Molecular analysis of multiresistant *Mannheimia haemolytica* isolates with particular reference to novel macrolide resistance genes and variants of the integrative and conjugative element ICE*Pmu1*

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by

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“The first gulp from the glass of natural sciences will turn you into an atheist, but at the bottom of the glass God is waiting for you.”

Werner Heisenberg
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   * both authors contributed equally to this study


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   Elevated minimum inhibitory concentrations of tildipirosin and gamithromycin among bovine *Pasteurella multocida* and *Mannheimia haemolytica* that carry the genes *erm*(42) and/or *msr*(E)-*mph*(E).

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   Do newly identified macrolide resistance genes have an effect on very recently approved macrolides?
Presentation of the complete genome sequence of Mannheimia hameolytica, a major causative agent of bovine respiratory disease.
2nd Junior Scientist-Symposium FLI, 21. – 24.08.2013, Jena, Germany, oral presentation

Analysis and comparison of the first integrative and conjugative element (ICE) of Mannheimia haemolytica, the ICEMh1.
International Pasteurellaceae Conference 2014, 13. – 16.05.2014, Prato, Italy, poster #46

Distribution of antimicrobial resistance genes via integrative and conjugative elements in Pasteurellaceae, the ICEMh1.
3rd Junior Scientist-Symposium FLI, 19. – 22.08.2014, Mariensee, Germany, poster #04

ICEMh1, the first integrative and conjugative element (ICE) of Mannheimia haemolytica.
Seventh International Conference on Antimicrobial Agents in Veterinary Medicine (AAVM), 16. – 19.09.2014, Berlin, Germany, poster
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<thead>
<tr>
<th>Abbreviation</th>
<th>Long form</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>avg</td>
<td>average</td>
</tr>
<tr>
<td>B. trehalosi</td>
<td>Bibersteinia trehalosi</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>BRD</td>
<td>bovine respiratory disease</td>
</tr>
<tr>
<td>CDS</td>
<td>coding sequences</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>et al.</td>
<td>et alii</td>
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<td>H. somni</td>
<td>Histophilus somni</td>
</tr>
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<td>IS</td>
<td>insertion sequence</td>
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<tr>
<td>ICE</td>
<td>integrative and conjugative element</td>
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<tr>
<td>kb</td>
<td>kilo base pair(s)</td>
</tr>
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<td>M. haemolytica</td>
<td>Mannheimia haemolytica</td>
</tr>
<tr>
<td>Mb</td>
<td>mega base pair(s)</td>
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<tr>
<td>MIC</td>
<td>minimal inhibitory concentration</td>
</tr>
<tr>
<td>MLSB</td>
<td>macrolides/lincosamides/streptogramin B</td>
</tr>
<tr>
<td>P. multocida</td>
<td>Pasteurella multocida</td>
</tr>
<tr>
<td>PBPs</td>
<td>penicillin-binding proteins</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RTX</td>
<td>repeats in toxin</td>
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<tr>
<td>SMRT</td>
<td>single-molecule real-time</td>
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<td>Tn</td>
<td>transposon</td>
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1. Summary (English)

Christopher Eidam

Molecular analysis of multiresistant *Mannheimia haemolytica* isolates with particular reference to novel macrolide resistance genes and variants of the integrative and conjugative element ICE*Pmu1*

For many decades *Mannheimia haemolytica* has been an important veterinary pathogen and subject of numerous studies, due to its involvement in the bovine respiratory disease (BRD) complex. However, a growing number of isolates show resistance to one or more antimicrobial agents, making the treatment of BRD increasingly difficult. The aims of this study were (i) to determine the effect of the new macrolide resistance genes *erm*(42), *msr*(E) and *mph*(E) on the latest approved macrolides gamithromycin and tildipirosin, (ii) to completely sequence the genome of *M. haemolytica* 42548, and (iii) to analyze the multiresistance-conferring integrative and conjugative element (ICE) ICE*Mh1* identified in *M. haemolytica* 42548.

