SCIENTIFIC OPINION

Scientific Opinion on Carbapenem resistance in food animal ecosystems[1]

EFSA Panel on Biological Hazards (BIOHAZ)[2, 3]

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ABSTRACT

Carbapenems are broad-spectrum β-lactam antimicrobials used for the treatment of serious infections in humans. To date only sporadic studies have reported the occurrence of carbapenemase-producing (CP) bacteria in food-producing animals and their environment. The bacteria and enzymes isolated include VIM-1 producing *Escherichia coli* and *Salmonella* Infantis from pigs and poultry in Germany, OXA-23-producing *Acinetobacter* spp. from cattle and horses in France and Belgium, and NDM-producing *Acinetobacter* spp. from pigs and poultry in China. In the German *S.* Infantis and *E. coli* isolates, the VIM-1-encoding genes were located on IncHI2 plasmids. A methodology including selective culture is proposed for the detection of CP strains of Enterobacteriaceae and *Acinetobacter* spp. The choice of selective media for the surveillance of carbapenem resistance for testing animal and food samples needs to be experimentally evaluated and validated. Biochemical and phenotypic tests for the confirmatory identification of CP bacteria are available. For CP bacteria in animals and food, active/passive monitoring and/or targeted surveys should cover key zoonotic agents, animal pathogens and indicator organisms. Priority should be given to broilers, fattening turkeys, fattening pigs, veal calves and meat thereof. Because there are no data on the comparative efficacy of individual control options, prioritisation is complex. Continued prohibition of the use of carbapenems in food-producing animals would be a simple and effective option. As genes encoding carbapenemase production are mostly plasmid-mediated, and co-resistance may be an important issue in the spread of such resistance mechanisms, decreasing the frequency of use of antimicrobials in animal production in the EU in accordance with prudent use guidelines is also of high priority. The effectiveness of any control measures should be monitored by targeted surveys, using selective isolation methods and pre-enrichment of samples. Control measures should be proactively implemented at national and international levels to prevent CP strains become widespread in livestock.

KEY WORDS

carbapenemases occurrence, detection , transmission, animals, control options

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SUMMARY

The European Food Safety Authority (EFSA) asked the Panel on Biological Hazards (BIOHAZ) to provide a scientific opinion on the carbapenem resistance in food animal ecosystems. Carbapenems are broad-spectrum beta (β)-lactam antimicrobials mostly used for the treatment of serious infections in humans, frequently in hospitalised patients, and are considered last-line therapy for infections caused by multidrug-resistant Gram-negative bacteria. In particular, the panel was asked: (i) to define the carbapenemase-producing (CP) bacterial strains and genes relevant for public health and linked to food-producing animals or food-borne transmission; (ii) to review the information on the epidemiology of acquired resistance to carbapenems, including the genes coding for such resistance, in food-producing animals and food; (iii) to perform a critical analysis of the methods (phenotypic and genotypic) and the interpretive criteria currently used for detection (isolation and identification) and characterisation of CP bacterial strains; (iv) to make recommendations for the harmonised monitoring and reporting of resistance (phenotypic and genotypic) caused by carbapenemases in food and food-producing animals in the EU; and finally, (v) to identify possible means of preventing or minimising the further emergence and spread of CP bacterial strains transmitted *via* the food chain, including consideration of the advantages and disadvantages of different options.

The BIOHAZ Panel concluded that the production of carbapenemases may confer diverse β-lactam resistance phenotypes depending on a variety of factors such as the bacterial species, variant of the enzyme, expression level due to different promoters, copies of carbapenemase-encoding genes and the presence of additional non-enzymatic resistance mechanisms. Although a wide variety of CP strains with several different mechanisms of resistance to carbapenems and with a diversity of acquired genes encoding carbapenem-hydrolyzing ß-lactamases have been identified in bacterial isolations from cases of human infection worldwide, to date only sporadic studies have reported the occurrence of CP bacteria in food-producing animals and their environment. The bacteria and enzymes therein include VIM-1-producing Enterobacteriaceae (*Escherichia coli* and a putative strain of *Salmonella* Infantis from pigs, poultry and their environment in Germany), OXA-23-producing *Acinetobacte*r genomospecies 15TU from cattle in France and *Acinetobacter* spp. from horses in Belgium, and NDM-producing *Acinetobacter* spp. from pigs and poultry in China.

Genes encoding carbapenemase production in *Acinetobacter* spp. from animals have been located on both the chromosome (*Acinetobacter* genomospecies 15TU from cattle in France) and on plasmids (*A. lwoffii* and *A. baumannii* from pigs in China). In the putative *S.* Infantis and *E. coli* isolates associated with food animals in Germany, the VIM-1-encoding genes were located on IncHI2 plasmids.

Factors that favour the emergence of carbapenem resistance include the increased consumption of carbapenems in humans driven in part by the worldwide spread of ESBLs in Enterobacteriacae, the location of carbapenem-encoding resistance genes on mobile genetic elements, and positive selection due to co-resistance with other commonly-used antibiotics.

For the detection of carbapenemase-producing Enterobacteriacae (CPE) the screening cut-off values of 0.125 mg/l for meropenem, 1 mg/l for imipenem and 0.125 mg/l for ertapenem are recommended. If disk diffusion is used, applying zone diameters of 25 mm for meropenem, 23 mm for imipenem, and 25 mm for ertapenem would be indicative of non-susceptibility to carbapenems. The Panel further noted that pre-enrichment by incubation of samples in selective broth containing a carbapenem at a low concentration (e.g. meropenem, 0.125 mg/L) may increase sensitivity. This methodology has not yet been validated, and any method proposed would have to be subjected to thorough experimental verification.

A methodology including selective culture is proposed for the detection of CP strains of Enterobacteriaceae and *Acinetobacter* spp. A variety of in-house and commercially available selective media has been used for the active surveillance of carbapenem resistance in hospitals. The choice of the media for testing animal and food samples needs to be experimentally evaluated and validated.

Biochemical and phenotypic tests for the confirmatory identification of CP bacteria among isolates exhibiting non-susceptibility to carbapenems are available. The sensitivity and specificity of these assays may vary considerably in different settings. The identity of the genes responsible for the carbapenemase production should be determined by molecular methods. The Panel further considers that plasmid and strain typing should be undertaken to acquire better knowledge on the epidemiology of genes encoding carbapenemase production among bacteria from food-producing animal populations, food thereof and evironmental samples.

The Panel noted that there are comprehensive requirements for the collection and reporting of antimicrobial resistance (AMR) data, including resistance to carbapenems, laid down in European legislation. Technical specifications and reporting manuals prepared by EFSA recommend reporting of isolate-based data, and recommend mandatory phenotypic monitoring of *Salmonella* spp. and indicator *E. coli* with a broadened test panel also covering meropenem. Active monitoring and/or additional targeted surveys for CP bacteria in animals and food should cover key zoonotic agents and indicator organisms of the commensal flora. Priority should be given to broilers, fattening turkeys, fattening pigs, veal calves, and the derived fresh meat of domestic origin. Dairy cattle, raw milk and aquaculture products may be also included in targeted surveys. For active monitoring all isolates of *Salmonella* spp. and *E. coli* collected within the compulsory monitoring programme, as required by European legislation, should be screened for meropenem resistance using standardized microdilution methods. Specific targeted surveys for the detection of CP organisms in the food animal ecosystem should be implemented at the EU level. For passive monitoring, diagnostic isolates of veterinary origin (at least those classified as microbiologically resistant to 3rd- or 4th-generation cephalosporins on the basis of epidemiological cut-off values) should be subjected to phenotypic testing for carbapenem resistance and carbapenemase production, and subsequent molecular identification and characterization of the carbapenemase production genes present. For correct interpretation of results relating to carbapenem resistance, the sampling strategy (active vs. passive monitoring) and the selection procedure applied for each isolate (randomly selected isolate vs. isolate from selective media) should be reported. Results of phenotypic methods used for testing for carbapenemase production as well as the results from further characterisation of resistance genes should be reported for each isolate. Methods involving pre-enrichment and selective plating should be used in specific surveys to increase sensitivity for populations with a low prevalence of CP microorganisms.

Because there are no data on the comparative efficicacy of individual control options in reducing the potential public health risks caused by CP bacteria related to food-producing animals, prioritisation is complex. At present, carbapenems are not licensed for use in food-producing animals in the EU and other parts of the world, and therefore one simple and effective control option to minimise the further emergence and possible spread of such strains transmitted *via* the food chain would be to continue to prohibit the use of carbapenems in food-producing animals. Furthermore, as genes encoding carbapenemase production are mostly plasmid-mediated, and co-resistance may be an important issue in the spread of such plasmid-mediated resistance mechanisms, decreasing the frequency of use of antimicrobials in animal production in the EU in accordance with prudent use guidelines is also of high priority. The effectiveness of any control measures should be monitored on a regular basis by targeted surveys of food-producing animals and foods for CP bacteria, using selective isolation methods and pre-enrichment of samples as necessary.

Finally the Panel strongly recommends that control measures to contain the spread of CP bacteria in food-producing animals should be proactively implemented at national and international levels. Such plans should be agreed to prevent CP strains become widespread in livestock.

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BACKGROUND AS PROVIDED BY EFSA

Carbapenems are last-resort antimicrobials in the treatment of highly drug-resistant Gram-negative infections in humans. Carbapenem resistance is much feared in human medicine since it results in loss of activity of nearly all β-lactam antibiotics. Moreover it is usually present in bacteria already resistant to a range of critically important antimicrobials (e.g., aminoglycosides, fluoroquinolones), leaving few therapeutic options.

Carbapenemases are now seen as a new and potentially emerging problem in food-producing animals. The prevalence of such resistance in bacteria from animals is largely unknown. To date three publications have reported the presence of carbapenem resistance in bacteria from animals, including food-producing animals (pigs, bovines and horses) in three different EU Member States (Germany, France and Belgium). Most significantly from a food safety perspective, a carbapenemase has been recently identified in *Salmonella* in different pig and broiler farm origins in Germany. This is most probably only the tip of the iceberg since there is limited surveillance on carbapenem resistance amongst bacteria from food-producing animals. Carbapenems are usually not included in the antibiotic panels of national surveillance programmes, nor in the panels of diagnostic laboratories dealing with veterinary samples.

EFSA (BIOMO Unit) has recently produced Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in *Salmonella*, *Campylobacter* and indicator *Escherichia coli* and *Enterococcus* spp. bacteria transmitted through food[4]. In this report there is reference already to the methods needed for screening for this type of resistance:

"Detection of the existence and spread of carbapenem-resistant bacteria in animal populations is considered extremely important for the assessment of potential zoonotic risks. It is therefore recommended that phenotypic testing for carbapenem resistance in Salmonella and E. coli should be performed consistently. The detection of carbapenem resistance is not straightforward, since carbapenemases belong to several different classes of beta-lactamases and no single test is likely to give high sensitivity as well as high specificity for all types of enzymes.

Therefore, as an additional check for the presence of carbapenemases, and in an attempt to circumvent some of these methodological difficulties, the inclusion of meropenem at two concentrations in the harmonised panel is suggested. Meropenem is considered optimal for inclusion as the single compound for detection of carbapenem resistance, with ertapenem being included in the optional third panel which MSs may voluntarily choose to include in their monitoring programme".

Actions to limit the spread of resistance are only possible when the prevalence of such resistance is low. This is particularly relevant to carbapenems. It is therefore of utmost importance to initiate studies to determine when and where this resistance is present, and to limit its dissemination throughout the food chain.

In view of the above, there is a need to:

- Assess the public health risk posed by carbapenemase-producing bacterial strains in food-producing animals and food.
- Assess the need to establish harmonised monitoring of carbapenemase-producing bacterial strains in food- producing animals and food in the EU.
- Recommend measures to limit the spread of strains exhibiting resistance to carbapenems in the food chain.

[4] http://www.efsa.europa.eu/en/efsajournal/doc/2742.pdf

It is the intention to request participation of ECDC and EU Reference laboratories in this working group.

TERMS OF REFERENCE AS PROVIDED BY EFSA

EFSA requests the BIOHAZ Panel to review the topic of carbapenem resistance in bacteria of animal origin (with a focus on food-producing animals) and to assess the public health implications of such resistance. In particular to:

1. Define the carbapenemase-producing bacterial strains and genes relevant for public health and linked to food-producing animals or food-borne transmission;

2. Review the information on the epidemiology of acquired resistance to carbapenems, including the genes coding for such resistance, in food-producing animals and food.

3. Perform a critical analysis of the methods (phenotypic and genotypic) and the interpretive criteria currently used for detection (isolation and identification) and characterisation of carbapenemase-producing bacterial strains.

4. Make recommendations for the harmonised monitoring and reporting of resistance (phenotypic and genotypic) caused by carbapenemases in food and food-producing animals in the EU.

5. Identify possible means of preventing or minimising the further emergence and spread of carbapenemase-producing bacterial strains transmitted *via* the food chain, including consideration of the advantages and disadvantages of different options.

ASSESSMENT

1. Introduction

Carbapenems are broad-spectrum beta(β)-lactam antimicrobials mostly used for the treatment of serious infections, frequently in hospitalised patients, and are considered last-line therapy for infections caused by multidrug-resistant (MDR) Gram-negative bacteria, e.g. the extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae, *Acinetobacter* spp. and *Pseudomonas* spp.

1.1. Structure of and activity of penems and carbapenems

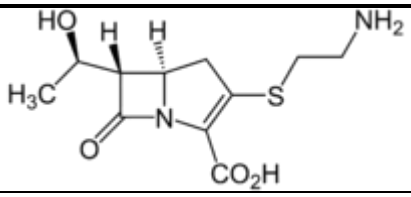
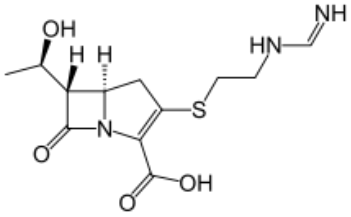
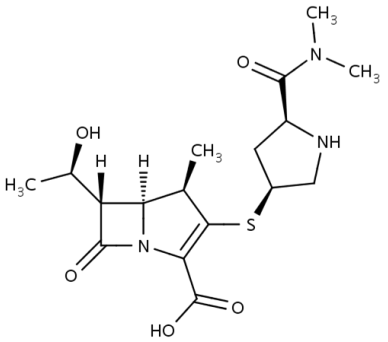
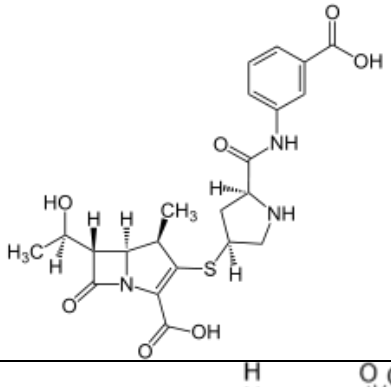
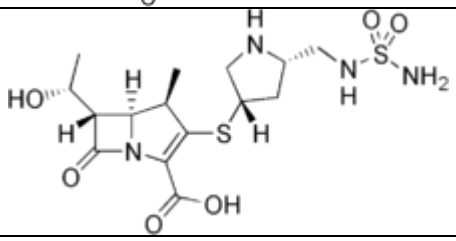
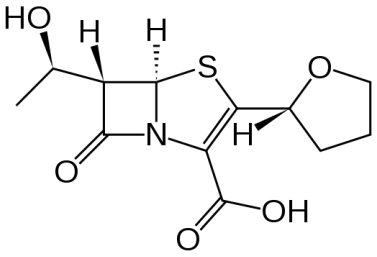
Penems and carbapenems are β-lactams sharing similar core structures (Table 1). The β-lactam ring is characteristically fused to an unsaturated five-membered ring that, in the case of penems, contains sulphur (2,3-dihydrothiazole ring), while in carbapenems the unsaturated ring includes a carbon at position 1 (2,3-dihydro-1H-pyrrole ring).

Carbapenems have been isolated from fermentation products of various streptomycetes. Thienamycin, isolated from *Streptomyces cattleya*, was the first among the naturally occurring carbapenems. The compound exhibited excellent activity against both Gram-positive and Gram-negative bacteria but was unstable in aqueous solutions. Thus, thienamycin derivatives with adequate stability have been developed by the pharmaceutical industry. The latter group includes all clinically available carbapenems, such as imipenem, meropenem, ertapenem and doripenem.

All carbapenems exhibit broad antimicrobial spectra against a variety of Gram-positive, Gram-negative and anaerobic microorganisms. Compared with imipenem, meropenem and doripenem, the spectrum of activity of ertapenem is slightly more limited, lacking activity against non-fermenters (*Pseudomonas* spp. and *Acinetobacter* spp.).

Carbapenems are vital for the effective therapy of community-acquired and healthcare-associated infections caused by MDR bacteria. Resistance to this class of antibiotic compromises the therapeutic options for patients by leaving only a few or in some cases no other antimicrobials with which to treat. These may often exhibit variable effectiveness and frequently provoke significant adverse reactions. There are only a very limited number of new antimicrobial agents in the antibiotic pipeline; thus the problem resulting from the lack of availability of effective antibiotics for therapy is considerably accentuated (ECDC and EMEA, 2009).

Table 1: Structure of carbapenems and penems

Carbapenems	Thienamycin	
	Imipenem	
	Meropenem	
	Ertapenem	
	Doripenem	
Penems	Faropenem	

1.2. Carbapenem resistance

Intrinsic resistance to carbapenems is attributed to a variety of mechanisms mediated by inherent properties, including low permeability of bacterial membranes, low affinity of penicillin binding proteins (PBPs) in different species, presence of multidrug efflux pumps and species-specific carbapenem-inactivating enzymes.

Acquired carbapenem resistance is due to the acquisition of exogenous genetic material containing gene(s) coding for carbapenemase production (CP) (i.e. enzymes capable of hydrolysing carbapenems and conferring detectable increase in levels of resistance to carbapenems). In this respect, carbapenem Minimal Inhibitory Concentration (MIC) may vary widely, ranging from full susceptibility to high-level resistance according to the Clinical Laboratory Standards Institute (CLSI) or the European Union Committee for Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints and according to bacterial species (see Section 3.2 below).

For the purpose of this document, carbapenem-non-susceptible (CNS) bacteria are defined as those exhibiting MICs above the epidemiological cut-off values (ECOFF) established by EUCAST (see Table 5 below), and the term 'acquired carbapenemases' is used to indicate carbapenemases produced by bacteria as a result of the acquisition of gene(s) coding for their production.

1.2.1. Resistance due to production of carbapenemases

Carbapenemases hydrolyse all ß-lactams including the carbapenems and frequently aztreonam. Based on their hydrolytic mechanism, carbapenemases are classified into two distinct groups, the serine carbapenemases (with an active site serine) and the metallo-β-lactamases (MBL) (with Zinc [Zn] ions in the active site). The group of serine carbapenemases includes enzymes that belong to the molecular classes A and D while the metallo-enzymes belong to class B of the Ambler classification (Ambler et al., 1991). According to the updated functional classification scheme (Bush and Jacoby, 2010), carbapenemases belong to subgroups 2f (class A), 2df (class D), 3a and 3b (class B) (Table 2).

