.

Further, we exchanged the commercial selective agar chromID® CARBA agar with an in-house prepared McC + CTX and McC + CTX + MEM. Our results support a previous study on the suitability of chromID® CARBA agar for detecting CPE with only slightly reduced susceptibility to carbapenems [22]. Within this study, it is presumed that the concentration of different ingredients and thus the selectivity is too high for CPE originating from animals or food. CPE from livestock often exhibit lower MIC values for the respective carbapenems than clinical human isolates [22,24]. Our previous study investigated the use of chromID® CARBA after the end of the shelf life expecting a possible decrease in antimicrobial concentration. As the selective agar is not specified in the EURL-AR reference method (https://www.eurl-ar.eu/protocols.aspx, December 2019), the change to adjusted agar might be easy adaptable. In this study, we analyzed the use of an in-house McC agar, supplemented with MEM and MEM+CTX, and commercial agar for the detection of CPE from feces. The experiments were carried out with fresh prepared in-house agars (two weeks). However, the comparison in this study is limited to only one commercial selective agar. For commercial agars, a wide variation regarding their suitability for the isolation of CPE from different samples has been reported [18,20,41,42]. For the chromID® CARBA agar, sensitivity in the literature ranges from 30 to 100%, and specificity from 50 to 100%. Still, the chromID® CARBA agar is described as one of the most sensitive media [18,20,41,42] and is therefore commonly used by German laboratories in the monitoring according to CID 2013/652/EU.

4.2. The Comparison of Both Microbiological Methods

Although the isolation of CPE from livestock and food is rare in monitoring programs, the number of reports on successful recovery is increasing in recent years [6]. The isolation methods described differ from direct plating to different incubation conditions, supplements, and enrichments [23,43,44]. These non-harmonized detection methods in research projects in the animal sector complicate a realistic assessment of the occurrence of CPE in livestock. The EURL-AR has developed and provided a harmonized method for CP *E. coli* isolation from meat and caeca samples, which is meant to be used in monitoring according to CID 2013/652/EU. However, it is currently rarely used beyond the EU monitoring programs. Several publications reported on difficulties in detecting CPE with the EURL-method [22–24]. We observed a sensitivity of 75% and specificity of 100% for the EURL-AR protocol with a commonly used commercial agar. This implies that 25% of CPE-positive samples were not recognized, which demonstrates the need for an improvement of the

reference method. Our results indicate that using the validated EURL-protocol in combination with the non-commercial McC agar supplemented with 0.125 mg/L MEM, and with 0.125 mg/L MEM and 1 mg/L CTX significantly improved the sensitivity compared to all other approaches. Selective enrichment under microaerobic conditions did not achieve the desired effect. This microaerobic incubation was promising because of the ability of Enterobacteriaceae to grow under this condition, in contrast to e.g., Aeromonads, which are often part of the accompanying microbiota [38]. Other options to favor Enterobacteriaceae over other bacteria have been investigated. An incubation temperature of 44 °C was described in the report on the validation of selective McC agar supplemented with 1 mg/L CTX for monitoring of ESBL- and AmpC producing *E. coli* in meat and caeca samples [45] and in a previous study of Irrgang et al. 2019 [23]. The authors recognized that the growth of CP Enterobacteriaceae other than CP *E. coli* might also be inhibited [23]. In terms of harmonization, a method should be capable of also detecting other bacterial species, as all CPE are of interest regarding the distribution of the resistances [6]. Our study focused on *E. coli* as the EU monitoring for ESBL/pAmpC and carbapenemases targets this species.

The reported problems during detection of CPE-containing samples indicate a low occurrence of CPE in samples of animal origin [23,24]. Another rarely discussed reason for reduced detection of CPE from feces and caecum could be that the bacteria in this environment have lost the ability to grow on routine media. It is described that *E. coli*, *Klebsiella pneumoniae*, *Salmonella enterica* serovar Typhimurium, and other human/zoonotic pathogens could enter a distinct state, a so-called viable but non-culturable (VBNC) state [46,47]. In combination with the physical stress exerted by caeca samples, the transition to a VBNC status would be useful for the survival of CPE [47]. Overall, we need to be able to detect positive samples and to isolate these CPE for a further characterization and risk assessment.

Another approach to improve sensitivity might be a more sensitive molecular screening method. Using pre-screening by PCR, efforts can focus on the cultivation of the isolates from PCR-positive samples. This would decrease the costs and the personal time necessary for the microbiological isolation. The multiplex Real-Time PCR applied in this study showed good results when screening the second enrichment (a sensitivity and specificity of 94.4%). It might be possible that the use of a quantitative Real-Time PCR will provide more reliable results as a direct increase of the bacterial growth can be detected and used for adapting the time points for selective cultivation. The Real-Time PCR performed was not effective when applied on the initial BPW enrichment. To avoid the isolation step of a target bacterium and to immediately analyze the entire sample, the option of metagenomics becomes more attractive. Metagenomics would allow a simultaneous identification and typing of CPE and other pathogens [47]. It would be a powerful tool for monitoring purposes and might be considered in the long term to become part of the routine method. Currently, this method is too expensive for routine use and requires additional bioinformatic expertise.

5. Conclusions

After various approaches to optimize the recovery rate of CPE in pig feces, it could be concluded that potential additives did not have a positive effect. However, a positive effect can be achieved by processing the samples in a timely manner. Moreover, our results confirmed the influence of the chosen media. Typically used commercial agar plates are optimized for the isolation of CPE from human clinical samples with usually high MIC values for carbapenems. Therefore, we recommend the EURL-method with a selective medium corresponding to the searched target bacterium. A second enrichment under microaerophilic conditions reduced the accompanying microbiota, but the sensitivity of the whole procedure was decreased from 100% to 86.1%. The PCR-screening from a second enrichment showed good results. Therefore, we recommend the modified method if a presumptive CP *E. coli* cannot be isolated. However, a sensitive method is essential to avoid an underestimation of the CPE occurrence in livestock in Europe.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms9051105/s1, Supplemental Material Table S1: Detailed information about the used CPE. Supplemental Material Table S2: The repeated isolation of CPE from the spiked faecal samples. Supplemental Material Table S3: Summarized Real-time PCR results on the repeated detection of carbapenemase genes from the spiked faecal samples.