In the first part of this Ph.D. project, 40 *Pasteurella multocida* and 29 *M. haemolytica* isolates that harbor the genes *erm*(42) and/or *msr*(E)-*mph*(E) or no macrolide resistance genes were screened for their minimum inhibitory concentrations (MICs) of gamithromycin and tildipirosin (CHAPTER 4). Susceptibility testing revealed, that *erm*(42) distinctly increased the MIC to tildipirosin, but had only little effect on the MIC to gamithromycin in *M. haemolytica* and *P. multocida*. However, *msr*(E)-*mph*(E) had a different effect. They caused a prominent increase in the gamithromycin MICs, but only a slight increase in the tildipirosin MICs.

In the second part of this Ph.D. project, several multiresistant, plasmid-free *M. haemolytica* isolates were screened for conserved components of an ICE structure. The *M. haemolytica* isolate 42548 was then subjected to whole genome sequencing by 454 and Illumina, including subsequent gap closure and analysis (CHAPTER 5). The genome of *M. haemolytica* 42548 has a size of 2,731,870 bp, harbors 2,888 genes, including 6 rRNA gene clusters and 61 tRNA genes. The 2,807 protein-encoding genes include 55 putative transposases, 5 antimicrobial
resistance genes and one CRISPR region of the I-C/Dvulg subtype. Additionally, an ICE, designated ICE\textit{Mh1}, was identified.

In the third part of this Ph.D. project, the first ICE from \textit{M. haemolytica}, the ICE\textit{Mh1}, was characterized and analyzed (CHAPTER 6). ICE\textit{Mh1} has a size of 92,345 bp and harbors 107 genes, including five resistance genes within two resistance gene regions of approximately 7.4 kb and 3.3 kb in size. These resistance gene regions comprise the following genes for resistance to kanamycin/neomycin (\textit{aphA1}), streptomycin (\textit{strA} and \textit{strB}), sulfonamide (\textit{sul2}) and tetracycline [\textit{tetR-tet(H)}]. The first 28,497 bp of ICE\textit{Mh1} match with a putative ICE region found in another \textit{M. haemolytica} genome, while the remaining 63,848 bp of ICE\textit{Mh1} correspond to sequences found in ICE\textit{Pmu1}. This suggests the possibility of a recombination event between a common ancestor that ICE\textit{Mh1} shares with ICE\textit{Pmu1}, and a putative ICE region of another \textit{M. haemolytica} genome. Furthermore, the mobility of ICE\textit{Mh1} could be shown by transferring the element to \textit{P. multocida} by conjugation.
2. Zusammenfassung (German)

Christopher Eidam

**Molekulare Analyse multiresistenter *Mannheimia haemolytica*-Isolate unter besonderer Berücksichtigung neuer Makrolid-Resistenzgene sowie neuer Varianten des integrativen und konjugativen Elements ICE*Pmu1*

Seit vielen Jahrzehnten ist *Mannheimia haemolytica* ein veterinärmedizinisch ausgesprochener relevanter Krankheitserreger und das Thema vieler wissenschaftlicher Studien, da der Organismus als einer der wesentlichen bakterielten Krankheitserreger boviner Atemwegsinfektionen gilt. Allerdings werden immer häufiger *M. haemolytica*-Isolate entdeckt, die gegen ein oder mehrere Antibiotika resistent sind, was die Behandlung boviner Atemwegsinfektionen erschwert. Die Ziele der vorliegenden PhD-These waren es (i) die Effekte der Makrolidresistenzgene *erm*(42), *msr*(E) und *mph*(E) auf die neusten Makrolidantibiotika Gamithromycin und Tildipirosin, zu untersuchen, (ii) das Genom von *M. haemolytica* 42548 vollständig zu sequenzieren und (iii) das in diesem Stamm fundene und Multiiresistenz vermittelnde integrative und konjugative Element (ICE) ICE*Mh1* zu analysieren.