Table 2: Classification of carbapenemases

Molecular class	Functional group/subgroup	Enzymes	Bacterial species
Class A	2f	Acquired	
		SME	*Serratia marcescens*
		NMC	*Enterobacter cloacae*
		IMI	*Enterobacter cloacae*
		KPC	*Klebsiella pneumoniae* *Klebsiella oxytoca* *Escherichia coli* *Enterobacter* spp. *Salmonella* Cubana *Citrobacter freundii* *Serratia marscescens* *Pseudomonas aeruginosa* *Pseudomonas putida*
		GES	*Klebsiella pneumoniae* *Escherichia coli* *Pseudomonas aeruginosa*
		SFC	*Serratia fonticola*
Class D	2df	Acquired	
		OXA-CHDLs*	
		OXA-23, -24/40, -58-type	*Acinetobacter baumannii* *Acinetobacter* genomospecies 15TU *Proteus mirabilis*
		OXA-48-type	*Klebsiella pneumoniae* *Klebsiella oxytoca* *Escherichia coli* *Enterobacter* spp. *Serratia marscescens* *Citrobacter freundii* *Providencia rettgeri* *Salmonella enterica*
		Intrinsic	
		OXA-CHDLs*	
		OXA-51-type	
		OXA-54	*Acinetobacter baumannii*
		OXA-55	*Shewanella oneidensis*
		OXA-62	*Shewanella algae*
		OXA-23-type (-103, -105)	*Pandoarea pnomenusa*
		OXA -134	*Acinetobacter radioresistens* *Acinetobacter lwoffii*
Class B			
B1	3a	Acquired	
		VIM	Enterobacteriaceae *Pseudomonas aeruginosa* *Acinetobacter baumannii*
		IMP	Enterobacteriaceae *Pseudomonas aeruginosa* *Acinetobacter baumannii*
		NDM	Enterobacteriaceae

Molecular class	Functional group/subgroup	Enzymes	Bacterial species
			Pseudomonas aeruginosa *Acinetobacter baumannii* *Acinetobacter lwoffii*
		SPM	*Pseudomonas aeruginosa*
		GIM	*Pseudomonas aeruginosa*
		SIM	*Acinetobacter baumannii*
		Intrinsic	
		BcII	*Bacillus cereus*
		CcrA	*Bacillus fragilis*
		BlaB	*Chryseobacterium meningosepticum*
		IND-1	*Chryseobacterium indologenes*
		SFB-1	*Shewanella frigdimarina*
		SLB-1	*Shewanella livingstonens*
B2		Intrinsic	
		CphA ImiS Sfh-1	*Aeromonas hydrophila* *Aeromonas veronii* *Serratia fonticola*
B3	3b	Intrinsic	
		GOB-1	*Chryseobacterium meningosepticum*
		FEZ-1	*Legionella gormanii*
		THIN-B	*Janthinobacterium lividum*
		CAU-1	*Caulobacter crescentus*
		BJP-1	*Bradyrhizobium japonicum*
		L1	*Stenotrophomonas maltophilia*

*CHDLs: Carbapenem hydrolysing class D β-lactamases

Production of carbapenemases may confer diverse β-lactam resistance phenotypes depending on a variety of factors such as the bacterial species, variant of the enzyme, expression level due to different promoters (conferred by insertion sequence (IS) elements preceding genes coding for the production of carbapenemases), copies of carbapenemase-encoding genes and the presence of additional non-enzymatic resistance mechanisms.

Many carbapenemase-producing (CP) strains frequently carry additional resistance determinants to other non-β-lactam antibiotics, making these organisms 'extensively drug-resistant' (XDR) or 'pan drug-resistant' (PDR) (Magiorakos et al., 2012). They commonly remain susceptible to only a few classes of antimicrobials, for example the polymyxins, tigecycline, fosfomycin, and nitrofurantoin.

A diversity of acquired genes encoding carbapenemhydrolyzing ß-lactamases of Ambler class A (such as KPC, GES types), class B (such as VIM, IMP, GIM, SPM, and NDM types) and class D (such as OXA-23, -40, -48, and -58 types) exist. These enzymes confer reduced susceptibility or resistance to almost all ß-lactams, including carbapenems. Low levels of resistance to carbapenems are more often observed among Enterobacteriaceae than in other bacterial species exhibiting such resistance.

One of the milestones in the emergence of carbapenemases in Enterobacteriaceae was the detection of a novel carbapenemase, designated as '*Klebsiella pneumoniae* carbapenemase' (KPC) according to the bacterial species from which it was originally isolated (Yigit et al., 2001). Such carbapenem-hydrolysing ß-lactamases confer resistance to almost all ß-lactams (Nordmann et al., 2011a). Subsequently, most acquired carbapenemases have been detected and reported in carbapenemase-

producing Enterobacteriaceae (CPE) globally (Nordmann et al., 2009; Bush and Jacoby, 2010; Nordmann et al., 2011a). The broad resistance profile in bacteria that is associated with the production of carbapenemases is significant in human infections, and poses a major on-going public health threat.

Multidrug resistance is now increasingly emerging in Enterobacteriaceae, not only amongst nosocomial but also community-acquired infections. One of the most important emerging resistance traits corresponds to the production of the carbapenemases (see above). The current and extensive worldwide spread of genes encoding for such enzymes in Enterobacteriaceae is a particularly important cause of concern (CDC, 2013a). This is because such genes are, in most cases, also associated with genes coding for resistance to non-ß-lactam antibiotics, therefore leading to MDR isolates (Nordmann et al., 2011a). The high rate of transmissibility of the genes encoding CP, which are for the most part located on self-conjugative plasmids carrying co-resistance determinants, and of bacteria that produce carbapenemases, underscores the need to rapidly identify the carbapenemase producers, to correctly implement infection control measures, and to prevent the horizontal transfer of these, as well as secondary transmission.

1.2.2. Resistance due to other mechanisms

Decreased susceptibility to carbapenems among Gram-negative bacteria is mostly attributed to enzymatic inactivation of carbapenems by carbapenemases. Carbapenem non-susceptibility (CNS) can also be due to other resistance mechanisms such as (a) ESBL or AmpC hyperproduction, (b) reduced hydrolysis of carbapenems, particularly ertapenem, by ESBLs e.g. CTX-M-15, (c) outer membrane impermeability due to porin alteration or loss by point mutations or deletions, (d) decreased permeability due to pleiotropic mutations that influence the expression levels of porins or non-specific efflux, (e) presence of efflux pumps, and (f) reduced affinity of penicillin-binding proteins.

1.3. Scope of opinion

In this opinion the focus will be on CP bacteria with acquired genetic elements encoding the production of carbapenemases that could be transferred from food-producing animals (and derived foods) to humans either directly, or indirectly through other pathways. The rationale for this approach is discussed in 1.2.1.

It is important: (i) to assess whether animals, particularly food-producing animals, and more generally the environment around animal production, might also provide the conditions required for the emergence of carbapenemase-mediated resistance; (ii) to evaluate whether specific reservoirs for CP bacteria or carbapenemase-encoding genes exist; and (iii) to identify factors that could favour the emergence and/or spread of carbapenemase-mediated resistance in food-producing animals and derived foods, and thus to humans through the food chain.

1.4. Significance and public health threat of human infections with carbapenemase-producing bacteria

Acquired carbapenemases have been found in all screened species of Enterobacteriaceae and also in non-fermenters, such as *Acinetobacter* spp. and *Pseudomonas* spp. (Patel and Bonomo, 2013).

Enterobacteriaceae are a family of Gram-negative bacilli that are part of the normal flora of the human and animal intestine and are the cause of community-acquired and healthcare-associated infections. Amongst the Enterobacteriaceae, certain species, within which resistance to antimicrobials is of particular concern, require special mention.

Of these, *Escherichia coli* is a cause of community-acquired infections (e.g. community-acquired urinary tract infections), commonly occurs in the intestinal tract of warm-blooded animals and is shed in their faeces, and may therefore spread throughout the ecosystem. A further genus that should be highlighted is non-typhoidal *Salmonella* spp., organisms of which are, for the most part, transmitted zoonotically. Although in general antimicrobial treatment is not recommended for human salmonellosis, in cases of extraintestinal development of the illness, or when patients are

immunologically compromised, treatment can be necessary. *Salmonella* spp. can also act as a reservoir of resistance genes.

Klebiella pneumoniae is the first bacterial species in which bla_{KPC} was found. This organism is a frequent cause of healthcare-associated infections e.g. pneumonias, bacteraemias, and has a well-documented propensity to accumulate antibiotic resistance determinants (Paterson and Bonomo, 2005).

Pseudomonas aeruginosa, a ubiquitous, non-fermenting Gram-negative rod commonly found in water and soil, is an opportunistic human pathogen and a major cause of healthcare-associated infections and infections in the immunosuppressed.

Another genus of bacteria, which has recently emerged as an organism of clinical importance, is *Acinetobacter* spp., most frequently *Acinetobacter baumanni*, especially strains that are MDR. *Acinetobacte*r spp. are typically soil and water organisms. They have also been isolated from food and the environment; they were previously mostly reported as the cause of infections in tropical climates and in victims of natural disasters, as well as war casualties and were previously considered colonisers in hospital environments. *Acinetobacte*r spp. have now emerged as an important cause of healthcare-associated infections, especially because of their resistance profile and their ability to accumulate mechanisms of resistance, thereby making them resistant to most, if not all antimicrobials, including the carbapenems. Such resistance compromises the treatment of patients, since there are often no effective antibiotics with which to treat them, or may result in delayed appropriate therapy, when such therapy is possible (Munoz-Price and Weinstein, 2008). This development is extremely worrisome, as such organisms can survive environmental desiccation for long periods of time, making eradication from hospitals difficult thus promoting transmission to patients and causing healthcare-associated infections and outbreaks of infection in hospitals (Maragakis et al., 2008; Munoz-Price and Weinstein, 2008).

A variety of carbapenemases have now been reported in Enterobacteriaceae and non-fermenters such as *P. aeruginosa* and *Acinetobacter* spp. These include the MBL, class A carbapenemases such as KPC, and carbapenem-hydrolysing class D β-lactamases (CHDLs) oxacillinases (OXA) (Souli et al., 2008; Patel and Bonomo, 2013).

1.5. Risk factors

Various risk factors have been identified as being associated with the emergence and increasing rate of carbapenem resistance in many parts of the world. One such risk factor is the increased consumption of carbapenems (ECDC, 2011). Causes of this increase in consumption are multifactorial, but one of the strong drivers is the worldwide spread of ESBLs in Enterobacteriacae during the last 15 years (Pitout, 2009; Woodford et al., 2013b). This global and apparently uncontrolled phenomenon has led to a dissemination of enterobacterial strains that are resistant to broad-spectrum ß-lactams and often co-resistant to numerous other antibiotic families. Carbapenems are the antibiotics of choice for treatment of infections with such MDR microorganisms, leading to a vicious cycle of antibiotic use and resistance (Paterson and Bonomo, 2005).

High and increasing rates of ESBL-producing bacteria represent an indirect risk for the spread of carbapenem resistance mechanisms. This is because carbapenems are the antibiotics of choice with which to treat patients infected with these types of MDR microorganisms. Uncontrolled use and misuse of antimicrobial agents in addition to the high rates of MDR microorganisms (e.g. ESBL-producing Enterobacteriaceae), all drive the large use of carbapenems (ESAC-Net interactive database[5]). The result is an interminable cycle of antimicrobial use, antimicrobial selection pressure and high rates of antimicrobial resistance (AMR).

[5] http://ecdc.europa.eu/en/activities/surveillance/esac-net/database/Pages/database.aspx

The emergence and spread of carbapenem-non-susceptible Enterobacteriaceae (CNSE), and CPE has also been identified as a public health threat for a number of reasons. As discussed in 1.2.1 above, CPE are commonly susceptible only to the polymyxins (e.g. colistin), fosfomycin and variably susceptible to tigecycline, although there have been recent reports of fosfomycin-resistant (Karageorgopoulos et al., 2012) and colistin-resistant Enterobacteriaceae (Marchaim et al., 2011). This limits the antimicrobial armamentarium to be used to treat an infected patient, since only a few and sometimes no effective antimicrobials remain, but also causes inadequate choice or delayed administration of antimicrobial therapy which are associated with poorer patient outcomes, increased morbidity, mortality, increased length of stay and increased costs in MDR organisms (Ibrahim et al., 2000; Cosgrove et al., 2003; Anderson et al., 2006; Maragakis et al., 2008; Roberts et al., 2009; de Kraker et al., 2011).

An additional concern is that these bacteria, and the mobile genetic elements (MGE) therein, are highly transmissible, rapidly disseminating within hospitals after being introduced. This secondary transmission can quickly lead to outbreaks, epidemics and, if left unchecked, to endemicity (Grundmann et al., 2010; Nordmann et al., 2011a).

Lastly, studies performed for CPE and CNSE have shown that infection or colonization has been associated with in-hospital mortality rates of 40-50% (Patel et al., 2008; Gasink et al., 2009; Marchaim et al., 2010; Chitnis et al., 2012). The risk posed by infections with these MDR microorganisms becomes even greater when considering the very limited number of new antimicrobial agents that are in the developmental pipeline (Boucher et al., 2009; ECDC and EMEA, 2009; Livermore et al., 2011).

1.6. Incidence of human infection

CPE have, in little more than a decade, spread globally (Gupta et al., 2011; Nordmann et al., 2011a; Canton et al., 2012) and they are now categorised as "microorganisms with a threat level of urgent" by the Centers for Disease Control and Prevention (CDC) (CDC, 2013a). The dissemination of MGE within these bacteria (e.g. transposons and plasmids), and epidemic strains that harbour such resistance elements, occurs through human population mobility and, more specifically through patient transfer between healthcare facilities, not only within the same country, but also across borders (ECDC, 2011).

Complete knowledge of the geographical distribution and magnitude of CNSE and CPE is unavailable for many reasons. Heterogeneous processes of clinical culturing, microbiological practices, detection, surveillance, reporting, notification of CNSE and CPE as well as when and if molecular testing is performed, likely contribute to the lack of complete data that exist worldwide. Most surveillance data report carbapenem resistance only for *K. pneumoniae.* Since molecular testing is not always performed and CNSE are frequently found to be CP, carbapenem resistance (or CNS) is frequently used as a surrogate marker for the presence of carbapenemases.

Data from Europe originate from that collected and reported by the European Antibiotic Resistance Surveillance System (EARS-Net)[6]. EARS-Net reports annual rates of AMR in bloodstream infections (BSI) and infections of cerebrospinal fluid (CSF) from hospitals in Europe, and brings together data on drug-resistant isolates and isolates with intermediate resistance[7]. These data originate from clinical microbiological laboratories across Europe, reporting antimicrobial susceptibility results only (no molecular mechanisms are reported) for seven invasive bacterial pathogens, including *K. pneumoniae* and *E. coli*. EARS-Net classifies carbapenem-resistant and carbapenem-intermediate isolates as 'carbapenem-resistant', so carbapenem-resistant Enterobacteriaceae (CRE) will be used with regard to EARS-Net data. Data from EARS-Net likely represent "the tip of the iceberg" of the total burden of CRE and CPE, for a number of reasons. EARS-Net collects only data on invasive bacterial isolates and since these bacteria also cause non-invasive infections, EARS-Net data probably correspond to only a fraction of the total number of infections.

[6] EARS-Net: http://www.ecdc.europa.eu/en/activities/surveillance/EARS-Net/Pages/index.aspx
[7] http://www.ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database/Pages/database.aspx

1.6.1. Carbapenem-resistant *Klebsiella pneumoniae* from EARS-Net data

A trend analysis on EARS-Net data from 2005-2010 looked at carbapenem resistance in *K. pneumoniae* isolates in Europe (Magiorakos et al., 2013). In this six-year period, eighteen countries reported at least one carbapenem-resistant *K. pneumoniae* isolate. Of these, six countries were included in the trend analysis since they reported carbapenem susceptibility data for *K. pneumoniae* isolates for at least three years during 2005–2010 and had a 1% or higher percentage of reported resistance for any of these years and for which a significantly increasing or decreasing trend was observed. The number of countries with ≥1% carbapenem resistance amongst invasive *K. pneumoniae* isolates, increased from two in 2005 (Greece, 27.8%; Germany, 3.1%) to five in 2010 (Greece, 49.8%; Cyprus, 16.4%; Italy, 12.5%; Hungary, 5.9%; Portugal, 2.2%). Significantly increasing trends were observed for Greece, Cyprus, Hungary and Italy ($p < 0.01$). Germany showed a decreasing trend ($p < 0.01$), since that country had not reported any carbapenem-resistant *K. pneumoniae* isolates in 2010.

Twenty-eight countries reported to EARS-Net in 2011, out of which 15 reported one or more carbapenem-resistant isolates. Twenty-three countries reported percentages of carbapenem-resistant isolates under 1%, two between 1 and 5%, one between 10-25% and two above 25%, the highest being Greece with 68.2%. Trends for 2008-2011 for 22 countries showed a significant increase for three countries, Greece, Hungary and Italy (ECDC, 2012).

1.6.2. Carbapenem resistance in the United States

Data from the USA on CRE are reported by the National Nosocomial National Healthcare Safety Network (NHSN) (formerly National Nosocomial Infection Surveillance System, NNIS) and from other surveillance systems such as the Surveillance Network-USA (TSN) and Meropenem Yearly Susceptibility Test Information Collection Program (MYSTIC). The 2009 MYSTIC summary showed a significant increase in carbapenem resistance of clinical isolates of *K. pneumoniae* from 0.6% in 2004 to 5.6% in 2008 (Rhomberg and Jones, 2009). The overall proportion of carbapenem resistance in Enterobacteriaceae in acute-care hospitals in the U.S. increased from 1.2% in 2001 to 4.2% in 2011 and more specifically carbapenem resistance in *K. pneumoniae* increased from 1.6% to 10.4% during the same time period (CDC, 2013b).

1.6.3. Carbapenem resistance worldwide

Reports of autochthonous, community cases and environmental isolations of CPE are being increasingly published, demonstrating that there is likely dissemination and penetration of CPE beyond the hospital into the community and environment in many countries and particularly in the South-East Asia (Kumarasamy et al., 2010; Picao et al., 2013).

1.7. Reservoirs of carbapenemase-producing bacteria and encoding genes

1.7.1. Reservoirs of bacteria

Variants of KPC, VIM, IMP, NDM, and OXA-48 families have been the predominant types identified among human isolates (Nordmann et al., 2011a; Tzouvelekis et al., 2012). Most of these enzyme family types have been encoded by plasmid-located genes from Enterobacteriaceae (mainly *K. pneumoniae*), and by chromosomally- or plasmid-located genes from *P. aeruginosa*, *A. baumannii* or *Aeromona*s spp. (Walsh et al., 2005; Poirel and Nordmann, 2006; Nordmann et al., 2011a; Nordmann et al., 2011b; Canton et al., 2012; Poirel et al., 2012b). Sporadic reports of *Vibrio cholerae* carrying bla_{NDM} have recently been documented (Darley et al., 2012). Available information is summarised in Table 3.

An outstanding public health threat is the possible large-scale dissemination of carbapenemase producers from the hospital to the community, and more widely to the environment. So far, only sporadic studies have reported the occurrence of carbapenemase-encoding genes in bacteria isolated from animals (VIM-1, OXA-48 and NDM-1 producing Enterobacteriaceae and NDM-1 or OXA-23-producing *Acinetobacter* spp.) or environmental samples (Fischer et al., 2012; Kempf et al., 2012;

Poirel et al., 2012a; Wang et al., 2012; Fischer et al., 2013a; Shaheen et al., 2013; Stolle et al., 2013; Zhang et al., 2013; Zurfluh et al., 2013).

Bacterial communities from aquatic environments including hospital sewage, wastewater treatment plants (WWTP), lakes, or rivers may also contribute to the dissemination of carbapenemase-encoding genes of clinical relevance, such as class A β-lactamase KPC-2, which is increasingly recovered from *Aeromonas* and enterobacterial species at hospital sewage in different countries (Valverde et al., 2012; Picao et al., 2013; Zurfluh et al., 2013), GES-5 identified from a bacterial community of activated sludge in a WWTP (Girlich et al., 2012a), BIC-1 from *P. fluorescens* (Girlich et al., 2010a), and IMI-2 from *Enterobacter asburiae* recovered in rivers of different countries (Aubron et al., 2005); class B β-lactamases VIM-1, VIM-2, VIM-13 from *P. aeruginosa*, *P. alcaligenes*, *K. pneumoniae* and diverse environmental species from rivers and lakes (Quinteira and Peixe, 2006; Scotta et al., 2011); IMP-8, IMP-10, and IMP-13 from Tunisian polluted rivers (Chouchani et al., 2013; Zurfluh et al., 2013), NDM-1 from diverse Enterobacteriaceae, *Pseudomonas* spp. and *Aeromonas* spp., *and V. cholerae* from wastewaters, and *Achromobacter* spp., *Kingella denitrificans*, and *P. aerugin*osa from tap water (Walsh et al., 2011); and class D OXA-23 from *A. baumannii* isolates recovered from the Seine river at a hospital wastewater discharge site (Girlich et al., 2010b).