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3 Discussion

3.1 Characterization of new CPE isolates

One aim of the IMPART project was the detailed characterization and the analysis of new CPE isolates. From 2014 until 2020, the monitoring of CPE in food-producing animals was an optional part in the mandatory monitoring of ESBL/AmpC producing *E. coli* in Europe. It was enforced with the Commission Implementing Decision 213/652 on the monitoring and reporting of AMR in zoonotic and commensal bacteria (European Commission, 2016). As a part of these monitoring programs, six CP *E. coli* were detected in Germany, of which four are part of this doctoral thesis (the VIM-1 producing *E. coli* 17-AB02384 in **publication 1** (Pauly et al. 2020a), the VIM-1 producing *E. coli* 19-AB01133 in **publication 2** (Pauly et al. 2021a), the OXA-48 producing *E. coli* 19-AB01443 (Irrgang et al. 2020a) and the GES producing *E. coli* 19-AB02908 (Irrgang et al. 2020b)). Pigs and poultry are sampled in annual rotation (Schrijver et al. 2018). All six monitoring CP *E. coli* were isolated from pig samples (Pauly et al. 2021a; EFSA and ECDC 2020; Irrgang et al. 2020a; Irrgang et al. 2020b; Pauly et al. 2020a). In addition, two *Salmonella* isolates were also detected in pig samples over the last years as a part of the corresponding *Salmonella* monitoring (EFSA and ECDC 2020). This suggests that pigs have become a livestock reservoir for CPE. One possible reason could be the homology between pigs and humans. The similarities of the digestive tract of humans and pigs led to the use of pigs as model organisms (Roura et al. 2016). For example, gut barrier functions and the functional permeability were studied using pigs as model organism (Roura et al. 2016). Due to their role as food-producing animals, pigs as reservoirs could lead to transmission of CPE between livestock and humans through meat products. Moreover, a new animal reservoir could play an important role for their spread into other ecological niches. The role of livestock as a reservoir for multidrug resistant bacteria or corresponding plasmids has been discussed in several studies (Fischer et al. 2014; Carattoli 2008). Therefore, for the six CP *E. coli* isolates, the location of first isolation (farms or slaughterhouses) were re-investigated. Only one isolate could be detected repeatedly (Irrgang et al. 2017b). The absence of further CPE isolations from composite fecal samples and environmental samples (including meat, cobwebs, dust, water trough, wet residues and sock swabs) contradicts a stable occurrence in pigs. This raises the question about the repeated spillover of isolates from humans or other potential entry sources. The low number of CPE in the monitoring can have several reasons. On one hand, it can be due to the lack of sensitivity of the recommended method. On the other hand, this may indicate that humans remain their main reservoir. Thus, repeated entry into the food production chain could be the main source of CPE occurrence.

Humans as well as birds and rodents are discussed as likely entry vectors. For instance, Fischer et al. (2013) (Fischer 2013b) described the detection of an NDM-1 producing *Salmonella enterica* subsp. *enterica* serovar Corvallis in a wild bird. They discussed the molecular analysis, which revealed that the presence of the NDM-1 carbapenemase in a black kite, a long-distance migrating bird, is most likely related to its wide distribution in humans in its wintering grounds (Fischer 2013b). Other studies referred to the proximity of CPE-positive animals (e.g. rats or gulls) to human or human environment (Desvars-Larrive et al. 2019; Vergara et al. 2017). Likewise, the colonization of animals and farmers with the same resistant bacteria was described for ESBL producers and MRSA (Fischer et al. 2017). Conversely, the possible route of transmission from animals to humans through the food chain could also pose a major risk to consumers. Depoorter et al. (2012) designed a model to estimate the exposure of humans to third generation cephalosporin resistant *E. coli* through broiler meat (Depoorter et al. 2012). Moreover, the location of the $bla_{\text{VIM-1}}$ gene with other human-associated resistance genes (e.g. like $bla_{\text{CMY-13}}$) on the same plasmid could be a hint for a human source (Caratolli 2009). Carbapenems are not licensed for the treatment of livestock animals in Europe. Nevertheless, with the use of other antimicrobial agents, a co-selection of carbapenemase encoding genes may have been possible since carbapenemase genes are mostly located on multi-resistance cassettes (Prescott 2008). Considering a potential link between CPE emergence in the animal and human sectors as well as other vectors (i.e. birds and rodents), the “One Health” approach should be deepened in future research. Possible transmission of resistance between humans, animals and the environment may occur by dissemination of resistant bacteria or mobile genetic elements, which can be transferred to other strains or even other bacterial species (Day et al. 2016; Leverstein-van Hall et al. 2011). Therefore, this approach about the global collaborative work from human and veterinary medicine is needed nowadays (Calistri et al. 2013). Based on the small number of CPE reports in food-producing animals, it is premature to discuss the assumption of two different convergent evolving reservoirs. Instead, it should be considered as a complex relation between AMR in humans, food and food-producing animals.

Despite the arguments in favour of a spontaneous human entry, earlier findings and the increasing trend of reports about CPE in livestock animals, especially in pigs, give rise to the assumption that the food and livestock sector might emerge as a reservoir for carbapenemase encoding genes or bacteria. Two studies in 2011 and 2012 by Fischer and her colleagues suggested a clonal relationship between two VIM-1 producing *E. coli* and three *S.* Infantis from three different farms (Fischer et al. 2012b).

The high similarity of the strains and plasmids and the long study period may indicate the ability of these strains to survive in the farm environment. Alternatively, an epidemiological link between CPE-positive farms was suspected.

