Veterinary Microbiology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Prevalence of carbapenemase producing Enterobacteriaceae isolated from German pig-fattening farms during the years 2011–2013

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ARTICLE INFO

Article history:
Received 1 September 2015
Received in revised form 20 November 2015
Accepted 21 November 2015

Keywords:
Livestock
Multidrug resistance
Beta-lactam antibiotics
CPE

ABSTRACT

Since recently Enterobacteriaceae carrying bla_{VIM-1} genes have been isolated in German animal husbandries, the monitoring of carbapenemase producing Enterobacteriaceae (CPE) in livestock became a major topic within the European Union. Nevertheless, due to missing surveillance studies the worldwide situation in livestock and livestock associated surroundings might still be underestimated. The here described study provides an overview of the CPE-prevalence in German pig-fattening farms during the years 2011–2013 (period when previously described bla_{VIM-1} findings occurred on pig-fattening farms (Efsa, 2011; Fischer et al., 2012, 2013a)). Therefore, a collection of 238 bacterial anacultures derived from pooled faeces and boot swab samples, collected in a cross-sectional study including 58 pig-fattening farms throughout Germany, were investigated.

The bacteria were selected on MacConkey agar plates containing 0.125 μg/ml meropenem. Enterobacteriaceae which were able to grow on these plates were further investigated for the presence of carbapenemase genes. Out of eight CPE-suspicious strains, two *Escherichia* (*E.*) *coli* strains—deriving from the same farm—contained the carbapenemase gene bla_{VIM-1}. For the remaining six Enterobacteriaceae it seems to be likely that they possess other resistance mechanisms, leading to reduced carbapenem susceptibility. Based on the obtained results, the overall CPE prevalence for German pig-fattening farms, sampled during the years 2011–2013 was 1.7%; 95% CI: 0–10. However, as it is of great importance to prevent a further spread of these bacteria between farms and livestock populations as well as their introduction into the food chain, an understanding of their routes of introduction and spread in combination with intensified monitoring programs are considered necessary.

1. Introduction

Worldwide increasing numbers of multidrug resistant Enterobacteriaceae lead to growing problems in infection control (Nordmann et al., 2011,2012a). Especially the global spread of bacteria displaying carbapenem resistance is of great concern. Carbapenems belong to the group of broad-spectrum beta-lactam antibiotics. They are considered as one of the most critically important drugs for the treatment of serious infections in humans (WHO, 2011). Formerly, solely known from cases of human infection, first reports recently described the occurrence of carbapenem resistant Enterobacteriaceae in livestock as well as wildlife and pets (Fischer et al., 2012; Efsa, 2013; Fischer et al., 2013a,b; Stolle et al., 2013). These findings raised the question whether carbapenem resistant bacteria are more widely spread among German livestock populations.

When talking about carbapenem resistance it has to be considered that different kinds of mechanisms can lead to this phenotype. In case of the Gram-negative Enterobacteriaceae carbapenem resistance or decreased carbapenem susceptibility have been associated with the expression of carbapenemase genes (Queenan and Bush, 2007), the expression of efflux-pumps (Bornet et al., 2003) or a combinations of the production of an extended-spectrum beta-lactamase-/Amp C-type beta-lactamase associated with outer membrane permeability defects (loss/modification of outer membrane proteins) (Nordmann et al., 2012a). However, in the context of infection control, the presence and spread of carbapenemase genes is the most crucial mechanism. Resultant enzymes possess the ability to hydrolyse almost all beta-lactam

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http://dx.doi.org/10.1016/j.vetmic.2015.11.030
0378-1135/

antibiotics and therefore, their occurrence dramatically limits the therapeutic options. In addition most of the enzymes are not inhibited by currently available beta-lactamase inhibitors (Queenan and Bush, 2007). Given that most of the resistance genes are located on plasmids or are associated with transposable elements or integrons, the spread among different bacterial species is possible. Whereas in clinical isolates from human infections a wide variety of carbapenemases has been detected, due to a lack of bigger surveillance studies, the current frequency of their occurrence in livestock and livestock associated surroundings is unknown. Nevertheless, investigations within the last years indicated the presence of carbapenemase producing *E. coli* and *S. enterica* in livestock and the farm surrounding (Fischer et al., 2012, 2013a). The therein detected metallo-beta-lactamase gene bla_{VIM-1} was included in the variable region of a class 1 integron, located on either a 220 or 300 kb IncHI2 plasmid. This localisation supports the potential of an increased carbapenemase-gene spread throughout the bacterial population.

To get more information about the number of CPE-positive pig-fattening farms during the years 2011 to 2013, 238 stored mixed bacterial cultures were systematically screened for the presence of carbapenem resistant Enterobacteriaceae. Due to the fact that the here described study was based on samples, taken during the first period of the national RESET project (www.reset-verbund.de), also primary cultures from those pig-farms where Fischer et al. described previous bla_{VIM-1} findings (Fischer et al., 2012,2013a), were a part of the investigated study population. Never the less, it has to be pointed out that the investigations described by Fischer et al., were based on isolates which were picked for ESBL/AmpC confirmation in the first instance and due to the observation of a noticeable resistance pattern, some of the isolates were subsequently identified as carbapenemase producers (Fischer et al., 2012). Therefore, the previously VIM-positive tested pig-farms depict the only intersection of the here and by Fischer et al. described study.