Contamination of vegetables, manure and farm facilities with contaminated water of anthropogenic origin might facilitate the penetration of highly promiscuous broad-host range plasmids in specific settings where some of these species may occur (e.g. *Salmonella* spp. in poultry, *E. coli* in livestock, *Klebsiella* spp. in dairy cattle, *Aeromonas* spp. and *Vibrio* spp. in aquaculture)[8]. Similarly aquaculture using water from contaminated rivers would result in a risk for the emergence and spread of carbapenemase producers in such settings.

Aeromonas spp. possess naturally-occurring carbapenemases, the genes for which could, in theory, be mobilised by genetic elements. The same considerations apply to other environmental species also possessing intrinsic carbapenemases (*Stenotrophomonas* spp., Flavobacteriaceae, etc). *Aeromona*s is unique in that the species has often been shown to be an intermediate reservoir of acquired clinically-relevant resistance genes (Girlich et al., 2011); for instance there have been many examples of plasmid-encoded ESBLs found in *Aeromonas* spp., and a plasmid-borne NDM-1 carbapenemase-encoding gene has also been identified in this species (Walsh et al., 2011). This phenomenon has not been described for other genera. Thus other environmental species (e.g., *Vibrio* spp., *Acinetobacter* spp. and *Pseudomonas* spp.) or even some Enterobacteriacae may also have to be considered in screening regimens. In this respect a strain of *P. fluorescens* with a new carbapenemase (BIC-1), so far never identified in strains from humans, was reported in 2010 (Girlich et al., 2010a).

Besides the risk of contaminated soil and water, the colonisation of wildlife by CP bacteria through contact with sewage, human faeces, or animal manure might facilitate the global spread of resistance genes, with consequences for public health, animal welfare, and ecosystem functions (Pesapane et al., 2013).

The occurrence, both in animals, including domestic pets and in healthy humans, of CP bacteria might be underestimated, taking into account the "silent" and successful dissemination of certain species of carbapenemase producers documented among humans (Gijon et al., 2012; Viau et al., 2012). Also, only a few studies performed by groups dealing with the environment or animals have taken this topic in consideration.

1.7.2. Reservoirs for carbapenemase production-encoding genes

Environmental bacteria are of clinical and veterinary concern as reservoirs of both acquired and also chromosomal genes coding for carbapenem resistance. The direct or indirect transmission of such genes from environmental bacteria to micro-organisms of clinical importance is a possibility, although the frequency of such transmission has not been quantified. A remarkable number and diversity of

[8] http://www.nnyagdev.org/index.php/dairy/research-projects/klebsiella-sources-transmission-control-points

chromosomal-encoded carbapenemases (mainly MBLs) are produced by a wide variety of environmental species of Proteobacteria, Firmicutes and Bacteroidetes such as, for example, *Bacillus cereus*, *Aeromonas* spp., *Stenotrophomonas* spp., *Chryseobacterium* spp., *Legionella gormanii*, *Janthinobacterium* spp., *Pseudomonas* spp., *Sphingobacterium* spp., *Acidovorax* spp., *Caulobacter* spp., *Cupriavidus* spp., *Sphingomonas* spp., among many others (Docquier et al., 2002; Aubron et al., 2005; Girlich et al., 2010b; Henriques et al., 2012). Functional metagenomics studies have disclosed carbapenemase orthologs from bacteria in an Alaskan soil never inhabited by humans highlighting the huge pool of carbapenem resistance genes in the environment, which seems to predate the introduction of carbapenems in the therapeutic arsenal (Allen et al., 2009; Gonzalez and Vila, 2012). Environmental bacterial species carrying acquired CP genes have also been reported (Scotta et al., 2011). These genes may be captured and mobilized among human and animal adapted populations by IS, class 1 integrons, or transposons (e.g.Tn*3*-like) that enable their expression in different bacterial hosts better adapted to humans and animals such as Enterobacteriaceae, *Pseudomonas* spp. and *Acinetobacter* spp., which are also hosts of acquired CP genes (Aubron et al., 2005; Walsh et al., 2005; Poirel and Nordmann, 2006; Bogaerts et al., 2010; Girlich et al., 2010a; Endimiani et al., 2011; Gundogan et al., 2011; Nordmann et al., 2011a; Nordmann et al., 2011b; Canton et al., 2012; Girlich et al., 2012a; Hamouda et al., 2012; Kempf et al., 2012; Poirel et al., 2012b; Smet et al., 2012; Wang et al., 2012; Bonnin et al., 2013; Naas et al., 2013; Zurfluh et al., 2013).

To date, the main ISs associated with the capture of CP genes such as IS*Kpn7* (bla_{KPC} genes), IS*1999* (bla_{OXA}), or IS*Aba125* and IS*Aba1* (bla_{OXA}, bla_{NDM}) have also been associated with other *bla* determinants in Enterobacteriaceae, *Acinetobacter* spp. and Pseudomonadaceae, highlighting their ability to acquire a wide diversity of genes of different origin (Aubert et al., 2006; Hamidian et al., 2013). Other ISs as IS*26*, and IS*1111*-like variants (e.g. IS*5045*, IS*4321*) which are widely spread among plasmids and genomes of species of the above mentioned species, have been frequently associated with different genetic context-carrying bla_{IMP}-, bla_{VIM}-, bla_{KPC}-, bla_{OXA}- or bla_{NDM}-genes generating composite transposons, thereby enhancing the possibilities of transfer among diverse bacterial communities by different mechanisms (Tzouvelekis et al., 2012). Some plasmids of Enterobacteriaceae (e.g. IncFI/FII, IncI, IncL/M, IncA/C, IncQ), *Pseudomonas* spp. and *Achromobacter xylosoxidans* (pNOR-like), or *Acinetobacter* spp. are able to spread in a broad host range of bacterial species, and are abundant among humans and animals, probably as a consequence of previous selection by antibiotics, heavy metals and other pollutants. They have been implicated repeatedly in recent independent acquisition events and thus are able to acquire new genes from environmental bacteria and to spread among humans, animals and the environment (Carattoli, 2009; Bertini et al., 2010; Girlich et al., 2010b; Andrade et al., 2011; Carattoli, 2011; Zhou et al., 2011; Chen et al., 2012; Girlich et al., 2012a; Bonnin et al., 2013; Di Pilato et al., 2013; Naas et al., 2013).

Exposure to antibiotics, metals and other environmental pollutants may enhance the mobilization of resistance genes (Kristiansson et al., 2011). Also, concentrations representing action limits used in environmental risk assessment may be enough to exert a significant selective pressure on genes of clinical relevance. Of particular concern is the release of antibiotics used in human and veterinary medicine into sewage and manure distribution systems as they can be associated with sewage sludge, contamination of rivers, liquid manure and farm soil (Tello et al., 2012).

Table 3: Bacterial reservoirs of acquired carbapenemases of clinical importance and identified sources

Enzyme family	Functional group or subgroup	Molecular class	Target species	Representative enzymes	Source
GES	2f	A	Enterobacteriaceae, *Pseudomonas* spp., *Acinetobacter* spp.	GES-4, -5, -6, -11, -14	HH, WWTP
KPC	2f	A	Enterobacteriaceae, *Pseudomonas* spp.	KPC-2 to KPC-13	HH, HV, SW,
SME	2f	A	*Serratia marcescens*	SME-1 to SME-3	HH
IMI	2f	A	*Enterobacter spp., Escherichia coli*	IMI-1 to IMI-3	R, HH
NmcA	2f	A	*Enterobacter cloacae*	NmcA	HH
SFC	2f	A	*Serratia fonticola*	SFC-1	E
BIC	2f	A	*Pseudomonas fluorescens*	BIC-1	R
OXA	2df	D	*Acinetobacter* spp.	OXA-23 group (OXA-23, -27, -49)	AN, R, HV, head lice
	2df	D	*Acinetobacter* spp.	OXA-24 group (OXA-2 -25, -26, -40, -72)	HH
	2df	D	*Acinetobacter* spp.	OXA-58 group (OXA-5 -97, -164)	HH
	2df	D	*Acinetobacter* spp.	OXA-143	HH
	2df	D	*Acinetobacter* spp.	OXA-235	HH
	2df	D	Enterobacteriaceae	OXA-48 group (OXA-48, -181, -204, -232)	HH, AN
IMP	3a	B	Enterobacteriaceae, *Pseudomonas* spp.	IMP-1 to IMP-33	HH, R
VIM	3a	B	Enterobacteriaceae, *Pseudomonas* spp., *Brevundimonas* spp., *Rhizobium* spp.	VIM-1 to VIM-34	HH, HV, AN, SW, R
NDM	3a	B	Enterobacteriaceae, *Pseudomonas* spp., *Acinetobacter* spp., *Vibrio* spp.	NDM-1 to NDM-8	HH, AN, SW, R
SPM	3a	B	*Pseudomonas aeruginosa*	SPM-1	HH
GIM	3a	B	*Pseudomonas aeruginosa*	GIM-1	HH
SIM	3a	B	*Acinetobacter baumannii*	SIM-1	HH
AIM	3a	B	*Pseudomonas aeruginosa*	AIM-1	HH
DIM	3a	B	*Pseudomonas stutzeri*	DIM-1	HH
KHM	3a	B	*Citrobacter freundii*	KHM-1	HH
TMB			*Achromobacter xylosoxydans*	TMB-1	HH
FIM	3a	B	*Pseudomonas aeruginosa*	FIM-1	HH

HH = hospitalized patients; HV = healthy human; AN = animal; SW = sewage; WWTP = wastewater treatment plant; R = rivers; E = environment

1.8. Summary and conclusions

- Carbapenems are broad-spectrum beta (β)-lactam antimicrobials mostly used for the treatment of serious infections in humans, frequently in hospitalised patients, and are considered last-line therapy for infections caused by multidrug-resistant Gram-negative bacteria.

- Production of carbapenemases may confer diverse β-lactam resistance phenotypes depending on a variety of factors such as the bacterial species, variant of the enzyme, expression level due to different promoters, copies of carbapenemase-encoding genes and the presence of additional non-enzymatic resistance mechanisms.

- Carbapenem MICs may vary widely ranging from full susceptibility to high-level resistance according to CLSI or EUCAST clinical breakpoints. Low levels of resistance to carbapenems are more often observed among Enterobacteriacae.

- A diversity of acquired genes encoding carbapenem-hydrolyzing ß-lactamases has been identified. Such enzymes confer reduced susceptibility or resistance to almost all ß-lactams, including carbapenems.

- The emergence and spread of carbapenem-non-susceptible Enterobacteriaceae and carbapenemase-producing Enterobacteriacae have been identified as major public health threats.

- Many carbapenemase-producing strains frequently carry additional resistance determinants to other non-β-lactam antibiotics, making these organisms 'extensively drug-resistant' or 'pan drug-resistant'.

- The focus of this Scientific Opinion is on carbapenemase-producing bacteria with acquired genetic elements encoding carbapenemase production and that could be transferred from food-producing animals (and derived foods) to humans directly, or indirectly through other pathways.

- Factors that favour the emergence of carbapenem resistance include the increased consumption of carbapenems in humans driven in part by the worldwide spread of ESBLs in Enterobacteriacae, the location of carbapenem-encoding resistance genes on mobile genetic elements, and positive selection due to co-resistance with other commonly-used antibiotics.

2. The epidemiology of carbapenemase-mediated resistance in food-producing animals and foods

Few studies have reported the presence of CP bacteria in food-producing animals and their environment (Fischer et al., 2012; Poirel et al., 2012a; Smet et al., 2012; Wang et al., 2012; Fischer et al., 2013a; Woodford et al., 2013b; Zhang et al., 2013). No reports of such bacteria from animal-derived food products are available.

Additional studies in which the isolation of CRE in livestock or food were reported, but presented inconsistencies in some of the methodologies. These reported: (i) *Salmonella* serovars Teko, Weltevreden and Saintpaul, from vegetables in India (Singh et al., 2007); (ii) *Salmonella* Paratyphi B variant Java, *S.* Saintpaul and *Salmonella* Virchow from buffalo calves and beef in India (Singh et al., 2012); and (iii) *E. coli* from oysters, water, shrimps and pond environment in Brazil (Vieira et al., 2008; Vieira et al., 2010). These studies have therefore not been considered further in this Opinion.

Most *Salmonella* spp. infections are considered to be directly or indirectly zoonotic in origin, and thus reports of CP isolates of this species from cases of human infections and other animal sources (i.e.

wildlife) have been included in this Opinion and are discussed below (Miriagou et al., 2003; Savard et al., 2011; Cabanes et al., 2012; Le Hello et al., 2013; Pfeifer et al., 2013; Rasheed et al., 2013; Woodford et al., 2013b). It has not been possible to elucidate if the carbapenemase-encoding genes described above were acquired in animal, environmental or clinical settings. In respect of wildlife, a CP *Salmonella* Corvallis isolate from a wild bird in Germany (Fischer et al., 2013c) has also been reported.

In addition, the isolation of NDM-1-producing *E. coli* isolates from companion animals (cats and dogs) in the USA (Shaheen et al., 2013), OXA-48 in *E. coli* and *K. pneumoniae* from dogs (Stolle et al., 2013), and OXA-23-like-producing *A. baumannii* from human head lice in Senegal (Kempf et al., 2012) have been described, but isolates from these origins are out of the scope of the Opinion and will not be discussed further.

The real prevalence of carbapenemase-encoding genes in zoonotic bacteria or commensals is unknown.

2.1. Carbapenemase-producing microorganisms relevant for public health that have been linked to food-producing animals, foodborne transmission and wildlife

The following CP microorganisms relevant for public health and linked to food-producing animals or foods have been described.

2.1.1. Carbapenemase-producing microorganisms from food-producing animals and their environment

Nine CP isolates (BY1-BY9) of *Acinetobacter* genomospecies 15TU were detected in 2010 on a dairy cattle farm in France (Poirel et al., 2012a). These isolates harboured the $bla_{\text{OXA-23}}$ gene and encoded the OXA-23 enzyme. With one exception, all isolates were resistant to penicillins, penicillins/β-lactamase inhibitors, carbapenems, but susceptible to cefotaxime and exhibited reduced susceptibility to ceftazidime (MICs 16-32 for both antimicrobials; BY1 showed higher MICs for carbapenems). All isolates were resistant to tetracyclines, kanamycin and fosfomycin. They were susceptible to fluoroquinolones, chloramphenicol, gentamicin, amikacin, tobramycin and sulphonamides. The isolates showed six distinct pulsed field gel electrophoresis (PFGE) patterns (6 genotypes). The isolate BY1 represented a single clone. *Acinetobacter* genomospecies 15TU is known to be phylogenetically related to *A. lwoffii*, and has been reportedly isolated from sewage, freshwater aquaculture habitats, trout intestines and frozen shrimps in a previous study (Guardabassi et al., 1999).

Nine Gram-negative non-fermentative *Acinetobacter* bacteria belonging to different species (see 1.4) resistant to imipenem (MIC>8 µg/mL) were identified in a study in different chicken farms (cloacal swabs) and one pig slaughterhouse (carcass swabs) in China. Among these isolates, an *A. lwoffii* isolate producing NDM-1 (isolate SGC-HZ9) recovered from a chicken anal swab was identified. The isolate was resistant to all tested β-lactams (excepting aztreonam), ciprofloxacin, tetracyclines, kanamycin, and chloramphenicol, and susceptible to gentamicin and polymyxin B (CLSI, 2008). No molecular typing data on the isolate were provided (Wang et al., 2012).

An *A. baumannii* isolate (GF216) which produced NDM-1 was recovered from a sick pig in a Chinese swine farm. The isolate was resistant to all tested β-lactams, ciprofloxacin, chloramphenicol, tetracyclines, kanamycin, tilmicosin and erythromycin, being susceptible to gentamicin and colistin. No molecular typing data on the isolates were provided (Zhang et al., 2013).

Two isolates of *Acinetobacter* spp. producing OXA-23 were collected from different hospitalised horses in a University Faculty in Belgium. One of these horses had been previously treated with penicillin. These isolates showed MICs for imipenem of 16 µg/ml, and together with other β-lactams, were also resistant to tetracyclines, sulphonamides, trimethoprim and gentamicin, but susceptible to colistin). Both isolates showed the same PFGE pattern (Smet et al., 2012).

Three CP *Salmonella* spp. isolates were obtained from two fattening pig farms and one broiler farm in Germany (Fischer et al., 2013a). The isolates from the two pig farms were from swine single faeces (isolates R27) or from the farm environment (isolate R25). The poultry-related isolate (R3) was collected from a dust sample. The isolates were classified as *Salmonella enterica* group C, antigenic formula "6,7:-:-"[9] and on this basis are herewith referred to as 'putative *S.* Infantis'. The multi locus sequence type ST32[10] and the two highly related PFGE-patterns are typical of *Salmonella* Infantis (6,7:r:1,5) (Hauser et al., 2012). The *Salmonella* isolates R3, R25 and R27 showed decreased susceptibility to carbapenems (non-wild type by the EUCAST ECOFF), but had MIC values below the clinical breakpoint (CLSI, 2011). The three isolates produced VIM-1 carbapenemase and carried both the AmpC-encoding gene $bla_{\text{ACC-1}}$ and $bla_{\text{VIM-1}}$. When *Salmonella* R3 was grown in liquid medium with carbapenems (1 to 16/8 µg/ml imipenem/ertapenem) the isolate was able to grow and showed clinical resistance (clinical breakpoints CLSI *vs.* EUCAST, imipenem ≥ 4 *vs.* >8 mg/L, ertapenem ≥ 1 *vs.* >1 mg/L). All isolates showed additional resistance to chloramphenicol, streptomycin, sulphonamides and trimethoprim and susceptibility to colistin, fosfomycin, nitrofurantoin and tigecycline (CLSI, 2011). *S.* Infantis is among the top ten *Salmonella* spp. serovars implicated in human salmonellosis worldwide (Hendriksen et al., 2011; EFSA and ECDC, 2013). The serovar has been implicated in several outbreaks, and broilers, layers and pigs are important reservoirs (Hauser et al., 2012; EFSA and ECDC, 2013). Isolates of this serovar have also caused disease with severe symptoms (septicaemia, fatal cases) and nosocomial outbreaks.

Several *E. coli* isolates (31) from pig faeces, flies, manure and bootsocks taken in one of the German swine farms cited above also produced VIM-1. These isolates showed slightly decreased susceptibility to carbapenems (above the EUCAST ECCOFFs), together with resistance to streptomycin, sulphonamides, and in all except one isolate, to tetracycline. Like the *Salmonella* isolate R27, the *E. coli* isolates R178 and R29 were also able to grow in medium with concentrations of carbapenems larger than or equal to the CLSI breakpoints. All isolates were positive for the $bla_{\text{ACC-1}}$ and the $bla_{\text{VIM-1}}$ genes. These isolates were identified as *E. coli* sequence type (ST) 88 which belong to the *E. coli* phylogenetic group A, and showed highly similar *Xba*I PFGE patterns and three different plasmid profiles. *E. coli* ST88 is commonly isolated from chickens, cattle, turkeys and humans in Germany (Fischer et al., 2012, 2013a; Fischer et al., 2013d; Fischer et al., 2013b).

2.1.2. Carbapenemase-producing *Salmonella* spp. isolates from cases of human infections

Reports of CP, non-typhoidal clinical isolates of *Salmonella* spp. from cases of human infection are still scarce. Information on such isolates is presented below.

The first description of a CP *Salmonella* spp. was a *S.* Cubana isolate (*S.* Cubana 4707) collected from a stool sample taken from a chronically ill 4-year old patient in 1998 in the USA (Miriagou et al., 2003). The patient had been hospitalised several times, and there was no history of recent travel before hospitalization. This isolate produced KPC-2 and exhibited resistance to most β-lactam antibiotics, including oxyimino-cephalosporins and imipenem. The isolate was also resistant to streptomycin, trimethoprim, and sulphamethoxazole. In the previous hospitalisations (three times in the six months previous to the isolation), the patient had been treated with ceftriaxone and ceftazidime, but not with carbapenems.