A possibility might be repeated entries from a common source, for example the piglet producer or food. Furthermore, the authors discussed an interspecies plasmid transfer regarding the presence of similar VIM-1 encoding plasmids in *S.* Infantis and *E. coli* of one farm (Fischer et al. 2012b). They showed that a self-transferable IncHI2 plasmid was detected in the *Salmonella* isolates and a smaller IncHI2 plasmid in the *E. coli* isolates, which were neither self-transferable nor mobilizable (Falgenhauer et al. 2017). Evidently, the CPE could not establish permanently; no further CPE have been reported from these farms in a follow-up study (Roschanski et al. 2017). Nevertheless, after the beginning of the CPE monitoring in food-producing animals, another related VIM-1 producing *E. coli* was isolated (*E. coli* 16-AB00585 in 2016) in Germany (Irrgang et al. 2017b). This isolate harbored the bla_{VIM-1} gene chromosomally (Irrgang et al. 2017b). More isolates of the same farm were similar to the one from the monitoring programme, although the bla_{VIM-1} genes were located on 180–200 kb IncHI2 plasmids (Irrgang et al. 2017b). Moreover, other CP *E. coli* have been isolated on pig farms. In 2017, two more CP *E. coli* were isolated within the monitoring programmes (*E. coli* 17-AB01027; unpublished but reported (EFSA and ECDC 2020) and the *E. coli* 17-AB02384; **publication 1** (Pauly et al. 2020a)). Especially the detailed characterized *E. coli* 17-AB02384 showed high similarities to the plasmids of earlier detected isolates. For instance, the plasmid pEC17-AB02384 was closely related to the VIM-1 encoding plasmids pSE15-SA01028 (90% identity) from *S. enterica* subsp. *enterica* and pRH-R178 (93% identity) from *E. coli*. Until the isolation of the *E. coli* 17-AB02384, all livestock-associated VIM-1-producers, whether monitoring isolates or previous ones, harbored the bla_{VIM-1} gene on a typical class 1 integron. These integrons were located on IncHI2 plasmids. In several countries, the same integron was described in different human pathogen bacteria, i.e. *Klebsiella* spp., *Enterobacter* spp., and *Pseudomonas* spp. and located on IncHI2 plasmids (Falgenhauer et al. 2017; Tato et al. 2010). Next to the high relation of the plasmids, the similarities include the sequence type (ST88), the affiliation to the phylogenetic group A according to Clermont et al. (2000) (Clermont et al. 2000) and the macrorestriction pattern. The two monitoring isolates from 2017 differed regarding these characteristics; the 17-AB01027 isolate belonged to the phylogenetic group B1 and the ST48, whereas the 17-AB02384 *E. coli* belonged to the phylogenetic group A, but to the ST7593 (Pauly et al. 2020a). Thereafter, no further isolate with such high consistency regarding the plasmids were detected.

Nevertheless, another *bla*$_{VIM-1}$ harboring *E. coli*, described in **publication 2** (Pauly et al. 2021a), was isolated in 2019. The *bla*$_{VIM-1}$ gene of the isolate 19-AB01133 was still a part of the described class 1 integron structure but located on an IncA/C$_2$ plasmid. It was one of the first reports of a *bla*$_{VIM-1}$ gene on an IncA/C$_2$ plasmid from animal samples. Four years before, in 2015, a similar strain was isolated from a Belgian pig meat sample by Garcia-Grealls and her colleagues (2020) (Garcia-Graells et al. 2020). Apart from the high similarity (99.99% identical over the entire sequence) of the plasmids, their origin (pig) and the MLST (ST5869) are the same. Thus, the possibility of repeated entries from a common source was considered.

Moreover, the *E. coli* isolate TZ 116, a backup strain for the validation of selective plates for the detection of CP *E. coli* (https://www.eurl-ar.eu/protocols.aspx), was isolated from a human sample. Its plasmid showed an equally high relationship to the plasmid pEC-19AB01133 (Pauly et al. 2021a) and the plasmid described by Garcia-Grealls (Garcia-Graells et al. 2020). In addition, other similar *bla*$_{VIM-1}$ harboring IncA/C plasmids of human origin were described (Esposito et al. 2017; Johnson et al. 2003). Johnson and Lang (2012) have written an overview about the history of such plasmids (Johnson and Lang 2012). They described plasmids of this Inc group as associated with the emergence of multidrug resistance in enteric pathogens. However, it was not possible to pursue this theory further. The detection of highly related *bla*$_{VIM-1}$ carrying plasmid in *E. coli* originated from different pig farms since 2011 indicated a persistence of such plasmids. In terms of trace back approaches, it should be considered that there is no legislative regulation for further investigations on CPE positive farms in Germany. The responsible authorities depend on the cooperation of the farmers.

In 2019, two additional CP *E. coli* were recovered during the CPE monitoring, with resistance based on other carbapenemases. The *E. coli* 19-AB01443 harbored a *bla*$_{OXA-48}$ located on a common ~60 kb IncL/M plasmid (Irrgang et al. 2020a). Such pOXA-48 plasmid-prototypes are globally distributed among several species and sample origin (Poirel et al. 2012). The third CP *E. coli* in 2019 carried a *bla*$_{GES-5}$ harboring plasmid (Irrgang et al. 2020b). As described in **publication 1** (Pauly et al. 2020a) **and 2** (Pauly et al. 2021a), a human entry source was speculated due to its strong similarity with human isolates. It can be assumed that the increasing variance of livestock-associated carbapenemase genes in the German food-production chain relates to the epidemiological situation of CPE in Germany (Brolund et al. 2019). Since 2010, the epidemiological situation in Germany is unchanged on stage 3 of 5, indicating a regional spread (Brolund et al. 2019).

In terms of numbers, the status is constant, but the diversity of genes is increasing. GES carbapenemases were detected only sporadically from human infections in Germany, in contrast to OXA-48, VIM, NDM and KPC carbapenemases (Pfennigwerth 2018). Moreover, NDM or KPC producing *E. coli* were not isolated within the monitoring programs in food-producing animals but in various bacteria (i.e. Enterobacteriaceae and pseudomonads) from human samples. However, the increasing variance of carbapenemase genes and bacteria is an interesting development. So far, VIM-1-producers and OXA-48-producers were present in food and livestock (Pauly et al. 2021a; Garcia-Graells et al. 2020; Irrgang et al. 2020a; Irrgang et al. 2020b; Pauly et al. 2020a; Fischer et al. 2012b), in wildlife (Duff et al. 2020; Vergara et al. 2017; Fischer 2013b), and in companion animals (Nigg et al. 2019; Boehmer et al. 2018; Pulss et al. 2018; Melo et al. 2017; Gonzalez-Torralba et al. 2016; Schmiedel et al. 2014; Stolle et al. 2013). Other carbapenemases seem to be less spread and only sporadically reported. For example, the carbapenemase KPC-2 was reported only once from fecal samples from yellow legged gull (Vergara et al. 2017) and the carbapenemase NDM-1 was reported only in a wild bird (Fischer et al. 2013b) and rats (Desvars-Larrive et al. 2019). The current distribution of carbapenemases in German livestock illustrates that the variability increased according to the development of human medicine, but only a part of them was detected in recent years.

