# Occurrence and features of chromosomally encoded carbapenemases in Gram-negative bacteria in farm animals sampled at slaughterhouse level

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The emergence and dissemination of carbapenem resistance in Gram-negative bacteria, both in fermenters (*Enterobacteriaceae*) and in non-fermenters (e.g. *Acinetobacter baumannii* and *Pseudomonas aeruginosa*) has been observed since the last years to a large extend. This resistance phenotype is often due to the production of carbapenemases, members of a versatile subset of β-lactamases belonging either to Ambler class A (e.g. KPC), class B metallo-β-lactamases, (e.g. IMP, VIM and NDM) or class D (e.g. OXA-variants), and most often encoded on transmissible plasmids (Bush, 2013). Carbapenemase-producing bacteria represent an increasing threat to the efficacy of carbapenems such as imipenem, ertapenem or meropenem, that are the drugs of choice in human medicine to treat serious infections due to extended-spectrum β-lactamase (ESBL)-producing strains (Nordmann et al., 2011). Beyond clinical settings, carbapenem-resistant isolates have been detected in the fecal flora of healthy humans (Ruppé et al., 2013; Zurfluh et al., 2015a), the aquatic environment (rivers and lakes) (Zurfluh et al., 2013), vegetables and seafood originating from Asia (Zurfluh et al., 2015b; Morrison and Rubin, 2015). Since carbapenems are not licenced for use in veterinary medicine (at least in Western countries), and a recent survey reported the lack of detection of carbapenemase-producing Enterobacteriaceae among farm animals, the risk of acquisition of such organisms from food producing animals is considered to be low (Stephan et al. 2013; Poirel et al., 2014). Nevertheless, carbapenemase producers have been isolated from the livestock in Germany and France, including commensal *E. coli* producing VIM-1 (Fischer et al., 2012a) and opportunistic, as well as zoonotic pathogens such as *Acinetobacter baumanii* producing OXA-23 (Poirel et al., 2012), and *Salmonella enterica* producing VIM-1 (Fischer et al., 2012b), raising concerns about the possibility of transmission via food-producing animals.

The main focus of antibiotic resistance monitoring is on plasmid-encoded carbapenemases. However, carbapenemases were originally described as species-specific, chromosomally encoded β-lactamases (Queenan and Bush, 2007), and environmental strains have repeatedly been identified as reservoirs for progenitors of globally disseminating plasmid-encoded carbapenemases. For example, *Acinetobacter radioresistens* has been identified as the source of a chromosomal $bla_{\text{OXA-23}}$-like gene, which is currently widespread as a plasmid-encoded $bla_{\text{OXA-23}}$ in *Acinetobacter baumannii* (Poirel et al., 2008). Further, *Schewanella xiamenensis* harbours a chromosomal $bla_{\text{OXA-181}}$, which has been detected on plasmids in other species e.g. in clinical isolates of *Klebsiella pneumoniae* and in *Klebsiella variicola* isolated from food (Potron et al., 2011; Zurfluh et al., 2015). The aim of this study was to screen for the presence of carbapenemases, including naturally occurring variants, in Gram-negative bacteria in fecal samples of food-producing animals at slaughterhouse level in Switzerland, using the SUPERCARBA II selective medium (Nordmann et al., 2012).

## Sampling

Fecal samples from 83 pigs (70 fattening pigs, 12 sows and 1 boar) as well as 160 cattle (11 calves, 36 cattle, 46 cows and 67 bulls) were collected in December 2014 and January 2015 from healthy food-producing animals at two major abattoirs in Switzerland. To prevent duplicate isolation, no more than 3 samples per livestock per slaughtering batch were collected. The providing farms were geographically distributed throughout Switzerland. Samples were taken from the jejunum part of the intestine with sterile swabs, placed in stomacher bags and processed on the same day.

## Microbiological analysis

Each sample was mixed in a stomacher machine with 10 ml of E.E. broth (Ltd, Basingstoke, Hampshire, England) and incubated at 37 °C for 24 hours for enrichment. The enriched samples (10 µl) were inoculated onto SUPERCARBA II medium selecting for bacteria with reduced susceptibility to ertapenem (Nordmann et al., 2012; Zurfluh et al., 2015a) and incubated at 37 °C for 24 hours under aerobic conditions. Resulting colonies were subcultured on TSI agar (Difco™, Becton Dickinson AG, Allschwil, Switzerland) and carbapenemase production was confirmed using the RAPIDEC® CARBA NP test (bioMérieux Ltd, Geneva, Switzerland), according to the manufacturer's instructions. Species identification was performed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Mabritec AG, Riehen, Switzerland).

Antimicrobial susceptibility testing was performed using the Kirby Bauer disk diffusion method and the antibiotics ampicillin, amoxicillin/clavulanic acid, cephalotin, cefotaxime, nalidixic acid, ciprofloxacin, azithromycin, gentamicin, kanamycin, streptomycin, tetracycline, sulfamethoxazole, trimethoprim and chloramphenicol (Becton Dickinson, Heidelberg, Germany). Results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2011).