CP *S.* Saintpaul (1 isolate) and *Salmonella* Kentucky (1) from a patient returning to France from Egypt in 2009 have also been described (Le Hello et al., 2013). The patient was co-infected with *S.* Kentucky resistant to ciprofloxacin and 3rd-generation cephalosporins (carrying $bla_{\text{CMY-2}}$), and with *S.* Saintpaul which produced OXA-48. One of the subsequent *S.* Kentucky isolates (obtained from the same patient more than one year later) also contained the $bla_{\text{OXA-48}}$ carbapenemase gene, although the isolate was phenotypically susceptible to imipenem (MIC 0.75 mg/L). This isolate was also resistant to azithromycin but susceptible to colistin and tigecycline. In the same study, *S.* Kentucky isolates (5) producing VIM-2 was described. All isolates were recovered from human clinical samples (one blood,

[9] http://www.pasteur.fr
[10] http://mlst.ucc.ie/mlst/dbs/Senterica

two urine and two faecal cultures) in 2010 in Morocco. They showed resistance to ciprofloxacin and 3rd-generation cephalosporins, and decreased susceptibility to imipenem (MIC 1-3 mg/L). Also *S.* Kentucky carrying bla_{OXA-48} was isolated from a patient from Morocco in a German hospital (Pfeifer et al., 2013).

Salmonella Westhampton resistant to imipenem and producing NDM-1 was isolated from urine and faecal samples from a French patient who was transferred from India to a hospital on Reunion Island in 2011. This isolate also carried $bla_{CTX-M-1}$ and bla_{TEM} genes. *E. coli* and *Klebsiella* isolates showing an ESBL phenotype, but no imipenem resistance were obtained from the same patient. No data on the presence of bla_{NDM-1} in these isolates were presented (Cabanes et al., 2012).

Monophasic *S.* Senftenberg (antigenic formula 3,19:g,s,t:−) resistant to imipenem and producing NDM-1 was isolated from clinical samples (perirectal culture) of a patient transferred from an Indian hospital to the USA. The isolate was only susceptible to tetracyclines, tigecycline, and trimethoprim-sulphamethoxazole. From the same patient, *K. pneumoniae* producing NDM-1 had been isolated one month previously, but the plasmids on which the gene was located in both isolates were different (Savard et al., 2011).

Although *Salmonella* spp. is in general regarded as a zoonotic pathogen, to our knowledge there are no indications that the infections involving CP strains described above were derived from food-producing animals directly or from foods thereof.

2.1.3. Carbapenemase-producing *Salmonella* spp. isolates from wildlife

Wildlife may also be a reservoir for CP *Salmonella* spp. In this respect, Fischer et al. (2013c) have recently reported the isolation in Germany of a NDM-producing *S.* Corvallis from a wild black kite. The isolate, NRL-Salm-12-1738, multilocus sequence type ST1541, showed carbapenem susceptibility values that suggested the presence of a carbapenemase (zone diameters of 24, 20 and 24 mm and MICs of 0.25, 0.5 and 0.125 mg/L for, respectively, imipenem, ertapenem and meropenem; non-wild-type by the EUCAST cut-off values, but susceptible or intermediate according to the CLSI clinical breakpoints). When the isolate was grown in liquid medium supplemented with imipenem (Luria-Bertani broth with 16 mg/L imipenem), it showed full resistance (clinical breakpoints CLSI versus EUCAST, imipenem ≥4 versus >8 mg/L). This isolate also exhibited resistance to chloramphenicol, kanamycin, tetracyclines, trimethoprim, streptomycin, sulphonamides and fosfomycin, carried the plasmid-mediated quinolone resistance gene *qnrS*, but was susceptible to tigecycline and nitrofurantoin.

2.2. Carbapenemase-encoding genes relevant for public health that have been linked to food-producing animals, foodborne transmission and wildlife

As described above, the following CP-encoding genes relevant for public health and which have been linked to food-producing animals or foods have been described:

The bla_{OXA-23} gene was found in isolates of *Acinetobacter* genomospecies 15TU from a dairy cattle farm in France (Poirel et al., 2012a), and was also detected in *Acinetobacter* spp. from two horses in Belgium (Smet et al., 2012).

The bla_{NDM-1} gene was found in *A. lwoffii* from a chicken in a broiler farm (isolate SGC-HZ9) (Wang et al., 2012) and *A. baumannii* from a pig farm (isolate GF216) (Zhang et al., 2013) in different regions of China.

The bla_{VIM-1} gene was found in *S.* Infantis from German broiler and pig farms, and *E. coli* from one of the same pig farms (Fischer et al., 2012, 2013a).

In other *Salmonella* spp. isolations from humans and other sources, the following genes have also been found:

The detection of *bla*$_{KPC-2}$ gene in a *S.* Cubana isolate made in 1998 in the USA (Miriagou et al., 2003). *bla*$_{VIM-2}$ in five *S.* Kentucky isolates from clinical human samples in Morocco (Le Hello et al., 2013). *bla*$_{OXA-48}$ in isolates of *S.* Saintpaul and *S.* Kentucky from the same patient, with more than one year between both isolation dates, supporting the "*in vivo*" transfer of the gene (Le Hello et al., 2013). This gene was also found in a *S.* Kentucky isolate from a patient from Morocco hospitalised in Germany (Pfeifer et al., 2013).

The *bla*$_{NDM-1}$ gene has been also found in *Salmonella* spp. from human clinical samples in USA and Reunion Island, in both cases in patients previously hospitalized in India (Savard et al., 2011; Cabanes et al., 2012; Rasheed et al., 2013), and *S.* Corvallis from a black kite in Germany – see above (Fischer et al., 2013c).

2.3. Mobile genetic elements involved in transmission of carbapenem resistance relevant for public health that have been linked to food-producing animals, foodborne transmission and wildlife

The following mobile genetic elements involved in the transmission of CP-encoding genes relevant for public health and linked to food-producing animals or foods have been so far described:

The *Acinetobacter* genomospecies 15TU isolate BY2, collected from cattle in France carried *bla*$_{OXA-23}$ located in the Tn*2008* transposon. In other eight isolates (BY-1, BY2-9) the IS*AbaI* element of Tn*2008* was truncated by the IS*Acsp2* IS. The gene appears to be chromosomally-located (Poirel et al., 2012a).

pAL-01 is a 270 kb self-transferable plasmid which carries the *bla*NDM-1 gene (partial sequence of the plasmid, accession number JN616388), isolated from *A. lwoffii* SGC-HZ9 from a chicken in China. The incompatibility (Inc) group of the plasmid could not be determined. The gene is attached to an intact ISAba125 insertion element that seems to play a role in the expression of the carbapenemase. The gene *aph*A6 is located upstream the IS*Aba125* (Wang et al., 2012).

pNDM-AB (accession number KC503911) is a 47 kb self-transferable plasmid found in an *A. baumannii* GF216 isolate that carries the *bla*$_{NDM-1}$ gene. The isolate was obtained from a pig in China. The Inc group of the plasmid could not be determined. Together with the *bla*$_{NDM-1}$ gene, the plasmid also harboured *aph*A6, *ble* and *msr*(E)-*mph*(E), and a copy of the IS*Aba125* element (Zhang et al., 2013).

pRHR-27 is a 300 kb incHI2 plasmid from the *S.* Infantis isolate R27 collected from a German pig farm (see above). The *bla*$_{VIM-1}$ was located in a class 1 integron together with *aac*A4 (*aac* (6')-Ib) and *aad*A1 in its variable region, and *qac*/*sul*1 in its 3'CS conserved segment. This integron forms part of a Tn*402*-like transposon. The plasmid also carries the *bla*$_{ACC-1}$, *str*A/B, *cat*A1, and a trimethoprim resistance gene. The pRH27 plasmid was neither self-transferable nor mobilisable under the conditions used (Fischer et al., 2013a). An indistinguishable plasmid has been found in other *S.* Infantis isolates from environmental samples from pig and poultry farms (Fischer et al., 2013a; Fischer et al., 2013d; Fischer et al., 2013b).

pRHR-178 (HG530658) is a 220 kb IncHI2 plasmid isolated from the *E. coli* isolate R178, collected in a German pig farm. Like in pRH27, the *bla*$_{VIM-1}$ is located in a class 1 integron forming part of a Tn*402*-like transposon (accession number HE663536). The plasmid also carries the *bla*$_{ACC-1}$, and *str*A/B genes, and was not transferable. The same plasmid has been found in other *E. coli* isolates collected within the same farm from pig faecal samples, manure and a fly (Fischer et al., 2012, 2013a; Fischer et al., 2013d; Fischer et al., 2013b).

In other *Salmonella* spp. isolates obtained from human clinical samples and from non-food producing animals, the following plasmids have been detected:

pST4707, a self-transferable plasmid of between 36 – 63 kb, in which both *bla*$_{KPC-2}$ and *bla*$_{TEM-1}$ genes were co-localized, from the isolate of *S.* Cubana described above. Genes encoding resistance to streptomycin, trimethoprim and sulphonamides were also present on the plasmid (Miriagou et al., 2003).

A 30 kb IncW plasmid carrying *bla*$_{VIM-2}$ within the In58 integron (containing also *aacC7*, *aacC1* and *aacA4* genes) in five isolates of *S.* Kentucky, and a 70 kb IncL/M plasmid in a *S.* Saintpaul isolate (Le Hello et al., 2013).

An IncL/M plasmid in a monophasic *S.* Senftenberg isolate. In this isolate the *armA* gene (aminoglycoside resistance) was also located on this IncL/M plasmid (Rasheed et al., 2013).

pRH-1738 is an ~180 kb IncA/C conjugative plasmid found in a strain of *S.* Corvallis (from a wild kite) in which both the *bla*$_{NDM-1}$ carbapenemase gene flanked by IS*Ab125* and the ble$_{BML}$ gene (accession number HG007972), and a *bla*$_{CMY-16}$ AmpC gene were located. On the same plasmid, two class 1 integrons (carrying *dfrA1-aadA5* or *aacA4*) as well as *floR*, *tet*(A), *strA/B*, and *sul1*, *sul2* were co-localized (Fischer et al., 2013c).

2.4. Data on occurrence of carbapenem resistance in food-producing animals and foods.

2.4.1. Harmonised EU monitoring of resistance: EFSA Community Summary Report.

To date carbapenems have not been included in the antibiotic panels of national surveillance programmes, nor in the panels of antibiotics used by veterinary diagnostic laboratories. Because screening for resistance to carbapenems has not been compulsory in official European Union (EU) surveillance activities, there are no data on resistance to this class of antimicrobial in the EFSA Community Summary reports.

2.4.2. Data from other published studies designed to detect carbapenem resistance

As stated above, few studies have reported the presence of CP bacteria from food-producing animals and their environment (Fischer et al., 2012; Poirel et al., 2012a; Wang et al., 2012; Fischer et al., 2013a; Fischer et al., 2013d; Fischer et al., 2013b; Woodford et al., 2013b; Zhang et al., 2013). The available data have come from studies conducted within several research projects in different countries, but not as a result of monitoring programmes. Thus, the methods used to detect the isolates were different. In some cases the studies focused on the isolation of ESBL/AmpC producers. When such isolates were subjected to tests for their susceptibility to carbapenems they were recognised as carbapenemase producers (Fischer et al., 2012, 2013a; Fischer et al., 2013d). In other investigations, the primary purpose was the identification of carbapenemase producers. The following studies have been published.

In a study conducted in one dairy farm in France in 2010, samples taken from 50 dairy cows were tested. The screening of the samples was done using selective Drigalski Agar plates (BioMerieux) with 1 µg/mL imipenem. Nine of the samples were positive for CPE *Acinetobacter;* all isolates produced OXA-23 (Poirel et al., 2012a).

In a survey on chicken farms (8), duck farms (6) and one pig slaughterhouse in Eastern China (Oct-Dec. 2010) in total 396 samples were collected. The samples were screened with selective brain heart infusion agar plates with 8 µg/ml imipenem, looking for non-fermenting Gram-negative isolates. In total, nine isolates (eight from pig samples, one from a chicken) from different bacterial genera (*Stenotrophomonas, Myroides, Chryseobacterium*, and *Acinetobacter*) were non-susceptible against carbapenems. One *A. lwoffii* producing NDM-1 was isolated from one of the broiler samples. The antibiotic usage records for the chicken farm indicated that penicillin, cefotaxime, cefradine, tilmicosin, doxycycline, and neomycin had been used (Wang et al., 2012).

Within a survey of Gram-negative bacteria from pig (5), broiler (3) and duck (1) farms conducted in South China (2011-2012), 1293 samples (single animals) were collected. The screening method used for isolation is not available. Isolates were tested for their susceptibility to carbapenems by growth on brain-heart infusion agar plates with 2 µg/ml meropenem. Isolates that grew on the media (276) were tested for the presence of bla_{NDM-1} and other carbapenemases. As described above one isolate from a pig with pneumonia and sepsis was positive for bla_{NDM-1}. Treatment records of the farm revealed the use of ß-lactams including 3rd and 4th generation cephalosporins, aminoglycosides and quinolones. Data on the remaining isolates have as yet not been published (Zhang et al., 2013).

Although testing for reduced susceptibility to carbapenems among isolates from food and food-producing animals has as yet not been included as part of the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP), two recent studies have been conducted on *E. coli* subpopulations to test for isolates with reduced susceptibility to carbapenems. In a collection from 2011, 138 cephalosporin resistant *E. coli* from pigs and cattle as well as meat from these animals and from poultry were subjected to phenotypic testing using discs containing meropenem, ertapenem and imipenem. None of these isolates showed reduced susceptibility to any of the three antimicrobials. Similar, in a collection from 2012, 249 cephalosporin-resistant *E. coli* from pigs and meat from pigs, cattle and poultry were tested and found negative for the presence of carbapenemase genes by Whole Genome Sequencing (WGS) by the use of the ResFinder Web tool (see below) (DANMAP, 2012).

From longitudinal studies conducted in swine (n=7) and broiler (n=7) farms in Germany (Laube et al., 2013a; Laube et al., 2013b), 2 fattening pig farms and 1 broiler farm were found to be positive for CP *Salmonella* spp. One of these farms was also positive for CP *E. coli*. The three farms were distributed in different locations in the same German Federal Region, with no apparent epidemiological link. The screening of the samples was done using selective MacConkey agar with 1 µg/mL cefotaxime, looking for ESBLs/AmpC producing isolates (Fischer et al., 2012, 2013a). When the isolates were tested for their susceptibility against a broad panel of ß-lactams, the production of carbapenemases was suspected and confirmed.

In a study carried out on hospitalised horses in Belgium in 2012, faecal samples collected from 20 horses were analysed for the presence of CP bacteria. The samples were screened using MacConkey agar plates with 1 µg/ml imipenem. In two of the samples *Acinetobacter* spp. isolates showing resistance to carbapenems were detected (Smet et al., 2012).

In a recent study, 184 *Salmonella* isolates belonging to the collection of the German National Reference Laboratory for Salmonella (mainly food and animal isolates) that showed clinical resistance to 3rd-generation cephalosporins (MICs $\geq$ 4mg/L for cefotaxime, and had been collected since 2006) were tested for their susceptibility to carbapenems (imipenem, meropenem and ertapenem). Only one of these isolates (*S.* Corvallis from a wild bird) showed susceptibility values (over the EUCAST cut off values) that suggested the presence of carbapenemases (in this case the presence of NDM-1 was confirmed) (Fischer et al., 2013c).

Kempf et al. (2012) performed a study in 2010 in Senegal, in which fecal samples from human head lice (354 samples), human faecal samples (717) and animal faecal samples (118) were screened for the presence of *A. baumannii* by means of real-time Polymerase Chain Reaction targeting the $bla_{OXA51\text{-}like}$ gene. Samples positive for *A. baumannii* were further screened for $bla_{OXA23\text{-}like}$ and $bla_{OXA24\text{-}like}$ genes. *A. baumannii* was detected in the head lice samples (4%), human stool samples (5.4%) and the animal stool samples (5%). No bla_{OXA24} gene was detected in the isolates (cultured from 14 head lice, 39 human and seven animal stool samples). Six of the isolates from the human faecal samples and three from the lice were positive for the $bla_{OXA23\text{-}like}$ gene. None of the *A. baumannii* from the animal samples was positive for any of the *bla* genes tested.

In a study in Switzerland (Stephan et al., 2013), investigations to determine the occurrence of CPE in food-producing animals in Switzerland were presented. Faecal samples from pigs (200), cattle (150)

and sheep (110), collected from individual healthy food-producing animals at slaughter (one animal per farm), as well as pooled faecal samples from poultry flocks (99 herd-level pooled faecal samples of chicken at the entry of a large slaughterhouse from the crates of 99 poultry flocks) were collected and investigated. In 16 of the samples (three sheep, five cattle and eight poultry) there was growth on Brilliance CRE agar. The isolates recovered were *Stenotrophomonas maltophilia*, *A. baumannii*, *E. coli* and *Citrobacter freundii*. The *E. coli* (4) and the *C. freundii* (4) isolates showed imipenem MICs between 0.19 and 1.5 µg/ml. None of the genes screened (genes encoding for VIM, GIM, Sim, NDM-1, IMI, KPC, OXA-48, OXA-40-like OXA-51-like and OXA-58-like) were present in these Enterobacteriacae.

Within another study conducted in Ethiopia (Kumsa et al., 2012) to determine the presence of *Acinetobacter* species in lice and *Melophagus ovinus* (sheep ked) of animals in 2011, cattle (207), sheep (85), dogs (47) and cats (16) were examined for ectoparasites. DNA from different *Acinetobacter* species was detected in lice (11.1%) and keds (86.4%): *Acinetobacter soli* in *Linognathus vituli* of cattle, *Acinetobacter lowffii* in *M. ovinus* of sheep, *Acinetobacter pittii* in *Heterodoxus spiniger* of dogs, one new *Acinetobacter* spp. in *M. ovinus* and two new *Acinetobacter* spp. in *H. spiniger* of dogs were detected. None of the carbapenemase resistance-encoding genes tested for $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-58}}$, $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-51}}$ were found in any of the lice and sheep keds.

2.5. Evidence of transmission of carbapenemase-producing strains and/or carbapenem resistance genes to humans by consumption or handling of contaminated food or through the food animal production environment.

As yet transmission of CP strains and/or resistance genes to humans by the handling of contaminated food or through the food animal production environment or food chain has not been reported. Nevertheless, as the bacterial species within which CP strains have been identified are common in food-producing animals and their environment, in foods, and in humans, the spread of such strains both from food producing animals to humans, and *vice-versa*, is a real possibility. Likewise, as the genes encoding resistance to carbapenems have been located on transmissible plasmids and are themselves transmissible by a variety of mechanisms (see 3.4.2 below), both intra- and inter-species transfer is also likely. Furthermore, when such genes are plasmid-located, linkage to other more common resistance genes such as those encoding co-resistance to tetracyclines, aminoglycosides, penicillins or cephalosporins (Fischer et al., 2012, 2013a) can favour their selection and spread when such antibiotics are used in a hospital or veterinary environment (EFSA Panel on Biological Hazards (BIOHAZ), 2011; Liebana et al., 2013; Woodford et al., 2013b).

A classic example of the rapidity of the spread, in the farm environment, of a plasmid encoding resistance to ESBL and other β-lactam antibiotics was that initially described by Liebana et al. (2006). In this study both the conjugative IncK, CTX-M-carrying plasmid, and strains carrying this pCT-like plasmid, were shown to spread rapidly among cattle on the farm. Later, they have also been reported among other farm animals and hospitals in different countries (Valverde et al., 2009; Cottell et al., 2011; Dhanji et al., 2012; Stokes et al., 2012). Results indicated that horizontal plasmid transfer between strains as well as horizontal gene transfer between plasmids contributed to the spread of resistance. Furthermore, some ESBL clones were shown to persist for several months, suggesting that clonal spread also contributes to the perpetuation of resistance. Although there is no suggestion that strains on this farm were resistant to carbapenem antibiotics, the study demonstrates how rapidly such ESBL-producing strains and plasmids coding for ESBL production can spread in a farm environment, and how unrelated antibiotic selection pressure can contribute to the transmission and establishment of such strains.

