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Lack of evidence so far for carbapenemase-producing *Enterobacteriaceae* in food-producing animals in Switzerland

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The increasing prevalence of *Enterobacteriaceae* that produce extended-spectrum-β-lactamases (ESBLs) undermines the efficacy of many β-lactam therapies based on penicillins and cephalosporins (Pitout and Laupland, 2008) and render carbapenems (imipenem, meropenem, ertapenem and doripenem) in human medicine crucial drugs of last resort for the treatment of infections due to multi-resistant gram-negative bacteria (Queenan and Bush, 2007). However, carbapenemase-producing *Enterobacteriaceae* have been increasingly reported worldwide (Nordmann et al., 2011), a development which is observed with great concern by the scientific and medical community and threatens to become a public health problem of global dimensions (Akova et al., 2012).

Carbapenemases are a diverse group of β-lactamases belonging to the Ambler classes A, B and D or Bush groups 2f, 3 and 2d, accordingly (Amber et al., 1991; Bush and Jacoby, 2010). Class A carbapenemases (Bush group 2f) include the serine β-lactamases NmcA, Sme, IMI-1 and SFC-1 which are chromosomally encoded, as well as the clinically common plasmid encoded KPC enzymes. Carbapenemases of this class are inhibited by clavulanic acid. Class B carbapenemases (Bush group 3) comprise the integron-encoded VIM-types, the IMP-, GIM-1, SPM-1- and SIM-types of enzymes, and the plasmid encoded NDM-1 carbapenamase. These metallo-β-lactamases are inhibited by EDTA but not by clavulanic acid. Class D (Bush group 2d) consists of OXA-48 type carbapenemases, which are plasmid encoded, and not inhibited by EDTA and not or only weakly inhibited by clavulanic acid.

Due to the heterogeneity of carbapenemases, their highly variable substrate spectra and their coexistance with other β-lactamases, the detection of carrier strains is a major technical challenge which is why the actual prevalence of carbapenemase-producers remains unknown (Nordmann et al., 2011).

Even though carbapenems are not licensed for the use in animals, carbapenem resistant bacteria, once introduced into the animal population, could be selected for by the use of other (licensed) beta-lactam antibiotics. Recent reports prove that the intestinal flora of pigs (Fischer et al., 2012) and cattle (Poirel et al., 2012) constitute a possible reservoir of carbapenemase producers. Because of possible transmission of resistance genes from livestock via the food chain into the human community, these findings are particularly alarming and constitute a crucial public health issue. As there are no novel β-lactams in development, early identification of carbapenemase producers in humans as well as in animals is of utmost importance.

Sampling

To screen for the occurrence of carbapenemase-producing *Enterobacteriaceae* in food-producing animals fecal samples were collected from March to May 2012 from 460 individual healthy food-producing animals at slaughter in Switzerland: 200 fattening pigs, 150 cattle (75 < 12 months; 75 > 12 months) and 110 sheep (97 < 12 months; 13 > 12 months). To prevent sample clustering, only one animal per farm was sampled. The farms were geographically distributed in the north western, central and north eastern part of Switzerland. Sampling was done with one swab per animal at a big slaughterhouse (on average 250 pigs per hours, 150 cattle per hour, 60 sheep per hour). Afterwards, the swabs were put into stomacher bags, transported under chilled conditions to the laboratory and processed within 3 hours. Furthermore, 99 herd-level pooled fecal samples of chicken were collected at the entry of a big poultry slaughterhouse (on average 50'000 animals per day) from the crates of 99 poultry flocks (approximately 6000 chicken per flock) distributed throughout Switzerland. These samples were sent directly from the slaughterhouse to the laboratory.