3.2 Modification of the current isolation method

So far, the detection of CPE in animals was reported sporadically. Nevertheless, the reports indicate that livestock could constitute as a potential source for the spread of CPE or carbapenemase gene harboring plasmids. As already discussed, some CPE were described as low concentrated in the samples and with only slightly reduced susceptibility. Both complicate the screening for these bacteria as a part of the antimicrobial monitoring in terms of an early warning system. Therefore, a reliable screening method is essential. The corresponding method optimization was one aim of the IMPART project. The current isolation method for CP. *E. coli* was elaborated by the EURL-AR. It was developed on the basis of the protocols for isolation of ESBL/AmpC producing from caecal samples of slaughtered animals and from meat. It is a culture-based method with the selection at the last step by using selective agar plates. As a part of this monitoring, only a few CPE isolates were detected. As one example for the lack of sensitivity of the recommended method, the CP *E. coli* 17-AB02384 is described in **publication 1** (Pauly et al. 2020a). This isolate was recovered within the monitoring of ESBL/AmpC producing *E. coli*. However, the CPE isolation method of the same sample failed.

Different approaches to optimize the current method were investigated. Moreover, the IMPART project aimed an effective isolation method for CPE in general, not only for *E. coli*. For this reason, different options to push Enterobacteriaceae were tested. It was difficult to include an Enterobacteriaceae-specific step, which may exclude the disturbing accompanying flora, what is due to the high variability of the family, which comprises 51 genera and 238 species (Octavia and Lan 2014). Enterobacteriaceae are ubiquitous in nature, are present in terrestrial and aquatic environments. Some of them are associated with animals, plants, or insects (Octavia and Lan 2014). Although such a large family is to be detected, the experiments were particularly constrained by the small number of strains and species. In recent years, only a few CPE were isolated during monitoring, and other studies also resulted in only a limited number of CPE. As another limitation, the available CPE were all VIM-1 producers. Thus, especially the first experiments were limited to seven CPE (four VIM-1 producing CP *E. coli*, two VIM-1 producing CP *S. enterica* subsp. *enterica* and one NDM-1 producing *S.* Corvallis). Furthermore, the method optimization had to focus on the handling within the monitoring for the performing routine laboratories. This means, for example that the first enrichment in buffered peptone water (BPW) remained unchanged in the investigated approaches, because it was also used for screening for other zoonotic pathogens (Amtsblatt der Europäischen Union, 2013). Considering these constraints, first some basic data had to be collected to optimize the method.

Therefore, the survival potential of seven different CPE (four *E. coli* and three *Salmonella enterica* subsp. *enterica* serovars) was observed in different matrices (food and faeces). They survived without any problems in the tested food matrices (milk, fish, poultry meat, pig meat and bovine meat). This survival is one of the current concerns of the public health authorities. Bacterial cross- and recontamination during food preparation could play an important role in the dissemination of such bacteria. For MRSA, the aspects of transmission dynamics and quantification of consumer exposure to MRSA through food consumption were addressed using a probabilistic model (Plaza-Rodriguez et al. 2019). The study showed a strong correlation between the prevalence of MRSA at retail and the likelihood of contamination of the final serving. Presumably, it would be similar for CPE. Since the rare detection of CPE in food suggested a very low prevalence (the prevalence of MRSA in German poultry in the supermarket is about 13 % (Pauly et al. 2019)), this risk can currently still be classified as unlikely. The same experiments using faeces (poultry faeces, pig faeces and bovine faeces) as matrix showed a reduction of two log-units within 24 h of the CPE. In addition to this reduction, the accompanying flora (i.e. *Pseudomonas* spp.) increased over time independent of the matrix and made an isolation of the target germ more difficult. This observation was described in previous studies (Irrgang et al. 2019), suggesting that rapid processing of samples is essential, preferred within 24 h. Nevertheless, sampling and transport are time-consuming, and a same-day-processing is almost impossible for routine laboratories. For this reason, attempts were made to stabilize CPE in faeces samples. However, if CPE are that unstable, the question is whether a new reservoir for CPE could emerge at all. It has been shown that CPE are not stable in faeces but may be stable in the environment of the animal. It is possible that the choice of sample material is unfavourable for monitoring. Environmental samples could be more informative. Nevertheless, in **publication 4** (Pauly et al. 2021b), a possible effect of any pre-addition (trehalose, glycerol, sodium acid) was tested due to their protective characteristics or the enhancive influence on recovery and growth to Enterobacteriaceae (Algara et al. 2019; Jordan and Davies 2001; Hollander and Nell 1954). Such an early addition would have the disadvantage that the sample could no longer be used for any other monitoring. This was a basic idea to keep the handling of the different monitoring as simple as possible. However, no positive effect was recognized by using such pre-additions. Because no increase in CPE isolation was achieved and the expected additional effort, this approach was not pursued further or recommended.

In addition, various attempts in terms of a second enrichment (i.e. different media, different supplements in different concentrations and some incubation conditions) and of molecular prescreening by real-time PCR were tested. Based on this, two secondary enrichments (LB+CTX and LB+MEM) were included in the modified method. However, a comprehensive method comparison (described in **publication 4** (Pauly et al. 2021b)) showed that this enrichment primarily improved the results of the associated real-time PCR, but not on the isolation itself. Conversely, PCR was only reliable after a second selective enrichment. PCR after initial enrichment in BPW cannot be used as a reliable evaluation tool. Nevertheless, molecular prescreening methods should also be considered in the future. If CPE-negative samples can be excluded prior to microbiological processing, significantly fewer samples would have to be processed in such an elaborate manner. In the future, promising methods such as i.e. metagenomics could play a major role in different monitoring. By processing a sample using metagenomics, the appropriate monitoring procedure could be selected (Grützke et al. 2019; Viau et al. 2016)