2.6. Summary and conclusions

- Harmonised monitoring of carbapenemase-producing bacteria in food-producing animals has as yet not been undertaken in the EU. EU-wide validated data on the occurrence of such resistance are therefore not available.

- Transmission of carbapenemase-producing strains and/or resistance genes to humans through the food animal production environment or food chain has as yet not been reported, but is considered likely should these strains/genes spread more widely in food-producing animals.

- Few studies have reported the presence of carbapenemase-producing bacteria in food-producing animals and their environment. Genes identified have included *bla*$_{\text{VIM-1}}$ in Enterobacteriaceae and *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-23}}$ in *Acinetobacter* spp. No such carbapenemase-producing isolates have been identified in food derived from food-producing animals.

- Carbapenemase-producing isolates of *Acinetobacter* spp. have been detected on a dairy cattle farm in France, in chickens on farms and pigs at slaughterhouse in China, and in horses in Belgium. The carbapenemase-encoding genes identified included *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-23}}$.

- Carbapenemase-producing isolates of putative *Salmonella* Infantis have been identified in food-producing animals (pig faeces) and their environment (pig and broiler farms) in Germany. The isolates carried both an acquired AmpC-encoding gene *bla*$_{\text{ACC-1}}$ and an acquired carbapenemase-encoding gene *bla*$_{\text{VIM-1}}$. *E. coli* producing VIM-1 carbapenemase was also identified in one of these pig farms (pig faeces and environment). These AmpC-encoding and carbapenemase-encoding genes were located on IncHI2 plasmids.

- Carbapenemase-producing isolates belonging to *Salmonella* Cubana, *S.* Kentucky, *S.* Saintpaul, *S.* Senftenberg and *S.* Westhampton producing KPC-2, VIM-2, OXA-48 or NDM-1 have been recovered from cases of human infection, although the origin of the genes (animal, environmental or clinical settings) cannot be determined. All these serovars are regarded as potentially zoonotic in origin.

- Carbapenemase-producing isolates from companion animals and from a wild bird have also been reported. Genes identified have included *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{NDM-1}}$ in Enterobacteriaceae and *bla*$_{\text{OXA-23}}$ in *Acinetobacter* spp.

- A wide variety of antibiotic resistance-encoding genes, including genes mediating resistance to a range of β-lactam-antibiotics, have been co-located on carbapenemase-encoding plasmids belonging to different incompatibility groups.

3. Critical analysis of the methods for detection of carbapenemase-producing bacteria (isolation and identification), and characterisation of carbapenemase-encoding genes and associated mobile elements.

3.1. Selective isolation of carbapenemase-producers

CP bacteria in food-producing animals have been so far identified in the context of existing surveillance systems for the detection of ESBLs or AmpCs. Specific surveys for the monitoring of carbapenemase producers have not been conducted and there is no experience on that issue. By analogy to what has been described in humans, the timely identification of carbapenemase producers is critical for the success of interventions (preventing introduction or reducing the incidence of carbapenemase producers in a specific setting). Thus, active surveillance is imperative in order to limit the dissemination of CP micro-organisms through (i) identification of reservoirs and (ii) early recognition of transmission events.

Active surveillance of cultures along with point prevalence surveys has been shown to be the most effective approach (Ben-David et al., 2010; Ciobotaro et al., 2011). Various versions of screening culture-based or molecular-based methodologies have been proposed (Landman et al., 2005; Schechner et al., 2009; Nordmann and Poirel, 2013). In-house prepared agar media (e.g. MacConkey,

SuperCarba) supplemented with a carbapenem (meropenem or ertapenem) or chromogenic media (e.g. CHROMAgar, chromID CARBA, Brilliance CRE) are suitable for this purpose (Landman et al., 2005; Samra et al., 2008; Adler et al., 2011; Moran Gilad et al., 2011; Girlich et al., 2013b; Kotsakis et al., 2013). The latter media have been evaluated and exhibit variable sensitivities and specificities for different Gram-negative bacteria and carbapenemase types. The different selective media are listed in Table 4, together with details on the carbapenem included in the media and their reported sensitivity and specificity.

Isolates obtained from the initial screening are considered as non-susceptible to carbapenems and then are further investigated by phenotypic, biochemical and molecular assays (Nordmann et al., 2012c; Nordmann and Poirel, 2013).

One of the in-house prepared selective agar media for the screening of carbapenemase producers is McConkey agar supplemented with meropenem (0.5µg/ml). Although the diagnostic values for this screening medium have not been determined it has been successfully used and exhibited high sensitivity during active surveillance (Sypsa et al., 2012; Adler et al., 2013). Colonies obtained on meropenem-containing MacConkey agar plates are visualized macroscopically as members of Enterobacteriaceae, or as non-fermenting Gram-negative bacilli; further identification is needed to the species level. One disadvantage of this medium is that the shelf-life of the plates is limited (one-two weeks) due to the instability of meropenem, and thus they should be prepared regularly.

CHROMagar KPC screening medium (CHROMagar company, Paris, France), which contains a carbapenem as the selective agent for resistance, was specifically designed for screening KPC producers. Using this medium, CP isolates with MIC values >16 µg/mL can be detected (Samra et al., 2008). The main problem associated with the use of this medium is that many carbapenemase producers, including those producing KPC-type enzymes, do not exhibit high MICs to carbapenems. Therefore the sensitivity of CHROMagar KPC screening medium appears to be quite low (Carrer et al., 2010).

SUPERCARBA medium has been developed to overpass the two main shortcomings of ChromID ESBL and CHROMagar KPC screening medium listed above, these being the lack of detection of isolates fully susceptible to broad-spectrum cephalosporins and of those exhibiting low MICs of carbapenems (Nordmann et al., 2012a). Hence, selection is based on a carbapenem (ertapenem) and not a cephalosporin. Furthermore the concentration of ertapenem is low (0.25 µg/ml), thus significantly enhancing sensitivity. Nevertheless, the specificity remains good, since SUPERCARBA medium has been supplemented with cloxacillin, which inhibits production of class C cephalosporinases, and that may confer reduced susceptibility to ertapenem when combined with some permeability defects. In addition, the SUPERCARBA medium contains zinc, thereby enhancing the production of MBLs and therefore constituting a significant advantage compared to the other media in order to detect those MBL producers showing low MICs to carbapenems.

Brilliance CRE (Oxoid, Basingstoke, U.K) is a chromogenic medium that contains a modified carbapenem and it is designed to detect carbapenem-non-susceptible bacteria. Results from several studies have shown that Brilliance CRE agar is a reliable selective medium, since it allows growth of the vast majority of the CP enterobacterial isolates, even those exhibiting low levels of resistance to carbapenems (Wilkinson et al., 2012; Girlich et al., 2013b; Kotsakis et al., 2013; Stuart et al., 2013). The specificity of Brilliance CRE is considered adequate and comparable to that of the SUPERCARBA. It should be noted that one of the advantages of this screening medium is its chromogenic ability to differentiate microorganisms to the genus or species level by colony color and morphology.

ChromID CARBA (bioMerieux) is a chromogenic medium that contains a mixture of antibiotics as selective agents for the detection of carbapenemase producers. This culture medium appears so far to show the best performance among the chromogenic media in terms of sensitivity and specificity (Wilkinson et al., 2012).

As reported by several investigators, the sensitivity of all three chromogenic media is affected by the difficulties in detecting OXA-48 producers which exhibit low carbapenem MICs. Therefore, a novel chromogenic medium, chromID OXA-48 (bioMerieux), has been developed for the efficient detection of OXA-48 producers. Indeed, this medium exhibits high sensitivity for the detection of OXA-48 positive micro-organisms. Because growth of isolates producing either class A or B carbapenemases is inhibited (Girlich et al., 2013a), in most instances chromID OXA-48 should be used in combination with an additional selective medium.

Table 4: Selective media for the screening of carbapenem-non-susceptible isolates.

Media	Company	Carbapenem included	Sensitivity (%)[a]	Specificity (%)[a]	References
McConkey +carbapenem	In house	Meropenem (0.125-0.5mg/L)	ND[c]	ND[c]	(Sypsa et al., 2012) (Adler et al., 2013)
SuperCarba	In house	Ertapenem (0.25mg/L)	96.5 100 (class A) 92 (class B) 100 (class D)	60.7	(Nordmann et al., 2012a) (Girlich et al., 2013b)
CHROMagarKPC/ Colorex KPC	CROMagar	NA[d]	88 43 70 (class A) 58.8 (class B) 11.6 (class D)	70 67.8	(Wilkinson et al., 2012) (Girlich et al., 2013b)
Brilliance CRE	Oxoid	NA[d]	82 94 85 76.3 85 (class A) 78.4 (class B) 69.8 (class D)	60 71 72 57.1	(Wilkinson et al., 2012) (Stuart et al., 2013) (Kotsakis et al., 2013) (Girlich et al., 2013b)
chromID CARBA	bioMerieux	NA[d]	96	76	(Wilkinson et al., 2012)
chromID OXA-48	bioMerieux	NA[d]	70.1 94.7 (class D) 0 (class A+B)	100	(Girlich et al., 2013a)

[a] sensitivity and specificity values are indicative and not comparable due to differences on the collections of isolates used for the evaluation, different types of carbapenemases, inoculum, and assay conditions.
[b] studies evaluating the optimal concentration are still ongoing.
[c] ND: not determined.
[d] NA: commercial information not available.

An algorithm has been proposed for the detection of carbapenemase-producing Enterobacteriacae by the "EUCAST subcommittee for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance"[11]. Since CPE may exhibit low level of resistance to carbapenems, the ECOFF values were taken into consideration in order to set a screening cut-off for the three carbapenems.

ECOFFs are determined from the MICs or inhibition zone diameter distributions of bacterial populations. They are defined as the values identifying the upper limit of the wild type population (i.e. characterized by the absence of acquired and mutational mechanism of resistance to a specific antimicrobial agent). The screening cut-off values are the lowest MICs or inhibition zone diameters identified in the non-wild type population possessing acquired mechanisms of resistance to a specific agent. For Enterobacteriaceae, the screening cut-off value is of 0.125 mg/l for meropenem, 1 mg/l for imipenem and 0.125 mg/l for ertapenem. If disk diffusion is used, applying zone diameters of 25 mm for meropenem, 23 mm for imipenem, and 25 mm for ertapenem would be indicative of non-susceptibility to carbapenems. Screening cut-off values for imipenem and meropenem have not yet been defined for *Acinetobacter* spp. Based on expert opinion, a decision has been made by the WG that values above the ECOFFs should be used as screening cut-offs for the latter micro-organism.

The use of ertapenem for screening confers high sensitivity but suffers from low specificity. On the other hand, for imipenem the MICs of wild-type population for several bacteria, such as Proteae, overlaps with the bacterial population bearing a carbapenem resistance mechanism. Screening with meropenem offers a good balance between sensitivity and specificity. Stability of carbapenem antimicrobials might be a problem because of their short active life.

A similar approach can be adopted for the monitoring of carbapenem resistance and identification of carbapenemase producers in animals and the environment. Specifically designed surveys for the detection of carbapenemase producers should be conducted. Pre-enrichment by incubation of samples in selective broth containing a carbapenem at a low concentration (e.g. meropenem 0.125 mg/L) may increase sensitivity (see also workflow in Figure 1). Cephalosporin-based pre-enrichment of the samples has been already recommended for the screening of ESBL-producers (EFSA Panel on Biological Hazards (BIOHAZ), 2011). A similar pre-enrichment approach using a carbapenem has not been validated for the screening of carbapenemase producers of animal origin, and any method proposed would have to be subjected to thorough experimental verification. In that respect, the proposed methodology is based on the CDC guidelines for screening of carbapenem resistance in Enterobacteriaceae (CDC, 2012) and on expert opinion.

Methods for application for two different uses are presented below:

(i) investigation of resistance for any given isolate from monitoring and/or surveillance schemes,

(ii) specifically designed surveys for detection of CP organisms

[11] http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Consultation/EUCAST_detection_of_resistance_mechanisms_Consultation_130711.pdf

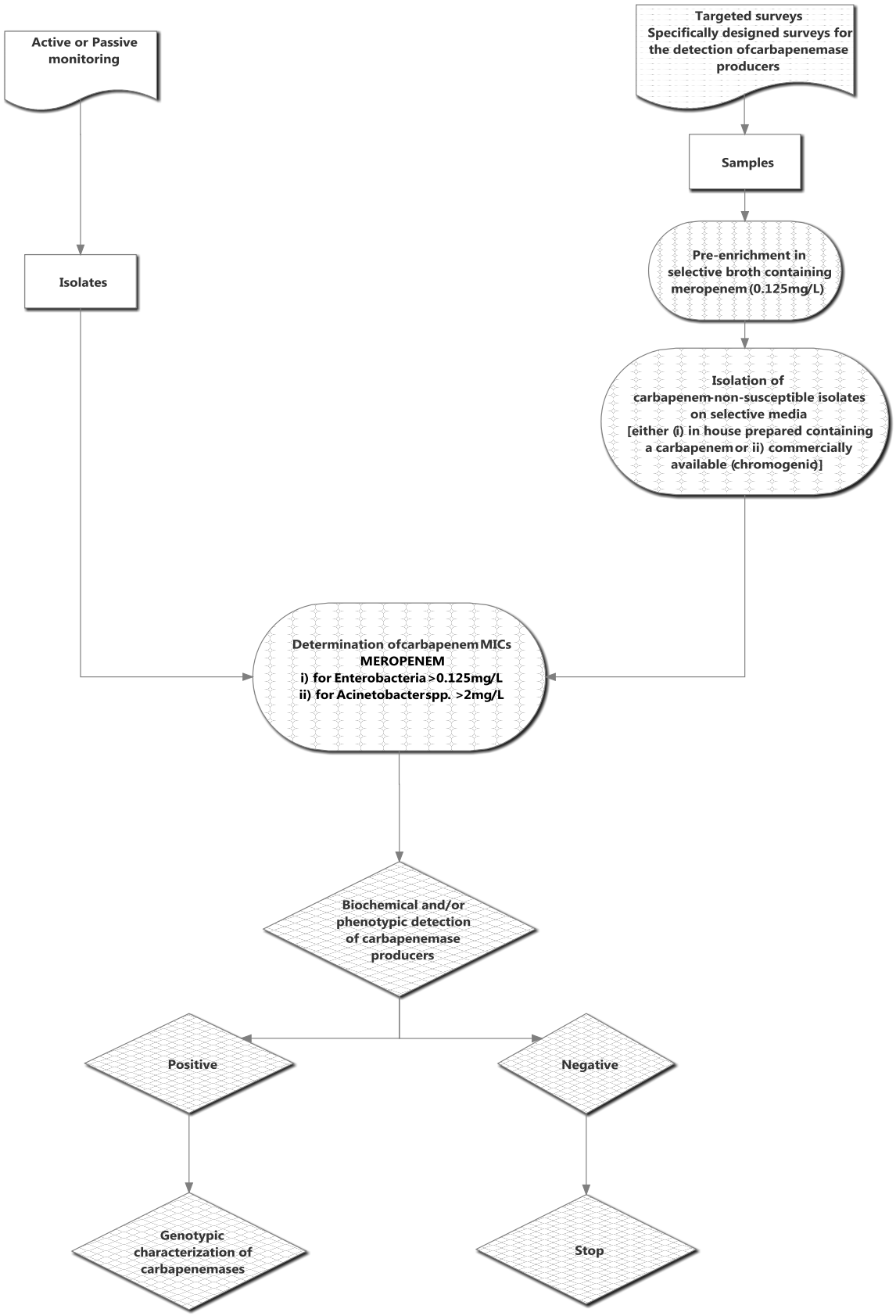


Figure 1: Proposed methodology for the detection of carbapenemase-producing non-susceptible Enterobacteriaceae and *Acinetobacter* spp.

3.2. Interpretative criteria for phenotypic testing for non-susceptibility to carbapenems.

Imipenem, meropenem and ertapenem breakpoints were established during the EUCAST harmonisation period in 2006, and doripenem breakpoints during the European Medicines Agency (EMA) registry process in 2008. In 2009 EUCAST reviewed all the relevant breakpoints on the basis of MIC distributions of wild-type organisms, MICs of MBL- and KPC-producing organisms, pharmacokinetic/pharmacodynamic (PK/PD) data and a review of current literature and concluded that there were not enough data to support a change in current breakpoints[12]. The point was made that breakpoints had been set as clinical breakpoints, and not for detection of carbapenem-inactivating enzymes.

In 2010, following a report from the US SENTRY Programme and analysing results from a study of 27,415 Enterobacteriaceae collected world-wide from a wide variety of infections, CLSI reduced breakpoint levels to ertapenem, imipenem and meropenem to levels similar to those of EUCAST (CLSI, 2010). The current EUCAST[13] and CLSI (2013) carbapenem breakpoints for Enterobactericaeae for imipenem, ertapenem and meropenem are shown in Tables 5 and 6 below.

Table 5: EUCAST[12] and CLSI (2013) MIC and zone diameters (ZD) for testing for phenotypic resistance to imipenem, ertapenem and meropenem in Enterobacteriaceae.

	CLSI			EUCAST			
MIC	**Breakpoints**			**Breakpoints**		**ECOFF**	**Screening cut-off**
	S	**I**	**R**	**S**	**R**	**(WT ≤ X mg/l)**	
Imipenem	≤ 1 mg/l	2	≥ 4 mg/l	≤ 2 mg/l	> 8 mg/l	≤ 1.0 mg/l	> 1 mg/l
Meropenem	≤ 1 mg/l	2	≥ 4 mg/l	≤ 2 mg/l	> 8 mg/l	≤ 0.125 mg/l	> 0.125 mg/l
Ertapenem	≤ 0.5 mg/l	1	≥ 2 mg/l	≤ 0.5 mg/l	> 1 mg/l	≤ 0.064 mg/l	> 0.125 mg/l
ZD	**Breakpoints**			**Breakpoints**		**ECOFF**	**Screening cut-off**
	S	**I**	**R**	**S**	**R**	**(WT ≥ Y mm)**	
Imipenem	≥ 23	20-22	≤ 19	≥ 22 mm	< 16 mm	≥ 24 mm	<23 mm
Meropenem	≥ 23	20-22	≤ 19	≥ 22 mm	< 16 mm	≥ 25 mm	< 25 mm
Ertapenem	≥ 22	19-21	≤ 18	≥ 25 mm	< 22 mm	≥ 29 mm	< 25 mm

Table 6: EUCAST[12] and CLSI (2013) MIC and zone diameters (ZD) for testing for phenotypic resistance to imipenem, and meropenem in *Acinetobacter* spp.

	CLSI			EUCAST			
MIC	**Breakpoints**			**Breakpoints**		**ECOFF**	**Screening cut-off**
	S	**I**	**R**	**S**	**R**	**(WT ≤ X mg/l)**	
Imipenem	≤ 4 mg/l	8	≥ 16 mg/l	≤ 2 mg/l	> 8 mg/l	≤ 1.0 mg/l	not defined*
Meropenem	≤ 4 mg/l	8	≥ 16 mg/l	≤ 2 mg/l	> 8 mg/l	≤ 2.0 mg/l	not defined*
ZD	**Breakpoints**			**Breakpoints**		**ECOFF**	**Screening cut-off**
	S	**I**	**R**	**S**	**R**	**(WT ≥ Y mm)**	
Imipenem	≥ 16	14-15	≤ 13	≥ 23 mm	< 17 mm	ND	not defined*
Meropenem	≥ 16	14-15	≤ 13	≥ 21 mm	< 15 mm	ND	not defined*

ND: not determined

*: screening cut-off values for imipenem and meropenem have not yet been defined. Values above the ECOFFs are proposed as screening cut-offs for *Acinetobacter* spp.

[12] http://www.eucast.org/antimicrobial_susceptibility_testing/previous_versions_of_tables

[13] http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Consultation/EUCAST_detection_of_resistance_mechanisms_Consultation_130711.pdf

The recently published technical specifications on the harmonised monitoring and reporting of AMR in *Salmonella* spp., *Campylobacter* spp., indicator *E. coli* and *Enterococcus* spp. transmitted through food (EFSA, 2012a) made a recommendation to include a carbapenem as a complementary antimicrobial to be inserted into the harmonised panel of antimicrobials used for test. Because of methodological difficulties a suggestion was made to include meropenem in the harmonised antimicrobial panel and then further test isolates which are non-susceptible to meropenem against imipenem and ertapenem in secondary panel. The point was also made that ertapenem is not advised as indicator of carbapenem resistance (Cohen Stuart et al., 2010).