## Identification of carbapenemase genes

DNA was extracted by a standard heat lysis protocol. Thereafter, isolates were screened for the presence of the carbapenemase genes *bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ by PCR using primers (custom-synthesized by Microsynth, Balgach, Switzerland) and conditions described previously (Ellington et al., 2007; Poirel et al., 2011). *Aeromonas veronii* isolates were tested for the *imiS* gene using primers described earlier (Sánchez-Céspedes et al., 2009). The *Pseudomonas alcaligenes* isolate was analyzed for the presence of *bla*$_{PAM-1}$ (Suzuki et al., 2014) using the newly designed primers PAM-1F 5'-CGT TTC CTC GCC AGC CTC G-3' and PAM-1R 5'-CGC GAC TTT GCC TGC TCG-3'. The *Stenotrophomonas maltophilia* isolate was tested for *bla*$_{L1}$ and *bla*$_{L2}$ with primers published previously (Avison et al., 2001). Amplicons were visualized by electrophoresis with 1% agarose gels stained with ethidium bromide under ultraviolet light.

**Table 1:** Strain identities, carbapenemases and antimicrobial resistance profiles of Gram-negative bacteria isolated from cattle at slaughterhouse level in Switzerland.

| Strain ID | Species | Carbapenemase | Resistance profile |
|---|---|---|---|
| S158 | *Aeromonas veronii* | ImiS | AM |
| S194 | *Aeromonas veronii* | ImiS | AM |
| S296 | *Aeromonas veronii* | ImiS | AM |
| S300 | *Aeromonas veronii* | ImiS | AM, NA, S |
| S308 | *Aeromonas veronii* | ImiS | AM, AMC, TE |
| S173 | *Pseudomonas alcaligenes* | PAM-1 | AM, AMC, CF, CTX, S |
| S164 | *Stenotrophomonas maltophilia* | L1, L2 | AM, AMC, CF, CTX, GM, S, TMP |

Abbreviations: AM, ampicillin; AMC, amoxicillin clavulanic acid; CF, cephalothin; CTX, cefotaxime; GM, gentamicin; NA, nalidixic acid; TE, tetracycline; TMP, trimethoprim; S, streptomycin.

## Results and Discussion

Fecal samples of 83 pigs and 160 cattle were investigated for the presence of carbapenemase producers. Two (2.4%) samples from pigs and 27 (16.9%) from cattle produced growth on the modified SUPERCARBA II agar medium. Subcultured colonies were subjected to the RAPIDEC® CARBA NP test to confirm for carbapenemase production. Thereafter, seven isolates, all from cattle, were retained for further analysis. Phenotypic resistance of the remaining 22 RAPIDEC-negative isolates was attributed to synergistic mechanisms such as hyperexpression of β-lactamase combined with modifications of outer membrane proteins (OMPs) and not subjected to further analysis. Species identification and PCR screening of the carbapenemase producers revealed five *A. veronii* harbouring *bla*$_{ImiS}$, one *P. alcaligenes* containing *bla*$_{PAM-1}$ and one *S. maltophilia* containing *bla*$_{L1}$ and *bla*$_{L2}$ (Tab. 1). None of the isolates harboured acquired carbapenemase genes such as *bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{NDM}$ or *bla*$_{OXA-48}$, thereby confirming a previous report on the absence of isolates harbouring transmissible carbapenemase genes in Swiss livestock (Stephan et al., 2013). The resistance phenotypes of the isolates are summarized in Table 1. Our survey shows that carbapenemase producers may be isolated from food producing animals. The detected isolates were neither indicators of resistance, nor common food-borne pathogens (e.g. *E. coli* or *Salmonella* spp.) and therefore would not have been identified by current surveillance programs. Rather, they represent ubiquitous, opportunistically pathogenic bacteria. Nevertheless, in particular *A. veronii* has been implicated in a broad range of human infections, predominantly biliary tract infections and secondary bacteraemia (Figueras, 2005). In this species, carbapenem resistance is due to chromosomally-encoded *bla*$_{ImiS}$, which is known to be co-transcribed with other chromosomally located β-lactamases with overexpression induced in the presence of β-lactam or carbapenem antibiotics (Walsh et al., 1995). Hence, the use of penicillins or penicillin-β-lactamase inhibitor combinations in veterinary medicine could create selective pressure for naturally occurring chromosomal carbapenemases, which, in theory, could eventually be mobilised

and disseminated by genetic elements (EFSA Panel on Biological Hazards (BIOHAZ), 2013). Veterinary and human clinicians need to be aware of the fact that treatment of animals and humans with β-lactam antimicrobials may be associated with the emergence of carbapenem resistant *A. veronii*, possibly representing a threat to human health, if disseminated from animals to humans. With regard to meat processing, it is of particular interest that *Aeromonas* spp. have the ability to grow at 4 °C as well as anaerobically. Food preservation techniques such as cooling and vacuum packing may facilitate the growth of *Aeromonas* spp. (Ørmen and Østensvik, 2001). This study shows that this species represents, together with other Gram-negative bacteria such as *P. alcaligenes* or *S. maltophilia*, a reservoir for carbapenemase genes in the fecal flora of food producing animals.

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