The modification that had a significant effect on the sensitivity of the isolation method was the choice of selective medium (described in **publication 3** (Pauly et al. 2020b)). In the official isolation protocol, selective agar is only suggested, not mandated (https://www.eurl-ar.eu/protocols.aspx). Due to this flexibility, it would be easy to implement. The chromID® CARBA agar (bioMérieux, Nürtingen, Germany), that was used for comparison, is described as one of the most sensitive ones (Dierikx et al. – submitted). Thus, it is commonly used in the German monitoring following CID 2013/652/EU and experiments in this doctoral thesis were focused on the chromID® CARBA agar. However, this selective agar was developed and tested predominantly for human isolates. **Publication 3** (Pauly et al. 2020b) compared the usability of this commercial agar with an alternative *in-house* prepared agar (MacConkey agar (McC) supplemented with 1 mg/L cefotaxime (CTX) and 0,125 mg/L meropenem (MEM) (McC+CTX+MEM)). A major disadvantage of the chromID® CARBA agar is that its composition is unknown. Based on previous experiments, it was assumed that contained antimicrobial agents lose activity over time or after expiry. Hence, the selectivity will decrease as well and CPE with only slightly reduced susceptibility to carbapenems might grow. In addition, a correlation between the growth and the resistance mechanism was suspected. In other studies, a different media performance corresponding to the Ambler classes of enzymes was reported. For example, Viau et al. (2016) (Viau et al. 2016) described a good detection for class A enzymes (i.e. KPC) by using ten different media. In contrast, the performance was variable for class B (i.e. NDM, VIM) and class D enzymes (OXA-48).

For chromID® CARBA agar evaluation (**publication 3** (Pauly et al. 2020b)), the used isolates represented different bacterial genera (*E. coli*, *S.* Corvallis, *V. parahaemolyticus* and *K. pneumoniae*) and carbapenem resistance genes (bla_{VIM-1}, bla_{NDM-1}, bla_{KPC-2}) as well as resistance mechanisms (zinc or serine based). The previous observations and assumptions were confirmed. These results supported the suspicion that one reason for the lack of sensitivity might be the choice of selective plates. All isolates grew well throughout the whole experimental period on the alternative *in-house* agar. This may be due to the low concentration of MEM in the agar (0,125 mg/L), which is lower than the current ECOFFs (0.25 mg/L). However, increased accompanying flora may occur due to the lower specificity. To simplify the isolation of CPE, the *in-house* plates should be modified to become a chromogenic agar.

Focusing the question of a suitable agar as well, a European wide ring trial was carried out as a part of the IMPART project. Various commercial agars were tested for their ability to isolate CPE from porcine faeces and meat. The authors described that all tested commercial agar plates had the same problems in detecting the *E. coli* strain R1180 (Irrgang et al. 2017b) with bla_{VIM-1} (Dierikx et al. – submitted). This strain showed just slightly reduced susceptibility to carbapenems and was originally derived from pig faeces. Therefore, realistic monitoring conditions were emulated with this isolate. However, it could not be detected with the commercially available agar plates, but with the *in-house* McC+CTX+MEM (Dierikx et al. – submitted). The previous results could therefore be confirmed within the framework of this interlaboratory study. The low MIC values of CPE with an animal origin are described in different publications. This observation may have several causes. A small exposure of a bacterium to the appropriate antibiotic may be one explanation. The gene was present and active but was not needed by the bacterium. In Europe, carbapenems are not approved for the treatment of livestock animals. Therefore, the previous exposure to carbapenems and the development of resistance is unlikely for bacteria in livestock animals and their direct environment. Nevertheless, the low MIC values could be due to the different, albeit possibly low, antimicrobial selection pressure to which the strains are exposed. As shown in **publication 1** (Pauly et al. 2020a) **and 2** (Pauly et al. 2021a)**,** CPE usually harbor several other resistance genes mostly on plasmids. Accordingly, other antimicrobial agents used in animal production enhance co-selection. Besides the potential co-selection, other molecular conditions could be a possible cause, i.e. the expression of the genes, a variable number of gene copies or a different genetic localization (plasmid or chromosome).

In summary, most of the approaches tested had little influence on the performance of the isolation method with one exception. The use of an appropriate selective agar significantly increased the sensitivity of the method (**publication 3** (Pauly et al. 2020b) **and 4** (Pauly et al. 2021b)). *In-house* prepared McC agar was shown to perform best and most constant over 20 weeks of storage. It has the advantage of known ingredients and can be easily implemented. However, apart from the increased sensitivity, consideration must be given to how and whether this modification can be implemented for routine laboratory performance. The selective agar is not recommended in the official protocol. In accredit laboratories, a lot of effort must be made to ensure that the agar complies with the various quality assurance guidelines. Commercial agars (i.e. chromID® CARBA SMART) do not need to be validated because the manufacturers provide quality certificates. *In-house* prepared agar plates (like the McC+CTX+MEM) must be validated by the laboratories. However, it is possible to have the recommended plate produced externally with the usual quality standards. The performance of the agar would have to be proven in comparison to the standard one. Another aspect to consider is that *E. coli* can be clearly distinguished from other species by its pink coloration, but other Enterobacteriaceae are not. A chromogenic reaction of other Enterobacteriaceae, such as *Salmonella* or *Klebsiella* would simplify to distinguish these pathogens from the accompanying microbiota.

Regarding the matrix effects on bacterial survival, samples should be processed on the day of sampling. Furthermore, the method gains in sensitivity through the choice of a suitable agar. In case of uncertainty, a second, selective enrichment can be helpful to reduce the accompanying microbiota and for molecular screening. To realistically assess the risk posed by CPE in the veterinary sector, a reliable, harmonized method must be used. The various CPE detection methods published, could cause an underestimation of the CPE occurrence. In the future, European monitoring should be a reliable measure of the occurrence of CPE. In addition, means of tracking CPE-positive farms should be considered. By prompt re-investigations of the farms and slaughterhouses, as well as gaining insights into piglet production, animal purchasing, slaughter cycles and more, potential entry routes might be identified.