3.3. Phenotypic and biochemical confirmation of carbapenemases

Once suspected, CP isolates are recovered on a plate, the next step is to characterize whether or not they are actually producing a carbapenemase. For this purpose, various phenotypic and biochemical methods for the detection of CP organisms have been described. A brief overview of these methods and their principles is presented below.

Modified Hodge test: The cloverleaf or modified Hodge test (MHT) has been extensively used for the detection of carbapenemase activity (Lee et al., 2001). The assay is based on the inactivation of imipenem or meropenem by whole bacterial cells or crude cell extracts.

MHT performs well for detection of KPC and OXA-48 producers (Pasteran et al., 2009; Nordmann et al., 2012c). However, i) its specificity is low against high-level AmpC producers or CTX-M producers accompanied also with porin loss (Pasteran et al., 2009) and ii) it exhibits relatively low sensitivity for producers of MBLs, especially NDM (though supplementing culture media with zinc may improve performance) (Girlich et al., 2012b). The assay is time-consuming and cannot distinguish the type of carbapenemase involved. MHT is still the only carbapenemase-detecting method proposed by the CLSI for screening purposes, although according to EUCAST the method is not recommended due to the aforementioned reliability problems and interpretation difficulties[14].

Detection of carbapenemase producers based on specific inhibitors: The relevant tests are based on inhibition of MBLs by various chelating agents capable of depriving the enzymes from the hydrolytically essential zinc ions. EDTA, dipicolinic acid, 2-mercaptopropionic acid and mercaptoacetic acid have been used as inhibitors, the former two being the most common (reviewed in (Miriagou et al., 2010; Nordmann et al., 2012c)). Phenotypic detection of class A carbapenemases (KPC) production is based on the inhibitory effect of tazobactam, boronic acid derivatives phenylboronic (PBA) and 3-aminophenylboronic acid (APBA) (reviewed by Miriagou et al.(2010) and Nordmann et al. (2012c)). Performance of tests utilizing PBA is considered superior to those with APBA (Tsakris et al., 2011; Miriagou et al., 2013).

The MBL-detecting assays are actually diffusion tests using disks containing a hydrolyzable β-lactam (typically a carbapenem, imipenem or meropenem) and/or a MBL inhibitor (Arakawa et al., 2000; Yong et al., 2002; Lee et al., 2003; Kimura et al., 2005). The double disk synergy test (DDST) is similar to that used for ESBL detection. Two disks containing predefined amounts of the β-lactam and the inhibitor are placed close to each other. Formation of a synergy image is indicative of MBL production. The method is considered highly sensitive even with isolates with low carbapenem resistance levels. The main drawback is that interpretation is subjective and results cannot be quantified. Therefore, the combined disk test (CDT) is currently preferred by most laboratories. In the CDT format, the β-lactam disk is potentiated with an inhibitor and the diameter of the inhibition zone is compared with that of the β-lactam disk alone. Increase of the inhibition zone diameter above a pre-defined cut-off value denotes MBL activity (Tsakris et al., 2010; Giske et al., 2011).

Detection of class A carbapenemases by the CDT synergy test using boronic derivatives combined with imipenem or meropenem exhibits high sensitivity for *K. pneumoniae* and *E. coli* (Tsakris et al.,

[14] http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Consultation/EUCAST_detection_of_resistance_mechanisms_Consultation_130711.pdf

2010; Giske et al., 2011). A major drawback of these methodologies is that boronates also inhibit cephalosporinases and in that respect the CDT performance is suboptimal (false positives), especially with AmpC hyperproducing enterobacterial isolates. This problem can be overcome by the use of cloxacillin-containing plates or disks (Giske et al., 2011; Nordmann et al., 2012c).

Carbapenemase detection by lowering carbapenem MICs using a chelating agent or a boronate derivative can be performed in either liquid or solid media (reviewed by Nordmann et al. (2012c)). In tests utilizing liquid media the suspected micro-organism is cultured in carbapenem-containing broth in duplicate (absence and presence of inhibitor). In tests using solid media gradient diffusion methodology (e.g. Etest bioMerieux, MIC Test Strip Liofilchem) is used and double sided strips are utilized containing a carbapenem on one side and 'carbapenem + EDTA or boronate' on the other. Strips containing different concentrations of carbapenems (imipenem or meropenem) and inhibitors are commercially available and can efficiently detect carbapenemase producers exhibiting both low and high level resistance.

Generally, the inhibitor-based assays are considered reliable for the identification of MBL-producers, although there have been studies reporting failures to detect VIM production among *A. baumannii* strains (Ikonomidis et al., 2008; Loli et al., 2008; Picao et al., 2008). False positive results have also been reported in *P. aeruginosa* due to the strong antimicrobial activity of EDTA against the species (Chu et al., 2005) and *A. baumannii* isolates due to the presence of OXA-type carbapenemases (Segal and Elisha, 2005). In addition, double carbapenemase producers (e.g. MBL plus KPC) may "deceive" the conventional CD tests frequently appearing negative for one or even both carbapenemases due to a masking effect. Inclusion of a carbapenem disk (imipenem or meropenem), potentiated with both inhibitors for class A (boronate) and class B (EDTA or DPA) carbapenemases, may facilitate the correct identification of carbapenemases in such isolates (Miriagou et al., 2003; Tsakris et al., 2011). An additional shortcoming of this type of methods is that there is no inhibitor-based method for the detection of class D CP bacteria.

Enzymatic properties of OXA-type carbapenemases have prevented the development of specific phenotypic tests. High-level resistance to temocillin has been suggested to be predictive of production of OXA-48 but the specificity of this feature remains to be more extensively evaluated (Nordmann et al., 2012c). For other authors, high level of temocillin resistance should be used as part of an algorithm to identify carbapenemases, rather than as a single test (Woodford et al., 2013a). Thus, the definitive identification of carbapenemase OXA producers requires molecular techniques (Nordmann et al., 2012c).

Biochemical-enzymatic detection of carbapenemases producers:

A recent development for an accurate identification of CPE corresponds to the Carbapenemase Nordmann-Poirel (Carba NP) test (Nordmann et al., 2012b). This biochemical test, which is applicable to isolated bacterial colonies, is based on in-vitro hydrolysis of imipenem. Hydrolysis of imipenem is detected by a change of the pH value of the indicator (red to yellow/orange). Although not as yet extensively used, this test has been found to be 100% sensitive and specific, as for molecular techniques. It detects not only all known carbapenemases (either belonging to Ambler A, B and D classes) in Enterobacteriaceae but, in contrast to molecular techniques, may also identify newly-emerging carbapenemases. The Carba NP test has the additional advantage of being both inexpensive and very rapid, and can be easily implemented in different laboratories, as there are no requirements for specific equipment or of specialized personnel.

A similar method, the Blue-CARBA, also based on in-vitro hydrolysis of imipenem, has been proposed by Pires et al. (Pires et al., 2013). In this case, the carbapenemase activity can be determined directly from bacterial culture without bacterial extraction. As described by the authors, sensitivity and specificity of the method was also 100% for *Enterobacteriaceae*, *Pseudomonas* spp., and *Acinetobacter* spp.

Detection of carbapenemase activity can be made using a UV spectrophotometer which is available in many microbiology laboratories. It is based on several steps including an 18-h culture (that can be shortened in some cases to 8 h), a protein extraction step followed by measurement of imipenem hydrolysis using an UV spectrophotometer (Bernabeu et al., 2012). This spectrophotometry-based technique has been shown to have a 100% sensitivity and a 98.5% specificity for detecting any kind of carbapenemase activity (Bernabeu et al., 2012). This technique can differentiate with accuracy carbapenemase- from non-carbapenemase-producers among CNS isolates (i.e. outer membrane permeability defect, overproduction of cephalosporinases or/and ESBLs). It can be implemented in any reference laboratory but this technique still requires time.

Recently the use of mass spectrometry for the detection of carbapenemase activity has been proposed, based on the analysis of the degradation of a carbapenem molecule (Burckhardt and Zimmermann, 2011; Hrabak et al., 2011). This method was shown to have a 97% sensitivity and 98% specificity (Hrabak et al., 2011). Although this technique has to be further evaluated, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-Tof) equipment is increasingly used in diagnostic bacteriology laboratory.

The recent EFSA report with technical specifications (EFSA, 2012a) further recommended that isolates with a carbapenemase phenotype (i.e. with apparent resistance to meropenem or imipenem) should be re-tested phenotypically against these antimicrobials to confirm such resistance using discs or gradient strips for synergy between carbapenems and EDTA (indicating a probable MBL) and for inhibition by boronic discs (indicating the possible presence of KPC enzymes).

3.4. Characterisation of carbapenemases and typing of plasmids carrying carbapenemase-encoding genes

3.4.1. Molecular detection and characterization of carbapenemase genes.

Apart from the above phenotypic or biochemical techniques, molecular technologies (either in-house or commercial) may be used as alternative methods. Molecular detection of CP genes can, in principle, be performed by various molecular methods such as microarrays, real-time PCR, multiplex PCR and WGS. There are two main problems related to such molecular tests: i) they may not be affordable for all laboratories and also require some specific expertise, and ii) such tests only detect 'known' genes. Thus any new and emerging carbapenemase-encoding gene will be missed unless the 'new' gene has been identified and corresponding sequence data are implemented in the test. Most importantly this potential lack of detection may result in false negatives, since isolates tested by such molecular techniques and for which negative results have been obtained will be flagged as "carbapenemase-negative" unless an additional accurate and definitive non-molecular test has been used. Favouring molecular detection methods is the fact that they are able to detect presence of variably expressed genes, which might not always be detected by phenotypic methods.

Regardless of which technique is chosen for detection and characterisation of carbapenemase genes, there will be both advantages and disadvantages in relation to costs, accuracy, speed and availability. Such methods are listed in Table 7 and further elaborated upon below.

Microarray, Real-Time PCR and multiplex PCR analysis are based on oligo or primer hybridization to conserved parts of a given carbapenemase gene group and will often only identify (sub-)groups of genes, rather than specific gene variants. In addition, these three methods rarely detect all known carbapenemase gene groups at the same time. An advantage of PCR-based methods is that they are relatively cheap and fast to perform, even on larger strain collections. Also, the instruments required to perform these analyses is often available in many microbial laboratories or can be acquired at relatively low costs. Identification of specific gene variants beyond (sub-group) level requires individual PCR protocols covering the complete sequence of the relevant genes followed by Sanger sequencing and bioinformatic sequence analysis as especially multiplex PCR protocols are prone to create false positive reactions. As opposed to this, WGS holds the possibility to detect and perform

bioinformatic analysis of all genes present in the isolate, including CP genes. Web-based tools such as ResFinder[15] have been developed in order to allow easy detection of resistance genes including carbapenemase genes based on WGS data. Bench top sequencers are not common in routine microbial laboratories, and the cost of sequencing equipment and WGS is currently higher than is the case for the other three methods, but the combined cost of the various phenotypic and genotypic tests that are usually required to fully characterise the resistance genes (and other relevant genes) present in the organism is usually greater than the cost of WGS, so investment in this technology is likely to lead to reduced longer-term costs.

Table 7: Methods for detection and characterization of carbapenemase-encoding genes

Method	Specification	Comments	References
Micro arrays	Commercially available DNA arrays have been designed. In these, the most common gene (groups) associated with carbapenemase production are detected.	Often only the gene related to the carbapenemase phenotype is detected at the group level. Further analysis using methods like PCR and sequencing (see below) is also required. Furthermore, many less common gene groups are not included in the array design at the moment. Micro arrays detection is more expensive than multiplex PCR. Analysis of sequence data from PCR products may therefore be necessary to identify any gene variants, in accordance with Genebank databases. Commercial arrays can be purchased from at least one source.	(Woodford et al., 2011)
Multiplex PCR	Several different multiplex PCR assays have been developed to detect carbapenemases. Multiplexing allows for parallel screening for several genes at the same time.	Often only the gene related to the carbapenemase phenotype is detected at the group level. Further analysis using methods such as PCR and sequencing (see below) are also required. Multiplex PCR assays are often not as robust as singular PCR and can in some instances produce unspecific DNA products. Multiplex PCR is relatively cheap and fast to run.	(Dallenne et al., 2010; Poirel et al., 2011; Hong et al., 2012; Kaase et al., 2012; Bogaerts et al., 2013)[a]

[15] http://cge.cbs.dtu.dk/services/ResFinder

(Single) Polymerase Chain Reaction (PCR) and sequencing	Specific PCR reactions have been designed for detection of all relevant carbapenemase genes. To identify individual carbapenemase variants, PCR products should be sequenced and compared to previously sequenced genes.	Single PCR protocols can be designed to cover a complete gene. This enables subsequent DNA sequencing. Because a strain might carry more than one carbapenemase gene, there is a risk of not detecting all genes present when using only one or few PCR assays. Single PCR assays are in general more robust than multiplex PCR, but they require more resources to cover the same spectra of genes as the multiplex PCRs.	
Whole genome sequencing (WGS)	Genomic DNA sequences can be generated on various sequencing platforms. Benchtop sequencers exist where smaller number of isolates can be sequenced within a few days.	WGS generates data ideally containing information on all genes present in an isolate, including carbapenemase production genes. Bioinformatic analysis is required to identify the relevant carbapenemase production genes.	(Zankari et al., 2012)

a) Other commercial methods based on hibridisation are also available.

3.4.2. Detection and characterization of plasmids harbouring carbapenemase genes.

Horizontal transfer of carbapenemase genes is an important factor contributing to the dissemination of such genes within the bacterial ecosystem. In Enterobacteriaceae, horizontal transfer of AMR is, in most cases, mediated by plasmids. In order to better understand the epidemiology of such plasmids, methods for their detection and characterisation are necessary. Some carbapenemase genes (bla_{VIMs}, bla_{GIMs}, bla_{GES-5}, bla_{SIM-1}, bla_{IMP-19}) have been shown to be located on integrons, but horizontal transfer of these will normally also be dependent on plasmid localization and co-mobilization. Enterobacterial and *Acinetobacter* plasmids can be classified through characterization of their replicons, relaxases and/or other genes in plasmid scaffold (plasmid multi locus sequence type) (Carattoli et al., 2005; Bertini et al., 2010; Alvarado et al., 2012)[16]. Genes conferring reduced susceptibility to carbapenems have been found located on many different groups of plasmids (recently reviewed by Carattoli (2013)). Many of these plasmid groups are the same as have been associated with genes conferring resistance to cephalosporins (ESBL- and AmpC- producing Enterobacteriaceae). Detection and characterization of these plasmids has been recently presented in considerable detail (EFSA Panel on Biological Hazards (BIOHAZ), 2011) and will therefore not be further described here.

3.5. Molecular typing of isolates.

The purpose of molecular typing is to determine genetic characteristics of isolates at the subspecies level in order to identify genetic relatedness and/or to allow source tracking/attribution and surveillance. Several genotyping methods are available. The principles on which these are based on and their discriminatory power are very different, which greatly influences the choice of the method according to the intended use.

Phenotypic tests are relatively easy to perform, but lack discriminatory power. Within serovars, and phage types of, e.g., *Salmonella* spp., many different genetically-related clusters of isolates can occur that can be identified by genotyping methods. Macrorestriction DNA analyses followed by PFGE

[16] See also http://pubmlst.org/plasmid/ and http://cge.cbs.dtu.dk/services/PlasmidFinder/

allow identification of lineages or clusters of epidemiologically-related isolates within. It is intended for tracing outbreaks in a limited time period and less suitable for performing phylogenetic analyses. Multi Locus Variable Number Tandem Repeat Analysis (MLVA) was developed as a sequence-based method with similar discriminatory power to PFGE. Multi Locus Sequence Typing (MLST) is a sequence-based method targeting five to seven highly conserved genes. This method has generally less discriminatory power than PFGE or MLVA but is the most reliable method to determine genetic relatedness of epidemiologically-unrelated isolates.

With the recent emergence of WGS, the full genetic information of a set of strains can be extracted and gene content (including MLST genes) or variations in chromosomal mutations (Single Nucleotide Polymorphisms, SNP) can be compared, which may be relevant for typing purposes (Didelot et al., 2012). In principle, SNP analysis allows for bacterial typing with high typeability and discriminative power, but also presents a challenge in relation to defining criteria for detecting outbreaks and clonality.

The choice of the molecular typing method to be used is determined by knowledge of the epidemiological relatedness of the isolates and the main purpose of typing – outbreak recognition and investigation, source attribution or surveillance. Next to phenotypic methods such as serotyping and phage typing, PFGE, MLVA and WGS can be used to identify clonal clusters of isolates that are related to a certain 'outbreak' in a restricted timeframe. MLST is the method of choice to identify relatedness of isolates of the same species from different backgrounds (e.g. animal *versus* human) and their evolutionary history.

Finally, new approaches allowing high-throughput bacterial typing as Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) coupled with chemometrics have been recently suggested as a reliable and alternative method to accurately discriminate particular *E. coli* clones (Sousa et al., 2013).

3.6. Summary and conclusions

- A methodology including selective culture is proposed for the detection of carbapenemase-producing strains of Enterobacteriaceae and *Acinetobacter* spp.

- Pre-enrichment by incubation of samples in selective broth containing a carbapenem at a low concentration (e.g. meropenem 0.125 mg/L) may increase sensitivity. This methodology has not yet been validated, and any method proposed would have to be subjected to thorough experimental verification.

- A variety of in-house and commercially-available selective media has been used for the active surveillance of carbapenem resistance in hospitals. The choice of the media for testing animal and food samples needs to be experimentally evaluated and validated.

- Meropenem offers a good balance between sensitivity and specificity and has been recommended to be included in the harmonised antimicrobial panel for the surveillance of AMR in isolates from food-producing animals, food thereof and environmental samples.

- Biochemical and phenotypic tests for the confirmatory identification of carbapenemase-producing bacteria among isolates exhibiting non-susceptibility to carbapenems are available. The sensitivity and specificity of these assays may vary considerably in different settings.

- The identity of the genes responsible for the carbapenemase production should be determined by molecular methods.

- Plasmid and strain typing should be undertaken to acquire better knowledge on the epidemiology of genes encoding carbapenemase production among bacteria from food-producing animal populations, food thereof and environmental samples.

4. Recommendations for a harmonised monitoring of resistance (phenotypic and genotypic) caused by carbapenemase-producing bacteria in food and food producing animals.

Pathogens possessing carbapenemases can cause significant problems for human health and as such have focused great interest amongst different stakeholders. Commensal bacteria encoding carbapenem resistance also provide a reservoir of resistance genes which may enter the food chain. Early detection of the presence of carbapenemase gene-carrying bacteria in the agricultural or veterinary sector will be very important to limit the spread at a very early stage. Targeted studies may provide crucial information in designing cost-effective measures to reduce the burden of disease in humans related to infection with CP bacteria.

As yet only a few findings of resistance to carbapenems in different livestock populations have been reported (See Chapter 2.1). The situation in both animals and humans is most likely underestimated, taking into account the "silent" and successful dissemination of certain species of CP bacteria reported among humans (Gijon et al., 2012; Viau et al., 2012). Therefore, monitoring of all sectors is considered important, especially given the considerable impact that CP bacterial infections may have on human health. This emphasises the need to ensure that methods are sufficiently sensitive to allow detection of low numbers of CP bacteria in the sample. Recently, the CDC has made recommendations for the control of the spread of CRE in hospitals (CDC, 2012). Selective pre-enrichment may be required to detect very low numbers of these types of organisms. A harmonized monitoring programme will deliver important baseline information on the presence and prevalence of resistance. Based on repeated monitoring, trends over time can be assessed. The basic general principles of harmonized monitoring of AMR in *Salmonella* spp. and *E. coli*, as laid down in the Community legislation (Directive 2033/99/EC[17] and implementing Decision 2013/652/EU[18]) and EFSA's recommendations (EFSA, 2012a), also apply to monitoring resistance caused by carbapenemases. Some additional considerations are important for this type of resistance, as currently knowledge is very limited and emergence of this new hazard needs to be adequately addressed. The following sections address these specific considerations.