Microbiological analysis

Each sample (about 1 g) was incubated for 24 hours at 37 °C in 10 ml of EE Broth (BD, Franklin Lakes, USA) for enrichment. The enriched fecal samples (10 µl) were inoculated onto Brilliance CRE agar (Oxoid, Hampshire, UK) and incubated at 37 °C for 24 hours under aerobic

conditions. From each sample with growth on Brilliance CRE agar, all colonies with different colony morphology were picked and subcultured on sheep blood agar (Difco laboratories; 5% sheep blood, SB055, Oxoid) at 37 °C for 24 hours. Oxidase-negative isolates were thereafter subjected to identification by API ID 32 E (bioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing and PCR search for *bla*$_{\text{carbapenemase}}$ genes

The isolated strains were subjected to antimicrobial susceptibility testing on Mueller-Hinton agar plates using E-Test strips containing imipenem alone and in combination with EDTA (bioMérieux, Marcy l'Etoile, France) in order to gain minimal inhibitory concentrations (MIC) for imipenem as well as preliminary discrimination between serine and metallo-β-lactamases.

Moreover, for all strains PCR assays for detection of *bla*$_{\text{MBL}}$ genes (*bla*$_{\text{VIM}}$, *bla*$_{\text{GIM}}$ *bla*$_{\text{SPM}}$, *bla*$_{\text{SIM}}$, *bla*$_{\text{NDM-1}}$) as well as *bla*$_{\text{serine-carbapenemase}}$ genes (*bla*$_{\text{IMI}}$, *bla*$_{\text{KPC}}$ *bla*$_{\text{OXA-48}}$, *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-40-like}}$, *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-58-like}}$) were performed. DNA was extracted by a standard heat lysis protocol. Thereafter, specific primer sets (custom-synthesized by Microsynth, Balgach, Switzerland) and the PCR conditions described were used (Woodford, 2010; Nordmann et al., 2011). Positive controls were integrated. Resulting amplicons were purified using the PCR Purification Kit (QIAGEN, Courtaboeuf, France) according to the manufacturer's recommendations. Custom-sequencing was performed by Microsynth (Balgach, Switzerland) and the nucleotide and protein sequences were analyzed with Codon Code Aligner V. 3.7.1.1. For database searches the BLASTN program of NCBI (http://www.ncbi.nlm.nih.gov/blast/) was used.

Results and Discussion

Fecal samples of 200 pigs, 150 cattle, 110 sheep, and 99 pooled fecal samples from crates of 99 poultry flocks were investigated to determine the occurrence of carbapenemase-producing *Enterobacteriaceae* in food-producing animals in Switzerland. From 16 samples (3 sheep, 5 cattle and 8 poultry) colonies growing on Brilliance CRE agar were found. From these samples 20 colonies were subcultured and subjected to an oxidase-test. Sixteen out of the 20 colonies were oxidase negative and used for further identification. Three isolates turned out to be *Stenotrophomonas maltophilia*, 4 to be *Acinetobacter baumanii*, 4 to be *E. coli* and 5 to be *Citrobacter freundii*. All isolates belonging to the *Enterobacteriaceae* (*E. coli* and *C. freundii*) were selected for further characterization. Minimal inhibitory concentrations for imipenem ranged between 0.19 and 1.5 µg/ml. Moreover, E-Test strips containing imipenem in combination with EDTA were used. This double synergy test is a simple method to detect metallo-β-lactamases (MLB). None of the tested isolates produced MLB's. Moreover, PCRs for all *bla* genes tested were negative for all 9 strains.

A technical aspect worth being discussed is why these non-carbapenemase-producing isolates were able to grow on Brilliance CRE agar. Three of the four *E. coli* isolates turned out to be ESBL-producers (data not shown), which was a plausible reason for their growth capacity on Brilliance CRE agar. For the *C. freundii* strains, it could be shown that the (relatively small) amount of imipenem supplemented to the Brilliance CRE agar was responsible for an induction of their chromosomal AmpC β-lactamase. This induction gave rise to a reversible augmentation of the MIC of imipenem from 0.19 to up to 1.5 µg/ml and thus allowed growth on CRE agar. Subculturing such induced bacteria on blood agar caused their reduced susceptibility to drop back to the normal MIC level of 0.19 µg/ml.

Based on the results of this study, no evidence for the occurrence of carbapenemase-producing *Enterobacteriaceae* in food-producing animals in Switzerland needs currently to be postulated. Further studies, however, covering all regions of Switzerland are necessary to get a complete picture and to assess future trends.

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