4 Summary

Optimization and harmonization of microbiological and molecular detection and typing methods for carbapenemase-producing Enterobacteriaceae

The increasing problem of antimicrobial resistances is recognized all over the world and in all compartments of the One Health principle. Especially resistances to carbapenems, which are categorized as Critically Important Antimicrobials, are of particular importance. For years, carbapenemase-producing (CP) Enterobacteriaceae (CPE) were almost exclusively reported from clinical samples. Nowadays, CPE from wild animals, domestic animals, livestock and food are reported worldwide. These resistances are mostly caused by the production of carbapenem-hydrolyzing enzymes, so called carbapenemases. The genetic information for their synthesis is often located on plasmids or other mobile genetic elements, which are transferable between different strains and even bacterial species. To detect the dynamics and occurrence of CPE, the EU has established a monitoring for CP *Escherichia* (*E.*) *coli* from food and livestock. In the past, some observations indicated a lack of sensitivity of the monitoring method. One example is an isolate from 2017 that was not detected in the German CP *E. coli* monitoring, but in monitoring for ESBL/AmpC β-lactamases. Therefore, one goal of this thesis was to optimize the microbiological isolation methods for CPE from meat and caecal samples. Next to this method optimization, the detailed characterization and comparison of recently isolated German CPE between 2017 and 2020 in frame of the EU wide CP monitoring programme was a second aim of this thesis. The phenotypic and genotypic characterization of such isolates included i.e. the assessment of MIC values and typing by pulsed-field gel electrophoresis and whole genome sequencing.

To improve the sensitivity of the isolation method, different approaches were tested to stimulate the growth of Enterobacteriaceae while reducing the interfering accompanying flora. These approaches included i.e. a second enrichment step (i.e. different media, different supplements in different concentrations and some incubation conditions), the choice of selective agar (*in-house* produced MacConkey agar supplemented with 1 mg/L cefotaxim and 0.125 mg/L meropenem (McC+CTX+MEM) and MacConkey agar supplemented with 0.125 mg/L meropenem (McC+MEM) or commercial chromID® CARBA SMART (bioMérieux, Nürtingen, Germany)) and the influence of pre-additions to the samples.

These approaches demonstrated a significant improvement in detectability when the recommended *in-house* selective agar was used instead of commercial agars. The original EURL-protocol in combination with *in-house* produced McC+MEM and McC+CTX+MEM agar plates led to a sensitivity and specificity of 100 % (by screening 54 samples, spiked with 100 cfu/ml of CPE), whereas the sensitivity decreased to ~75 % by using the same procedure in combination with the tested commercial agar. If this recommended method fails to isolate a putative colony due to the accompanying flora, an alternative method (including a second anaerobic selective enrichment followed by real-time PCR) should be used. Furthermore, a higher sensitivity of the isolation method can be assumed if the samples are prepared within a few hours. This is because a stepwise reduction of 2 log units within 24 hours after CPE could be demonstrated in faecal samples.

The detailed characterization of recently isolated CPE revealed several observations, which could support two conclusions. On one hand, CPE with high similarity regarding the sequence type, phylogenetic group and the plasmids have been repeatedly isolated in the German food chain. Repeated isolation of strains with such high similarity raises concerns about stability and a possible adaption of CPE to a new reservoir, i.e. pigs. On the other hand, the variance of detected CPE increased. While initially only VIM-1 producing *E. coli* and *Salmonella enterica* were detected, in 2019, OXA-48 and GES-5 producing *E. coli* from pig production were detected. In addition, certain similarities to human CPE were described for all isolates. These observations indicate a repeated, independent and possibly human entry, which underlines the One Health aspect.

In conclusion, the sensitivity of the isolation method for CPE from meat and ceacal samples could be increased. By using selective and sensitive cultivation methods, the number of isolated CPE may be more in line with the actual prevalence. The comparison of isolated CPE from animal origin could provide insights into possible adaptation, plasmid evolution, and other questions. The variance of isolates increased, whereas some persistence of VIM-1 producing *E. coli* isolates was observed at the same time.

5 Zusammenfassung

Optimierung und Harmonisierung von mikrobiologischen und molekularen Nachweis- und Typisierungsmethoden für Carbapenemase-produzierende Enterobacteriaceae

Die wachsende Problematik der Antibiotikaresistenzen wird weltweit und in allen Bereichen des One Health Prinzips beobachtet. Insbesondere Resistenzen gegen Carbapeneme, die als „Critically Important Antimicrobials" kategorisiert sind, sind von besonderer Bedeutung. Jahrelang wurden Carbapenemase-produzierende (CP) Enterobacteriaceae (CPE) fast ausschließlich aus klinischen Proben gemeldet, wobei es mittlerweile auch Nachweise bei Wild-, Haus- und Nutztieren sowie aus Lebensmitteln gibt. Diese Resistenzen basieren meist auf der Produktion von Carbapenem-hydrolysierenden Enzymen, den sogenannten Carbapenemasen. Die genetische Information für deren Synthese befindet sich häufig auf Plasmiden oder anderen mobilen genetischen Elementen, die zwischen verschiedenen Stämmen und sogar zwischen Bakterienspezies übertragbar sind. Um die Dynamik und das Vorkommen von CPE zu erfassen, hat die Europäische Union ein Monitoring für CP *Escherichia* (*E.*) *coli* aus Lebensmitteln und Nutztieren eingerichtet. In der Vergangenheit deuteten einige Beobachtungen auf eine mangelnde Sensitivität dieser Methode hin. So konnte beispielsweise ein Isolat aus dem Jahr 2017 im Rahmen des deutschen CP *E. coli*-Monitorings nicht nachgewiesen werden, wohl aber im Monitoring auf ESBL/AmpC β-Laktamasen. Deshalb war es ein Ziel dieser Arbeit, die mikrobiologische Isolierungsmethode für CPE aus Fleisch- und Zäkumproben zu optimieren. Neben dieser Methodenoptimierung war die detaillierte Charakterisierung und der Vergleich zwischen 2017 und 2020, im Rahmen des EU-weiten CP-Monitoringprogramms, isolierter deutscher CPE ein zweites Ziel dieser Arbeit. Die phänotypische und genotypische Charakterisierung solcher Isolate umfasste u. a. die MHK-Werte, Pulsfeld-Gelelektrophorese und Ganzgenomsequenzierung.