Besides *E. coli* and *Salmonella* spp., which are the main target species recommended by EFSA for monitoring AMR, other micro-organisms should also be considered. Spread of MDR *Klebsiella* spp. in farms can cause animal health problems, as reported in the USA and Canada (Kim et al., 2005). This bacterial species can be isolated from faeces of cows and other sites in farms. Besides problems with mastitis, an increase in the antibiotic resistance rates of *Klebsiella* spp in cattle has been reported[19]. Other members of the family of Enterobacteriaceae (e.g. *Morganella* spp., *Proteus* spp., *Enterobacter* spp., *Providencia* spp.) belonging to the normal flora of the gastro-intestinal tract should be considered in monitoring activities as they may carry (multidrug) resistance genes and can cause severe nosocomial infections. Similarly, *Acinetobacter* spp. has been isolated from several reservoirs, and has recently emerged as a pathogen of clinical importance due to its ability to accumulate resistance mechanisms, including to the carbapenems.

[17] OJ L 325, 12.12.2003, p.31-40.
[18] OJ L 303, 14.11.2013, p.26-39.
[19] http://www.nnyagdev.org/index.php/dairy/research-projects/klebsiella-sources-transmission-control-points/ and http://www.vet.cornell.edu/news/Klebsiella.cfm

4.1. Purpose of the suggested harmonised monitoring

Harmonised monitoring of carbapenem resistance caused by acquired carbapenemases has several objectives:

- to detect the existence, and determine the prevalence of CNS bacteria in food-producing animal populations and derived food, including imported from outside the EU;
- to describe the geographical distribution of CNS bacteria within the EU;
- to detect changes in presence and prevalence of CNS bacteria over time;
- to detect and identify CP bacteria amongst CNS bacteria;
- to identify the genes responsible for the CP amongst CNS bacteria;
- to detect emergence of new carbapenemase-encoding genes amongst CNS bacteria with a potential relevance to public health;
- to determine the effect of interventions on the prevalence of carbapenemase-mediated resistance.

These objectives may complement the general aims for monitoring AMR in bacteria of animal origin in the EU. In this document the drafted monitoring is restricted to livestock and food of animal origin. Other sources, like companion animals, environmental contamination and food of plant origin might be relevant, but out of the scope of this document.

4.2. General approaches of the suggested harmonised monitoring

EFSA specifications (EFSA, 2012a) recommend mandatory phenotypic monitoring of *Salmonella* spp. and indicator *E. coli* with a broadened test panel covering also meropenem. Furthermore, a two-step strategy has been recommended to further characterize those isolates of *E. coli* and *Salmonella* spp. showing resistance to extended-spectrum cephalosporins and carbapenems. To improve detection of ESBL/AmpC-producing bacteria, analytical methods using selective media have been recommended.

Decision 2013/652/EU foresees, among other requirements, compulsory testing of randomly selected isolates of *Salmonella* spp. and *E. coli* for resistance to meropenem and further characterization and classification of those isolates showing resistance to meropenem. Whereas specific monitoring of ESBL- or AmpC- producing *E. coli* using more sensitive selective methods will be compulsory according to this Decision, specific monitoring for CP microorganisms, using more sensitive detection methods (e.g. using selective pre-enrichment), is up to the individual Member State (MS).

Due to the high public health relevance of carbapenem resistance, several approaches may be combined to monitor the emergence of such resistance in livestock and food. The importance of the implementation of current recommendations needs to be highlighted and additional specific approaches need to be defined in order to overcome the problems of detection due to the heterogeneous expression of different enzymes coding for CP. EUCAST is in the process of developing some guidelines to improve the detection of different mechanisms of resistance, including those conferring CP[20].

In current monitoring activities, a sample size of 170 randomly selected isolates is regarded as suitable for detecting trends in changes of prevalence over time. This sample size is not sufficient for the timely detection of the emergence of new resistance patterns in the case of no reports or only sporadic

[20] http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Consultation/EUCAST_detection_of_resistance_mechanisms_Consultation_130711.pdf

findings (as observed for CP microorganisms in animals). Consequently, harmonised monitoring of resistance caused by the production of carbapenemases needs to go beyond the existing recommendations and legal requirements for routine phenotypic surveillance. Specifically, different approaches should be combined, including passive and active surveillance as well as targeted surveys and additional usage of sensitive and specific methods.

4.2.1. Targeted surveys

In order to increase the probability of detection of emerging AMR determinants, active surveys should target those animal and food categories where the risk of development and spread of resistant isolates are highest. For this, information on risk factors contributing to the presence of resistance caused by carbapenemases would be desirable, but is largely unknown. One approach would be to apply those criteria, already suggested for ESBL/AmpC-producing bacteria (EFSA Panel on Biological Hazards (BIOHAZ), 2011). In that document, the recommendation was to include those animal species and farms with the highest consumption of 3rd and 4th-generation cephalosporins in the survey, as carbapenems are not licensed for usage in animals. In case detailed data on antimicrobial consumption are not available, other risk factors such as type of production, health status or holding size could be used for a targeted selection of samples instead as a proxy. For animal categories where trade is suspected to be a risk factor, farms can be targeted according to the origin of the animals received (EFSA Panel on Biological Hazards (BIOHAZ), 2011; Nawaz et al., 2012). As these data are largely not available, a more practical approach could be to select those animal species and food products where resistance to β-lactams or cephalosporins has been most frequently observed, or those with the highest potential for consumers' exposure. This would address the public health risk related to those pathogens showing multiple drug resistance.

Targeted surveys can either be included into active monitoring programs, where additionally more sensitive methods are applied to screen samples taken for CP isolates, and/or running surveys in populations not covered by routine monitoring. For the latter, a combination of routine methods and more sensitive methods should be envisaged.

As recommended for ESBL/AmpC-monitoring (EFSA, 2012a), a way forward could be represented by organising an EU baseline survey with the major objective to determine the presence of CP *E. coli* in specific food-producing animals at slaughterhouse and in food samples at retail. Conducting such an EU baseline survey would in any case have the added value of building capacity in those countries that have no or very limited experience with the detection of carbapenem resistance.

4.2.2. Active monitoring

All isolates of *Salmonella* spp. and *E. coli* collected within the compulsory monitoring program, as required by Community legislation (Decision 2013/652/EU) or recommended in the technical specifications prepared by EFSA (EFSA, 2012a) should be screened for meropenem resistance using standardized microdilution methods. Other members of the family of Enterobacteriaceae (e.g. *Klebsiella* spp., *Morganella* spp., *Proteus* spp., *Enterobacter* spp., *Providencia* spp.) and *Acinetobacter* spp. may be considered in future monitoring activities. Microorganisms that are classified microbiologically resistant to 3rd or 4th generation cephalosporins or carbapenems based on the ECOFF should be subjected to phenotypic and molecular identification and characterization of the carbapenemase-encoding genes present.

4.2.3. Passive monitoring

Subjecting all Gram-negative diagnostic isolates of veterinary origin (at least those that are classified as microbiologically resistant to 3rd or 4th generation cephalosporins on the basis of ECOFF values) to phenotypic testing for CNS would be desirable. When CNS bacteria are identified, testing for CP, and further molecular identification and characterization of the genes involved is recommended. As diagnostic submissions are likely to originate from animals with a health problem and treated with

antimicrobials prior to sample collection, these samples could also be indicative of the emergence of carbapenemase-mediated resistance.

Furthermore, *Salmonella* spp. isolates collected within official control programs not subject to active monitoring for AMR as required by legislation (e.g. food, feed) may be tested for carbapenem resistance. Of special interest may be isolates from imported products or food types (e.g. seafood and meat) considered of higher risk and not covered by routine monitoring or specific surveys (Lavilla et al., 2008; Gundogan et al., 2011; Nawaz et al., 2012).

4.2.4. Combined monitoring activities

Immediate implementation of screening for CNS of all Enterobacteriaceae from active monitoring, and at least Enterobacteriaceae and *Acinetobacter* spp. with cephalosporin resistance from passive monitoring is recommended. Additionally, directing CNS bacteria to more specific phenotypic and/or genotypic characterization is recommended.

In a next step, targeted surveys using more sensitive methods for detection of CNS bacteria should be run in those populations where active monitoring is required by legislation. Finally, similar targeted surveys should be started to investigate the presence of CNS bacteria within other relevant populations. This should include imported foods from countries where carbapenem resistance is known to be prevalent in bacterial isolates from cases of human infection.

4.3. Bacterial species, food animal species and/or food products to be considered for monitoring of resistance caused by carbapenemase-producing bacteria from a public health perspective

In defining combinations of bacteria/animal/food to become subject to mandatory AMR monitoring, the approach followed by EFSA (2012a) was to prioritise potential consumers' exposure. Routine AMR monitoring focuses on domestic productions, covering *Salmonella* spp. and *E. coli* in poultry (separated by production type), cattle and pigs and products thereof on a regular basis. Complementary monitoring AMR in poultry meat imported from third countries at the EU level has also been proposed (EFSA, 2012b, a). For those food-producing animal species and their derived fresh meat (e.g. lamb, duck, geese, goats) for which consumption is more specific to certain MSs, a threshold mechanism, calculated on the basis of the animals slaughtered, has been introduced for the monitoring to become performed consistently in a given MS (EFSA, 2012a).

4.3.1. Prioritisation of bacterial species

Monitoring for CP bacteria in animals and food should cover zoonotic agents, animal pathogens and indicator organisms of the commensal flora. Monitoring of *E. coli* and *Salmonella* spp. for AMR has been recommended by EFSA (EFSA, 2007, 2008, 2012a, b, c) and will be compulsory starting in 2014 (Commission Implementing Decision 2013/652/EU). Other members of the family of Enterobacteriaceae (e.g. *Klebsiella* spp., *Morganella* spp., *Proteus* spp., *Enterobacter* spp., *Providencia* spp.) as well as *Acinetobacter* spp. might additionally be considered in carbapenemase monitoring.

4.3.2. Prioritisation of food-producing animals and food categories

As foreseen by the EU legislation and recommended in a number of EFSA scientific reports, AMR monitoring should be performed in *Salmonella* spp. and *E. coli* isolates in all major food-producing livestock species and products thereof. It should target those animal and food categories where the risk of development and spread of resistant isolates are highest. As information on risk factors contributing to the presence of resistance caused by carbapenemases is largely unknown, focus is put on the major food-producing animal species. This should take into account different production types, reflecting the widely differing treatment regimes, management practices, and hygienic conditions (EFSA, 2012a).

As a priority, animal species already covered by active monitoring for AMR due to current EU legislation, such as broilers, fattening turkeys, fattening pigs, veal calves, and the derived fresh meat of domestic origin, preferably sampled at retail, should be addressed in active monitoring and/or additional targeted surveys for CP bacteria. The inclusion of the breeding level is considered of special relevance for carbapenemase monitoring as occurrence of CP bacteria in the top level of the animal pyramid may contribute to a rapid and wide spread introduction into the production level, as observed for ESBL/AmpC-producing bacteria. Dairy cattle and raw milk samples should also be addressed in targeted surveys, due to the specific use of cephalosporins in dairy cattle, and to the risk posed by potential consumption of raw milk. Aquaculture products may also be included in targeted surveys, due to the possible presence of resistant bacteria in the aquatic environment. Although not the primary focus of this document, as carbapenem resistance genes may be spread via effluents (Picao et al., 2013; Zurfluh et al., 2013) the inclusion of vegetables in targeted surveys could be considered.

EFSA (2012a) recommended additional AMR monitoring in imported food from countries outside the EU. In the absence of specific data on CNS bacteria, and because of the high prevalence of cephalosporin resistance, priority should be given to poultry meat imported from countries outside the EU (EFSA, 2012a). Other types of imported food which may be of special relevance are fishery and aquaculture products, as well as vegetables and fruits, usually consumed raw. Finally, with regard to passive monitoring, isolates of Enterobacteriaceae and *Acinetobacter* spp. from all animal and food isolates should be included into the carbapenem resistance monitoring.

4.4. Analytical methods in routine monitoring

The current procedure of testing a randomly selected commensal *E. coli* or *Salmonella* spp. isolate recovered from non-selective media provides continuity with previous routine monitoring recommendations (EFSA, 2012a). Inclusion of meropenem into the test panel will provide continuous information on the possible spread of the resistance pattern.

More specific methods involving pre-enrichment and selective plating should be used in targeted surveys to increase sensitivity for populations with a very low prevalence of CP microorganisms. Current knowledge on the available methods has been summarised in Chapter 3.

The recommendations developed by EUCAST to improve the detection of different mechanisms of resistance, including those conferring resistance to carbapenems, should be taken into account to the best extent possible. Additionally, consideration should be given to a more detailed analysis of all isolates showing non-susceptibility to carbapenems above the ECOFF value.

4.5. Sampling design

The principles, already fixed for routine monitoring aiming to estimate the prevalence of AMR in the studied populations and changes thereof, apply also for active monitoring for carbapenem resistance. A randomised sampling strategy should be used for active monitoring programmes and targeted surveys which ensures the representativeness of the entire population (EFSA, 2012a) and facilitates estimation of the magnitude of the problem. Use should be made of the principles already described for routine AMR monitoring in *Salmonella* spp. and *E. coli* (EFSA, 2012a). This includes the selection of the epidemiological units, the stage of sampling, the type of sample and the selection of isolates for susceptibility testing. One sample for each epidemiological unit should be used for the targeted study using selective media.

As regards CP, additionally a risk-based approach should be used involving all isolates from diagnostic submissions showing cephalosporin resistance. Isolates from animals, previously treated with antimicrobials should not be excluded from testing but information should be recorded.

Furthermore, animal pathogens should be continuously screened for cephalosporin resistance and those isolates showing results above the ECOFF value should be subject to further testing for CP and more detailed characterisation and classification.

As regards frequency of sampling, annual sampling would be optimal, but more intensive sampling every second or third year was considered an option (EFSA, 2012a). As foreseen in Community legislation, active monitoring should be performed in the major food producing species every second year involving phenotypic testing for meropenem resistance. This should be complemented by a targeted survey searching with sensitive methods for CPE.

Targeted studies in other populations of special interest, including for example the breeding level for broilers and turkeys, should be run at least once and repeated if new information becomes available, e.g. from the production level, passive monitoring or other research activities.

4.6. Sample size calculation

Considerations for sample size calculations have been described in previous EFSA recommendations (EFSA, 2007, 2008, 2012a) and are not repeated here. In the current community legislation, a sample size of 170 randomly selected isolates is fixed for detecting trends in changes of prevalence over time. For the specific monitoring for ESBL/AmpC/carbapenemase-producing *Salmonella* spp. and *E. coli*, 300 samples (one from each epidemiological unit) should be tested using more sensitive methods (Commission Implementing Decision 2013/652/EU).

As regards carbapenem resistance, sample size calculation has to focus on the detection of this new resistance pattern and has to take into account the impact of imperfect tests. Two scenarios are discussed below:

- collection of a reasonable number of random isolates for routine screening for phenotypic resistance;
- testing of samples with selective media for the detection of the resistance above a certain prevalence level within an epidemiological unit (e.g. a flock) or population (e.g. broilers).

4.6.1. Sample size for detecting a certain prevalence level within the isolates collected

The number of biological samples to be collected from each animal population in order to achieve 170 isolates depends on the prevalence of the bacteria species monitored. Whereas for commensal bacteria this will be achieved by active monitoring of random samples, for other bacterial species targeted investigations are necessary. For feasibility reasons, a passive surveillance scheme may be implemented using isolates deriving from diagnostic testing or surveillance activities. At least 170 isolates from each *E. coli*, *Salmonella* spp. (if available) and other members of the Enterobacteriaceae family should be collected from each animal population and production type.

No screening system is available which will detect all CP isolates, and most probably those showing non-susceptibility to meropenem will be detected. Testing of 170 isolates would allow detection of at least one positive isolate, given a prevalence of around 2% within the isolates tested (95% C.I.). Testing of more isolates would be desirable, since it would allow the detection of positive isolates in conditions of a lower within-isolates prevalence level.

4.6.2. Sample size for the detecting a hazard in a population with a certain prevalence level

As currently only few findings of CP bacteria in livestock have been reported, targeted surveys may be designed with the purpose of confirming that the prevalence of CP bacteria is likely not above a certain level.

For example, when using a test with 90% sensitivity and 100% specificity, and assuming an underlying prevalence of 2%, the detection of at least one positive sample would require testing of 165 samples. In a similar scenario, using a test with a reduced sensitivity of 80% would require increasing the number of samples tested to 186 samples.

If the prevalence was lower (1% or 0.5%), using a test with 90% sensitivity and 100% specificity would require testing of respectively 332 or 665 samples in order to detect one positive sample. The number of samples required would further increase to 665 or 748 samples if the sensitivity of the test was reduced to 80%.

Similar considerations can be applied to detect the presence of a CP isolate within a farm or flock (as we assume that the within flock prevalence may be low), which highlights that testing of several pooled samples from the same epidemiological unit would increase the overall sensitivity.

4.7. Recommendations on data collection and reporting of data

There are already comprehensive requirements for the data collection and reporting of AMR data laid down in Directive 2003/99/EC, in the technical specifications and reporting manuals prepared by EFSA (EFSA, 2012a)) recommending reporting of isolate based data.

This type of reporting has to be developed further to cover results of testing of animal pathogens from passive monitoring activities as this is currently not covered by community legislation or EFSA recommendations. For isolates from diagnostic submissions, information on antimicrobial treatment before sampling should be provided.

All isolates with phenotypic CNS should be further analysed in the National Reference Laboratory and reporting of data should include these confirmatory results.

As regards reporting on CNS, for correct interpretation of the results it is important that the sampling strategy (active vs. passive monitoring) and the selection procedure for each isolate (randomly selected isolate vs. isolate from selective media) applied are reported. In cases where selective methods have been used, such methods should be described in detail. Results of phenotypic resistance testing for cephalosporins and carbapenems, synergy testing as well as further characterisation of resistance genes should be reported for each isolate.

4.8. Summary and conclusions

- Requirements for the collection and reporting of antimicrobial resistance data, including resistance to carbapenems, are laid down in European legislation.

- Technical specifications and reporting manuals prepared by EFSA recommend reporting of isolate-based data, and recommend mandatory phenotypic monitoring of *Salmonella* spp. and indicator *E. coli* with a broadened test panel also covering meropenem.

- Since some carbapenemase-producing strains have been identified in food animals and their environment, more detailed investigation is now required to determine the extent and distribution of such strains in the food animal ecosystem.

- Active monitoring and/or additional targeted surveys for carbapenemase-producing bacteria in animals and food should cover key zoonotic agents and indicator organisms of the commensal flora. Priority should be given to broilers, fattening turkeys, fattening pigs, veal calves, and the derived fresh meat of domestic origin. Dairy cattle, raw milk and aquaculture products may be also included in targeted surveys.

- For active monitoring, all isolates of *Salmonella* spp. and *E. coli* collected within the compulsory monitoring programme, as required by European legislation, should be screened for meropenem resistance using standardized microdilution methods.

- Specific targeted surveys for the detection of carbapenemase-producing organisms in the food animal ecosystem should be implemented at the EU level.

- For passive monitoring, diagnostic isolates of veterinary origin (at least those classified as microbiologically resistant to 3rd- or 4th-generation cephalosporins on the basis of epidemiological cut-off values) should be subjected to phenotypic testing for carbapenem resistance and carbapenemase production, and subsequent molecular identification and characterization of the carbapenemase production genes present.

- For correct interpretation of results relating to carbapenem resistance, the sampling strategy (active *vs.* passive monitoring) and the selection procedure applied for each isolate (randomly selected isolate vs. isolate from selective media) should be reported. Results of phenotypic methods used for testing for carbapenemase production as well as the results from further characterisation of resistance genes should be reported for each isolate.

- Methods involving pre-enrichment and selective plating should be used in specific surveys to increase sensitivity for populations with a low prevalence of carbapenemase-producing microorganisms.