2. Material and methods

2.1. Selection of bacterial isolates

Overall, 238 of the primary, mixed bacterial cultures isolated from pooled faeces and boot swab samples, taken from 58 pig-fattening farms throughout Germany, were investigated. All of the samples had been taken in terms of the first period of the national RESET project (www.reset-verbund.de), which was carried out during the years 2011–2013 (Hering et al., 2014; von Salviati et al., 2014). One of the major topics within this project was the estimation of the prevalence of ESBL- and AmpC-producing Enterobacteriaceae in German livestock farms. The sampling and processing of the material in the laboratory was described previously (Hering et al., 2014; von Salviati et al., 2014). Briefly, several samples (e.g., faeces, boot swab) were taken from each farm and selectively cultivated on MacConkey agar plates containing 1 μg/ml cefotaxim. Mixed sets of bacteria which were able to grow on these plates (primary anacultures) were stored at −80 °C for further investigations. As carbapenem resistance is often associated with resistance against the newer antibiotics like 3rd and 4th generation of cephalosporins, the collected anacultures performed a good basis for the adjacent screening.

2.2. Detection of isolates possessing reduced carbapenem susceptibility

The previously harvested bacteria were taken from the stock and re-cultured non selective in LB-broth (Luria/Miller), (Carl Roth, Karlsruhe, Germany) (37 °C, overnight). On the following day the overnight culture was spread on selective MacConkey agar plates (MacConkey agar No 3; OXOID, Hampshire, UK) containing 0.125 μg/ml meropenem, (Sigma–Aldrich, Seelze, Germany). The screening cut-off value was recommended by the European Food Safety Authority (Efsa, 2013). Bacterial species that grew on these plates were identified using MALDI-TOF mass-spectrometry (MALDI Microflex® LT and Biotyper® database, Bruker Daltronics, Bremen, Germany). As for *Proteus* spp. no unambiguous species identification was possible using MALDI-TOF MS, biochemical approaches were carried out, using the api® 20E (bioMerieux, Nuertingen, Germany) and the VITEK2 systems, version 07.01 (bioMerieux, Nuertingen, Germany). Per sample, one colony of each identified enterobacterial species was picked and further investigated by using the approaches described below.

2.3. Antimicrobial susceptibility testing

Identified Enterobacteriaceae were tested for their antimicrobial susceptibility using the disc diffusion method described by the clinical and laboratory standards institute CLSI-M02-A11(Clsi, 2012b). The following carbapenems were tested: meropenem (10 μg), imipenem (10 μg) and ertapenem (10 μg) (bestbion, Cologne, Germany).

In addition the minimal inhibitory concentration (MIC) was determined for the carbapenems meropenem and imipenem using the broth microdilution according to the protocol CLSI-M07-A9 (Clsi, 2012a). Cation-adjusted Mueller-Hinton broth was adjusted to the following carbapenem concentrations [μg/ml]: 0; 0.125; 0.25; 0.5; 1; 2; 4; 8; 16; 32 and 64 and the inoculation was performed in the microdilution format, containing 0.1 ml of broth per well. The final inoculum was adjusted to a concentration of approximately $2-8\times10^5$ CFU/ml and the plate was incubated at 37 °C for 16–20 h.

The obtained data were interpreted using the standard CLSI-M100-S24 (Clsi, 2014). In addition, the cut-off values mentioned within the *Scientific Opinion on Carbapenem resistance in food animal ecosystems* (Efsa, 2013) were considered.

2.4. Carba-NP test

All strains, which were able to grow on the meropenem containing agar plates, were tested by performing the Carba-NP test. This test represents a rapid detection method of carbapenemase-producing Enterobacteriaceae based on in vitro hydrolysis of imipenem by a bacterial lysate. The experiments were performed as previously described (Nordmann et al., 2012b).

2.5. Determination of carbapenemase genes using polymerase chain reaction (PCR)

The DNA preparation for the following PCR reactions was performed as previously described (Roschanski et al., 2014). The screening of the carbapenemase genes bla_{VIM},bla_{KPC}, bla_{NDM}, bla_{OXA-48} and bla_{GES} was performed by real-time PCR using the Roche Lightcycler 480II (Roche Diagnostics GmbH, Mannheim, Germany). The used primer-/probe- sequences for the detection of the tested carbapenemase genes were taken from previously published protocols (Swayne et al., 2011; van der Zee et al., 2014) and multiplexed in a new way. In addition the used probe-fluorophores were adjusted in accordance to the available Light-cycler channels (Table 1). Real-time amplification was performed in 25 μL reactions containing 12.5 μL ABsolute qPCR Mix (Thermo Scientific, St. Leon Roth, Germany), 1 μL of each forward and reverse primer (10 pmol), 0.1 μL NDM TaqMan probe (5 pmol), 0.2 μL of each of the other four TaqMan probes (10 pmol), 0.6 μL of

Please cite this article in press as: N. Roschanski, et al., Prevalence of carbapenemase producing Enterobacteriaceae isolated from German pig-fattening farms during the years 2011–2013, Vet. Microbiol. (2015), http://dx.doi.org/10.1016/j.vetmic.2015.11.030