Um die Sensitivität der Isolierungsmethode zu verbessern, wurden verschiedene Ansätze getestet, die Enterobacteriaceae in ihrem Wachstum anregen und gleichzeitig die störende Begleitflora reduzieren sollten. Diese Ansätze beinhalteten die Testung eines zweiten Anreicherungsschritts (d.h. verschiedene Medien, verschiedene Zusätze in verschiedenen Konzentrationen und unterschiedliche Inkubationsbedingungen), die Wahl des selektiven Agars (*in-house* MacConkey-Agar mit 1 mg/L Cefotaxim und 0,125 mg/L Meropenem (McC+CTX+MEM) und MacConkey-Agar mit 0,125 mg/L Meropenem (McC+MEM) oder kommerziellen chromID® CARBA SMART Agar (bioMérieux, Nürtingen, Deutschland)) und die Analyse des Einflusses von Vorab-Zugaben zu den Proben.

Es konnte eine signifikante Verbesserung der Sensitivität nachgewiesen werden, wenn der empfohlene, hauseigene Selektivagar anstelle des kommerziellen Agars verwendet wurde. Das ursprüngliche EURL-Protokoll in Kombination mit *in-house* McC+MEM- und McC+CTX+MEM-Agarplatten führte zu einer Sensitivität und Spezifität von 100 % (beim Screening von 54 Proben, die mit 100 cfu/ml angereichert waren). Die Sensitivität sank bei Verwendung des gleichen Verfahrens in Kombination mit dem getesteten kommerziellen Agar auf ~75 %. Gelingt es aufgrund der Begleitflora mit dieser Methode nicht eine mutmaßliche Kolonie zu isolieren, sollte eine alternative Methode, einschließlich einer zweiten anaeroben, selektiven Anreicherung mit anschließender real-time PCR, angewandt werden. Im Rahmen dieser Arbeit konnte außerdem eine schrittweise Verringerung der CPE um 2 log-Stufen in Fäkalproben innerhalb von 24 Stunden nachgewiesen werden. Dementsprechend kann von einer höheren Sensitivität der Isolierungsmethode ausgegangen werden, wenn die Proben innerhalb weniger Stunden bearbeitet werden.

Die detaillierte Charakterisierung der kürzlich isolierten CPE führte zu unterschiedlichen Beobachtungen, die zwei Schlussfolgerungen unterstützen könnten. Einerseits wurden in der deutschen Lebensmittelkette wiederholt CPE mit hoher Ähnlichkeit, hinsichtlich des Sequenztyps, der phylogenetischen Gruppe und der Plasmide, isoliert. Diese wiederholte Isolierung von Stämmen mit solch hoher Ähnlichkeit gibt Anlass zur Sorge über die Stabilität und eine mögliche Anpassung von CPE an ein neues Reservoir, beispielsweise Schweine. Andererseits hat die Varianz der nachgewiesenen CPE zugenommen. Während zunächst nur VIM-1 produzierende *E. coli* und *S. enterica* nachgewiesen wurden, wurden 2019 auch OXA-48 und GES-5 produzierende *E. coli* aus der deutschen Schweineproduktion isoliert. Darüber hinaus wurden für alle Isolate gewisse Ähnlichkeiten mit CPE humanen Ursprungs beschrieben. Diese Beobachtungen deuten auf einen wiederholten, unabhängigen und möglicherweise menschlichen Eintrag hin, was den One Health-Aspekt unterstreicht.

Zusammenfassend konnte die Sensitivität der Isolierungsmethode für CPE aus Fleisch- und Zäkumproben erhöht werden. Durch die Verwendung selektiver und sensitiver Kultivierungsmethoden könnte sich die Anzahl der isolierten CPE zukünftig der tatsächlichen Prävalenz nähern. Der Vergleich isolierter CPE tierischer Herkunft könnte Einblicke in eine mögliche Anpassung und die Plasmidevolution geben. Die Varianz der Isolate nahm zu, während gleichzeitig eine gewisse Persistenz von VIM-1-produzierenden *E. coli* Isolaten beobachtet wurde.

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III. List of publications

First author in publications:

Publication 1.

Pauly N, Hammerl JA, Schwarz S, Grobbel M, Meemken D, Malorny B, Tenhagen B-A, Käsbohrer A, Irrgang A (2020): Co-occurrence of the *bla*VIM-1 and *bla*SHV-12 genes on an IncHI2 plasmid of an *Escherichia coli* isolate recovered from German livestock. *J Antimicrob Chemother* 19;76(2):531-533.

Publication 2.

Pauly N, Hammerl JA, Grobbel M, Käsbohrer A, Tenhagen B-A, Malorny B, Schwarz S, Meemken D, Irrgang A (2021): Identification of a bla_{VIM-1}-Carrying IncA/C2 Multiresistance Plasmid in an *Escherichia coli* Isolate Recovered from the German Food Chain. *Microorganisms* 24;9(1):29. doi: 10.3390/microorganisms901002

Publication 3.

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Publication 4.

Pauly N, Klaar Y, Skladnikiewicz-Ziemer T, Juraschek K, Grobbel M, Hammerl JA, Hemmers L, Käsbohrer A, Schwarz S, Meemken S, Tenhagen B-A, Irrgang A (2021): Isolation Procedure for CP *E. coli* from Caeca Samples under Review towards an Increased Sensitivity. *Microorganisms* 2021, 9, 1105. doi: 10.3390/microorganisms9051105

Co-author in publications:

Publication 5

Irrgang A, Tenhagen BA, **Pauly N**, Schmoger S, Kaesbohrer A, Hammerl JA (2019): Characterization of VIM-1-Producing *E. coli* Isolated From a German Fattening Pig Farm by an Improved Isolation Procedure. Frontiers in Microbiology. 10(2256). doi: 10.3389/fmicb.2019.02256

Publication 6

Irrgang A, **Pauly N**, Tenhagen BA, Grobbel M, Kaesbohrer A, Hammerl JA (2020a): Spill-Over from Public Health? First Detection of an OXA-48-Producing *Escherichia coli* in a German Pig Farm. Microorganisms. 8(6). doi: 10.3390/microorganisms8060855

Publication 7

Irrgang A, Tausch SH, **Pauly N**, Grobbel M, Kaesbohrer A, Hammerl JA (2020b): First Detection of GES-5-Producing *Escherichia coli* from Livestock-An Increasing Diversity of Carbapenemases Recognized from German Pig Production. Microorganisms. 8(10). doi: 10.3390/microorganisms8101593

Publication 8

Dierikx C, Börjesson S, Perrin-Guyomard A, Haenni M, Norström M, Divon H, Ilag HK, Granier SA, Hammerum A, Sejer Kjeldgaard J, **Pauly N**, Randall L, Anjum M, Smialowska A, Franco A, Veldman K, Schau Slettemeås J (submitted): A European multicenter evaluation study to investigate the performance on commercially available selective agar plates for the detection of Carbapenemase-Producing Enterobacteriaceae.