- The recommendations developed by EUCAST to improve the detection of different mechanisms of resistance, including those conferring resistance to carbapenems, should be taken into account to the best extent possible. Additional consideration should be given to a more detailed analysis of all isolates showing non-susceptibility to carbapenems above the ECOFF value.

5. Possible control options for preventing or minimising the further emergence and spread of carbapenemase-producing bacterial strains transmitted *via* the food chain.

5.1. Introduction

Public health risks caused by CP bacteria are primarily determined by (i) the frequency of the occurrence (prevalence) and the quantity of these organisms in food-producing animals and food, (ii) the genetic characteristics of the carbapenemase genes involved, and (iii) the frequency and magnitude of transmission from animals/foods to humans. In the first instance proposed mitigation measures must therefore be targeted at preventing the introduction of such strains into food-producing animals, secondly, at reducing the prevalence and quantity of such organisms in food-producing animals and foods thereof, and thirdly, in reducing their transmission from contaminated animals/foods to humans.

In contrast to possible control options to reduce the public health risk caused by the transmission through the food chain or *via* the food-producing animal environment of ESBL- and/or AmpC-producing bacterial strains to humans (EFSA Panel on Biological Hazards (BIOHAZ), 2011; Liebana et al., 2013), the impact of similar measures for CP bacteria are difficult, if not impossible, to assess. This is primarily because, as discussed above, CP microorganisms are only just emerging in food-producing animals and their environment, and the prevalence of such strains in the food chain is supposedly very low.

In view of the importance afforded to carbapenems in the treatment of human infections with multiple drug-resistant or pan drug-resistant pathogens in health care settings or the community (see above), the control options discussed below should be considered.

5.2. Use of carbapenems and other antimicrobials in food-producing animals and human medicine

All efforts should be made to continue to regard carbapenems as Critically-Important Antimicrobials (CIAs) (WHO, 2005, 2007, 2011, 2012), that should be reserved specifically for the treatment of severe MDR disease in humans, and not used in food-producing animals.

At present carbapenem antibiotics are not licensed for use in food-producing animals in the EU, North America, and Australasia. Information on the permitted use of such antibiotics in food-producing animals in third countries is not readily available. In any event, the use of such antibiotics in food animals globally should be actively discouraged.

Mirroring what has been observed with ESBL-encoding genes, carbapenemase genes are often located on genetic structures (plasmids, transposons, integrons) bearing genes conferring resistance to other antibiotics, co-selection both in animals and in the environment is a real possibility. In addition, co-resistance is an important issue in CP microorganisms, and carbapenemases are mostly plasmid-encoded (with several antibiotic resistance genes co-located on such plasmids).

The importance of the prudent use of antibiotics in combating the development of resistance to antimicrobials in food-producing animals has been well-documented (Anthony et al., 2003; ECDC et al., 2009; EFSA Panel on Biological Hazards (BIOHAZ), 2011). Decreasing the frequency of use of antibiotics in animals is currently being afforded high priority in numerous fora. Efforts to minimise overall antibiotic usage, in particular of cephalosporins, in food-producing animals must be actively continued. This is also important in relation to carbapenem resistance in light of the co-resistance issues documented above.

Recommendations for reducing total antimicrobial use in animals have been made previously (EFSA Panel on Biological Hazards (BIOHAZ), 2011), and should be followed in accordance with prudent use guidelines. Furthermore, since carbapenemase production genes and genes encoding resistance to certain heavy metals such as zinc are sometimes linked, the use of compounds containing such elements should also be minimised.

5.3. Containment measures

Strains of bacteria exhibiting resistance to antibiotics and the resistance genes therein can spread from the hospital environment to the animal population by a variety of routes – waste-water, human/animal contact, etc. Measures addressing such routes of transmission, and which would be able to minimise the potential spill-over of CP organisms from humans to food-producing animals are therefore particularly important.

Control measures to contain the spread of CP bacteria in food-producing animals should be proactively implemented at both national and international levels, and should involve inter-departmental communication between human and veterinary authorities. Such plans should be agreed to prevent CP strains become widespread in livestock.

As an example, containment plans were formulated in several countries in the EU in the early 2000s to combat the possible introduction of AmpC-producing *Salmonella* Newport into the EU from the USA. Such plans included restrictions on the index case farm, intensive investigations of all trading links and contiguous premises, and risk management decisions on compensation. If necessary such plans could be revisited in anticipation of the appearance of CP strains in livestock.

Measures for the containment of CP organisms at the farm level may range from identification and isolation of carriers, animal quarantine through to destruction of infected flocks / herds. Restrictions in the movement of personnel (farm staff, veterinarians, produce delivery staff, etc.) between farms, implementing increased farm biosecurity, controls on animal trade (of CP carriers) and trade of animal by-products, or by improving hygiene throughout the food chain (directing products to heat treatment; specific harvesting requirements; implementing general post-harvest controls for food-borne pathogens) may also be considered.

5.4. Measures after targeted investigations (root-cause analysis)

Where the presence of a CP strain is confirmed, more detailed epidemiological investigations should be started immediately, as long as findings are rare events. Where possible such activities should be

targeted at identifying the point of introduction of the CP strain, the level of spread which has occurred within a farm, between farms, their environment and the staff. Farms from which animals have been received should be included in the analysis. In case of findings in foodstuffs, the farms from which these had originated should be included into the targeted search.

The most sensitive methods should be used for all investigations and isolates showing CP, and should be targeted for detailed genotypic comparison.

In cases where containment measures are taken as recommended in chapter 5.3., targeted surveys should be started to verify the efficiency of the measures taken.

Because of the importance of carbapenems in human medicine, should resistance to such antibiotics be identified in bacteria from food-producing animals in targeted surveys, then specific containment measures, such as the ones described in Chapter 5.3 above, may be considered.

5.5. Summary and conclusions

- Because there are no data on the comparative efficacy of individual control options in reducing the potential public health risks caused by carbapenemase-producing bacteria related to food-producing animals, prioritisation is complex.

- All efforts should be made to continue to regard carbapenems as Critically-Important Antimicrobials that should be reserved specifically for the treatment of serious infections with multidrug-resistant bacteria in humans, and not used in food-producing animals.

- As carbapenems are not licensed for use in food-producing animals in the EU and other parts of the world, one simple and effective control option to minimise the further emergence and possible spread of such strains transmitted *via* the food chain would be to continue to prohibit the use of carbapenems in food-producing animals.

- As already stated for reducing ESBL/AmpC resistance, restriction of usage of cephalosporins/ systemically active 3rd/4th generation cephalosporins to very specific circumstances and prohibition of off-label use might be a highly effective control option to reduce selection pressure.

- As long as findings of carbapenemase-producing strains are rare events in food-producing animals, each positive finding should be thoroughly investigated.

- As genes encoding carbapenemase production are mostly plasmid-mediated, and co-resistance may be an important issue in the spread of such plasmid-mediated resistance mechanisms, decreasing the frequency of use of antimicrobials in animal production in the EU in accordance with prudent use guidelines is also of high priority.

- The effectiveness of any control measures should be monitored on a regular basis by targeted surveys of food-producing animals and foods for carbapenemase-producing bacteria, using selective isolation methods and pre-enrichment of samples as necessary.

- Control measures to contain the spread of carbapenemase-producing bacteria in food-producing animals should be proactively implemented at national and international levels, and should involve inter-departmental communication between human and veterinary authorities. Such plans should be agreed to prevent carbapenemase-producing strains become widespread in livestock.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

General conclusions

- Carbapenems are broad-spectrum beta (β)-lactam antimicrobials mostly used for the treatment of serious infections in humans, frequently in hospitalised patients, and are considered last-line therapy for infections caused by multidrug-resistant Gram-negative bacteria.

- Production of carbapenemases may confer diverse β-lactam resistance phenotypes depending on a variety of factors such as the bacterial species, variant of the enzyme, expression level due to different promoters, copies of carbapenemase-encoding genes and the presence of additional non-enzymatic resistance mechanisms.

- Carbapenem MICs may vary widely ranging from full susceptibility to high-level resistance according to CLSI or EUCAST clinical breakpoints. Low levels of resistance to carbapenems are more often observed among Enterobacteriacae.

- A diversity of acquired genes encoding carbapenem-hydrolyzing ß-lactamases has been identified. Such enzymes confer reduced susceptibility or resistance to almost all ß-lactams, including carbapenems.

- The emergence and spread of carbapenem-non-susceptible Enterobacteriaceae and carbapenemase-producing Enterobacteriacae have been identified as major public health threats.

- Many carbapenemase-producing strains frequently carry additional resistance determinants to other non-β-lactam antibiotics, making these organisms 'extensively drug-resistant' or 'pan drug-resistant'.

- The focus of this Scientific Opinion is on carbapenemase-producing bacteria with acquired genetic elements encoding carbapenemase production and that could be transferred from food-producing animals (and derived foods) to humans directly, or indirectly through other pathways.

- Factors that favour the emergence of carbapenem resistance include the increased consumption of carbapenems in humans driven in part by the worldwide spread of ESBLs in Enterobacteriacae, the location of carbapenem-encoding resistance genes on mobile genetic elements, and positive selection due to co-resistance with other commonly-used antibiotics.

Answer to ToR1 and ToR2. Define the carbapenemase-producing bacterial strains and genes relevant for public health and linked to food-producing animals or food-borne transmission. Review the information on the epidemiology of acquired resistance to carbapenems, including the genes coding for such resistance, in food-producing animals and food.

- Harmonised monitoring of carbapenemase-producing bacteria in food-producing animals has as yet not been undertaken in the EU. EU-wide validated data on the occurrence of such resistance are therefore not available.

- Transmission of carbapenemase-producing strains and/or resistance genes to humans through the food animal production environment or food chain has as yet not been reported, but is considered likely should these strains/genes spread more widely in food-producing animals.

- Few studies have reported the presence of carbapenemase-producing bacteria in food-producing animals and their environment. Genes identified have included *bla*$_{VIM-1}$ in Enterobacteriaceae and *bla*$_{NDM-1}$ and *bla*$_{OXA-23}$ in *Acinetobacter* spp. No such carbapenemase-producing isolates have been identified in food derived from food-producing animals.

- Carbapenemase-producing isolates of *Acinetobacter* spp. have been detected on a dairy cattle farm in France, in chickens on farms and pigs at slaughterhouse in China, and in horses in Belgium. The carbapenemase-encoding genes identified included *bla*$_{NDM-1}$ and *bla*$_{OXA-23}$.

- Carbapenemase-producing isolates of putative *Salmonella* Infantis have been identified in food-producing animals (pig faeces) and their environment (pig and broiler farms) in Germany. The isolates carried both an acquired AmpC-encoding gene *bla*$_{ACC-1}$ and an acquired carbapenemase-encoding gene *bla*$_{VIM-1}$. *E. coli* producing VIM-1 carbapenemase was also identified in one of these pig farms (pig faeces and environment). These AmpC-encoding and carbapenemase-encoding genes were located on IncHI2 plasmids.

- Carbapenemase-producing isolates belonging to *Salmonella* Cubana, *S.* Kentucky, *S.* Saintpaul, *S.* Senftenberg and *S.* Westhampton producing KPC-2, VIM-2, OXA-48 or NDM-1 have been recovered from cases of human infection, although the origin of the genes (animal, environmental or clinical settings) cannot be determined. All these serovars are regarded as potentially zoonotic in origin.

- Carbapenemase-producing isolates from companion animals and from a wild bird have also been reported. Genes identified have included *bla*$_{OXA-48}$ and *bla*$_{NDM-1}$ in Enterobacteriaceae and *bla*$_{OXA-23}$ in *Acinetobacter* spp.

- A wide variety of antibiotic resistance-encoding genes, including genes mediating resistance to a range of β-lactam-antibiotics, have been co-located on carbapenemase-encoding plasmids belonging to different incompatibility groups.

Answer to ToR3. Perform a critical analysis of the methods (phenotypic and genotypic) and the interpretive criteria currently used for detection (isolation and identification) and characterisation of carbapenemase-producing bacterial strains.

- A methodology including selective culture is proposed for the detection of carbapenemase-producing strains of Enterobacteriaceae and *Acinetobacter* spp.

- Pre-enrichment by incubation of samples in selective broth containing a carbapenem at a low concentration (e.g. meropenem 0.125 mg/L) may increase sensitivity. This methodology has not yet been validated, and any method proposed would have to be subjected to thorough experimental verification.

- A variety of in-house and commercially-available selective media has been used for the active surveillance of carbapenem resistance in hospitals. The choice of the media for testing animal and food samples needs to be experimentally evaluated and validated.

- Meropenem offers a good balance between sensitivity and specificity and has been recommended to be included in the harmonised antimicrobial panel for the surveillance of AMR in isolates from food-producing animals, food thereof and environmental samples.

- Biochemical and phenotypic tests for the confirmatory identification of carbapenemase-producing bacteria among isolates exhibiting non-susceptibility to carbapenems are available. The sensitivity and specificity of these assays may vary considerably in different settings.

- The identity of the genes responsible for the carbapenemase production should be determined by molecular methods.

- Plasmid and strain typing should be undertaken to acquire better knowledge on the epidemiology of genes encoding carbapenemase production among bacteria from food-producing animal populations, food thereof and environmental samples.

Answer to ToR4. Make recommendations for the harmonised monitoring and reporting of resistance (phenotypic and genotypic) caused by carbapenemases in food and food-producing animals in the EU.

- Requirements for the collection and reporting of antimicrobial resistance data, including resistance to carbapenems, are laid down in European legislation.

- Technical specifications and reporting manuals prepared by EFSA recommend reporting of isolate-based data, and recommend mandatory phenotypic monitoring of *Salmonella* spp. and indicator *E. coli* with a broadened test panel also covering meropenem.

- Since some carbapenemase-producing strains have been identified in food animals and their environment, more detailed investigation is now required to determine the extent and distribution of such strains in the food animal ecosystem.

- Active monitoring and/or additional targeted surveys for carbapenemase-producing bacteria in animals and food should cover key zoonotic agents and indicator organisms of the commensal flora. Priority should be given to broilers, fattening turkeys, fattening pigs, veal calves, and the derived fresh meat of domestic origin. Dairy cattle, raw milk and aquaculture products may be also included in targeted surveys.

- For active monitoring, all isolates of *Salmonella* spp. and *E. coli* collected within the compulsory monitoring programme, as required by European legislation, should be screened for meropenem resistance using standardized microdilution methods.

- Specific targeted surveys for the detection of carbapenemase-producing organisms in the food animal ecosystem should be implemented at the EU level.

- For passive monitoring, diagnostic isolates of veterinary origin (at least those classified as microbiologically resistant to 3rd- or 4th-generation cephalosporins on the basis of epidemiological cut-off values) should be subjected to phenotypic testing for carbapenem resistance and carbapenemase production, and subsequent molecular identification and characterization of the carbapenemase production genes present.

- For correct interpretation of results relating to carbapenem resistance, the sampling strategy (active *vs.* passive monitoring) and the selection procedure applied for each isolate (randomly selected isolate vs. isolate from selective media) should be reported. Results of phenotypic methods used for testing for carbapenemase production as well as the results from further characterisation of resistance genes should be reported for each isolate.

- Methods involving pre-enrichment and selective plating should be used in specific surveys to increase sensitivity for populations with a low prevalence of carbapenemase-producing microorganisms.

- The recommendations developed by EUCAST to improve the detection of different mechanisms of resistance, including those conferring resistance to carbapenems, should be taken into account to the best extent possible. Additional consideration should be given to a

more detailed analysis of all isolates showing non-susceptibility to carbapenems above the ECOFF value.

Answer to ToR5. Identify possible means of preventing or minimising the further emergence and spread of carbapenemase-producing bacterial strains transmitted *via* the food chain, including consideration of the advantages and disadvantages of different options.

- Because there are no data on the comparative efficacy of individual control options in reducing the potential public health risks caused by carbapenemase-producing bacteria related to food-producing animals, prioritisation is complex.

- All efforts should be made to continue to regard carbapenems as Critically-Important Antimicrobials that should be reserved specifically for the treatment of serious infections with multidrug-resistant bacteria in humans, and not used in food-producing animals.

- As carbapenems are not licensed for use in food-producing animals in the EU and other parts of the world, one simple and effective control option to minimise the further emergence and possible spread of such strains transmitted *via* the food chain would be to continue to prohibit the use of carbapenems in food-producing animals.

- As already stated for reducing ESBL/AmpC resistance, restriction of usage of cephalosporins/ systemically active $3^{rd}/4^{th}$ generation cephalosporins to very specific circumstances and prohibition of off-label use might be a highly effective control option to reduce selection pressure.

- As long as findings of carbapenemase-producing strains are rare events in food-producing animals, each positive finding should be thoroughly investigated.

- As genes encoding carbapenemase production are mostly plasmid-mediated, and co-resistance may be an important issue in the spread of such plasmid-mediated resistance mechanisms, decreasing the frequency of use of antimicrobials in animal production in the EU in accordance with prudent use guidelines is also of high priority.

- The effectiveness of any control measures should be monitored on a regular basis by targeted surveys of food-producing animals and foods for carbapenemase-producing bacteria, using selective isolation methods and pre-enrichment of samples as necessary.

- Control measures to contain the spread of carbapenemase-producing bacteria in food-producing animals should be proactively implemented at national and international levels, and should involve inter-departmental communication between human and veterinary authorities. Such plans should be agreed to prevent carbapenemase-producing strains become widespread in livestock.

ADDITIONAL RECOMMENDATION

- Fruit and vegetables, particularly those which are more prone to bacterial contamination and are usually consumed raw, should be assessed for contamination with bacteria with acquired carbapenemases.

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ABBREVIATIONS

AMR	Antimicrobial drug resistance
BIOHAZ	Biological Hazards (EFSA Panel on)
BIOMO	Biological Monitoring (EFSA Unit on)
BSI	Bloodstream infections
CIAs	Critically-Important Antimicrobials
CDC	Centers for Disease Control and Prevention (USA)
CDT	Combined Disk Test
CHDLs	Carbapenem hydrolysing class D β-lactamases
CLSI	Clinical Laboratory Standards Institute (USA)
CNS	Carbapenem-non-susceptible / carbapenem non-susceptibility
CNSE	Carbapenem-non-susceptible Enterobacteriaceae
CP	Carbapenemase-producing / carbapenemase production
CPE	Carbapenemase-producing Enterobacteriaceae
CRE	Carbapenem-resistant Enterobacteriaceae
CSF	Cerebrospinal fluid
EARS-Net	European Antibiotic Resistance Surveillance System
ECDC	European Centre for Disease Prevention and Control
ECOFF	Epidemiological cut-off value
EFSA	European Food Safety Authority
EMA	European Medicines Agency
ESBL	Extended Spectrum Beta (β)-Lactamase
EU	European Union
EUCAST	European Union Committee for Antimicrobial Susceptibility Testing
FTIR-ATR	Fourier transform infrared spectroscopy with attenuated total reflectance
Inc	Incompatibility group
IS	Insertion sequence
KPC	'*Klebsiella pneumonia*e carbapenemase'
MALDI-Tof	Matrix-assisted laser desorption/ionization (MALDI)- Time-of-flight mass spectrometry (Tof)
MBL	Metallo β-lactamase
MIC	Minimal Inhibitory Concentration
MDR	Multidrug-resistant
MGE	Mobile genetic elements
MLST	Multi locus sequence typing
MLVA	Multiple-locus variable number tandem repeat analysis
MS(s)	Member State(s)
MYSTIC	Meropenem Yearly Susceptibility Test Information Collection Program (USA)
NHSN	National Nosocomial National Healthcare Safety Network
NNIS	National Nosocomial Infection Surveillance System
OXA	Oxacillinases
PBP	Penicillin binding protein
PDR	Pan drug-resistant

PK/PD	Pharmacokinetic/Pharmacodynamic (modelling)
PFGE	Pulsed field gel electrophoresis
PCR	Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism
ST	Sequence type
TSN	Surveillance Network-USA
WGS	Whole Genome Sequencing
WWTP	Wastewater treatment plant
XDR	Extensively drug-resistant
ZD	Zone diameter