Oral presentations:

1) **Pauly N**, Jäckel C, Strauch E, Hammerl JA (2019): Characterization of the phenotypic and genotypic properties of carbapenemase-producing *Vibrio* spp. isolates in Germany. One Health EJP ASM in Dublin, Ireland.

2) **Pauly N**, Grobbel M, Irrgang A (2019): IMPART - Improving phenotypic testing of AMR by development of sensitive screening assays for emerging resistance and setting missing ECOFFs. WP 2: Selective isolation, detection and characterization of carbapenemase-producing Enterobacteriaceae. NRL Symposium in Berlin, Germany

3) **Pauly N**, Hammerl JA, Grobbel M, Malorny B, Meemken D, Schwarz S, Käsbohrer A, Irrgang A (2019): Optimization and standardization of microbiological and molecular detection methods for carbapenemase-producing Enterobacteriaceae. Junior Scientist Zoonoses Meeting in Berlin, Germany

4) **Pauly N**, Grobbel M, Irrgang A (2019): EJP IMPART - Erkenntnisse aus dem Ringversuch des WP 2. Abteilungskolloquium der Abteilung Biologische Sicherheit am BfR in Berlin, Germany

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5) Irrgang A, **Pauly N**, Grobbel M, Hammerl JA, Käsbohrer A, Tenhagen B-A (2018): Carbapenemresistente Bakterien in der Lebensmittelkette. BfR-Symposium „Zoonosen und Lebensmittelsicherheit“. 2019. Berlin, Germany.

Poster presentations:

1) **Pauly A**, Hammerl J-A, Grobbel M, Malorny B, Käsbohrer A, Tenhagen B-A, Irrgang A (2018): Optimization and standardization of microbiological and molecular detection and typing methods for Carbapenemase-producing Enterobacteriaceae. DRS Doktorandensymposium in Berlin, Germany

2) **Pauly A**, Hammerl J-A, Grobbel M, Malorny B, Käsbohrer A, Tenhagen B-A, Irrgang A (2018): First results of optimization of microbiological and molecular detection and typing methods for Carbapenemase-producing Enterobacteriaceae. BfR PreDoc Symposium in Berlin, Germany.

3) **Pauly A**, Hammerl J-A, Grobbel M, Malorny B, Käsbohrer A, Tenhagen B-A, Irrgang A (2019): *Escherichia coli* producing VIM-1 carbapenemase isolated by antimicrobial resistance monitoring programs in food-producing animals. 71. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie e. V. 2019. Göttingen, Germany

4) **Pauly N**, Jäckel C, Strauch E, Hammerl JA (2019): Characterization of the phenotypic and genotypic properties of carbapenemase-producing *Vibrio* spp. isolates in Germany. One Health EJP ASM in Dublin, Ireland

5) **Pauly A**, Hammerl J-A, Grobbel M, Malorny B, Käsbohrer A, Tenhagen B-A, Irrgang A (2019): Comparison of two VIM-1-producing *Escherichia coli* isolated from food-producing animals within the German antimicrobial resistance monitoring. One Health EJP ASM in Dublin, Ireland

6) **Pauly A**, Hammerl J-A, Grobbel M, Malorny B, Meemken D, Schwarz S, Käsbohrer A, Irrgang A (2019): Optimization and standardization of microbiological and molecular detection methods for carbapenemase-producing Enterobacteriaceae. Junior Scientist Zoonoses Meeting in Berlin, Germany

7) **Pauly A**, Hammerl J-A, Grobbel M, Käsbohrer A, Tenhagen B-A, Irrgang A (2019): Characterization of new VIM-1 producing *Escherichia coli* from German pig production. 8th Symposium on Antimicrobial Resistance in Animals and the Environment in Tours, France

8) **Pauly A**, Hammerl J-A, Grobbel M, Tenhagen B-A, Käsbohrer A, Irrgang A (2019): Towards an improved microbiological detection method for carbapenemase-producing Enterobacteriaceae. International Symposium on Zoonoses Research in Berlin, Germany

9) **Pauly N**, Hammerl JA, Schmoger S, Grobbel M, Fischer J, Käsbohrer A, Irrgang A (2020): Change of usability of a commercial selective agar for isolation of carbapenemase-producing Enterobacteriaceae from samples of animal origin. 72. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie e. V. 2020. Leipzig, Germany

10) **Pauly N**, Hammerl JA, Schmoger S, Grobbel M, Fischer J, Käsbohrer A, Irrgang A (2020): Age-related changes of usability of a commercial selective agar for isolation of carbapenemase-producing Enterobacteriaceae from samples of animal origin. One Health EJP Annual Scientific Meeting 2020, online

11) **Pauly N**, Klaar Y, Skladnikiewicz-Ziemer T, Juraschek K, Grobbel M, Hammerl JA, Käsbohrer A, Irrgang A (2021): How to increase the sensitivity of CPE isolation method from caecal samples. One Health EJP Annual Scientific Meeting 2021, online

Co-author in poster presentations:

12) Irrgang A, **Pauly N**, Grobbel M, Käsbohrer A, Hammerl JA (2020): Occurrence of CPE in German livestock 2019 – an increasing diversity. One Health EJP Annual Scientific Meeting 2020, online

13) Perrin-Guyomard A, Granier S, Haenni M, Schau Slettemeås J, Ilag HK, Anjum M, Randall L, AbuOun M, **Pauly N**, Irrgang A, Hammerl JA, Svendsen CA, Kjeldgaard JS, Hammerum A, Franco A, Skarżyńska M, Kamińska E, Wasyl D, Dierikx C, Börjesson S, Geurts Y and Veldman K (2021): Multicentre Evaluation Of Culture Based Methods To Selectively Isolate Colistin-resistant Enterobacteriaceae From Food-producing Animals And Food Products. One Health EJP Annual Scientific Meeting 2020, online

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V. Conflict of Interest

In the context of this work, there are no conflicts of interest to declare due to third-party funding.

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VII. Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 13.12.2021 Natalie Pauly