Im zweiten Teil dieses Ph.D. Projektes wurden mehrere multiresistente, plasmidfreie *M. haemolytica*-Isolate hinsichtlich der Präsenz eines ICEs untersucht. Das *M. haemolytica*-Isolat 42548 wurde einer Gesamtgenom-Sequenzierung mittels 454 und Illumina, mit anschließendem Lückenschluss und Genomanalyse, unterzogen (KAPITEL 5). Das Genom

3. Introduction

3.1. The species *Mannheimia haemolytica*

*Mannheimia haemolytica* is a member of the family *Pasteurellaceae*, which is included in the class of Gamma-Proteobacteria (BRENNER 2005). It was formerly designated *Pasteurella haemolytica*, until 16S rRNA sequencing and DNA-DNA hybridization experiments led to the definition of the genus *Mannheimia* (ANGEN et al. 1999a). 16S rRNA sequencing is usually sufficient to determine monophyly of a genus, but might fail in distinguishing further between closely related species. However, DNA–DNA hybridization offers a more sensitive method to discriminate between species (CHRISTENSEN et al. 2007). Nevertheless, some scientists consider this method as too crude for the closely related species of the family *Pasteurellaceae* (KUHNERT and KORCZAK 2006). One possible approach is a species-specific PCR assay, like the multiplex PCR by ALEXANDER et al. (2008), which allows to distinguish between three species of *Mannheimia*, including *M. haemolytica*.

Bacteria of the species *M. haemolytica* are Gram-negative, facultative anaerobic and weakly haemolytic. Furthermore, they are non-motile and coccoid or rod shaped. *M. haemolytica* was initially divided into 17 different serotypes, which are determined using an indirect haemagglutination test (IHA) by BIBERSTEIN et al. (1960). Around the same time, SMITH (1961) described two biotypes, A and T, based on several phenotypic properties and named them according to their ability to ferment either L-arabinose or trehalose. Also, these typing methods could be associated with each other. Strains which were identified as biotype T belonged to serotypes 3, 4, 10 or 15, while all other serotypes register as biotype A. However, all strains belonging to biotype T were reclassified in 1990 as *Pasteurella trehalosi* (SNEATH and STEVENS 1990) and again in 2007 as *Bibersteinia trehalosi* (BLACKALL et al. 2007), leaving a classification into 14 serotypes of biotype A.

As with most taxa of *Pasteurellaceae*, the main habitat of *M. haemolytica* is considered to be the upper respiratory tract of ruminants (ANGEN et al. 1999b). Considered as a commensal inhabitant, it is well adapted to its specific host. However, *M. haemolytica* is also an opportunistic pathogen and can cause mild to severe respiratory tract infections. Bovine *M. haemolytica* isolates were shown to be able to bind transferrin from cattle, goats and sheep, indicating specificity for ruminants in general (YU and SCHRYVERS 1994). Also,
M. haemolytica is able to produce a leukotoxin which is considered to be a RTX homologue. The leukotoxin operon consists of four genes, \( lktCABD \), of which \( lktA \) is the toxin itself. The LktA of M. haemolytica is specific for ruminant lymphoid cells, with the highest leukotoxic activity towards bovine leukocytic cells. Furthermore, bovine isolates were found to be more likely of serotype A1, while ovine isolates are mainly classified as serotype A2 (SHEWEN and WILKIE 1982). Recent discoveries show that isolates of bovine origin nowadays mainly comprise the serotypes A1, A2 and A6 (KATSUDA et al. 2009).

3.2. Bovine respiratory disease complex

M. haemolytica is considered the major bacterial agent of the multifactorial bovine respiratory disease (BRD) complex, also called ‘shipping fever’ (RICE et al. 2007). BRD is responsible for losses of more than three billion U.S. dollars per year for the global cattle industry (WATTS and SWEENEY 2010). Primary viral infections are believed to play an important role in weakening the immune system of the host and thereby allowing for subsequent bacterial infections. Aside from M. haemolytica, Pasteurella multocida and Histophilus somni are the most important bacterial pathogens involved in BRD. Other factors promoting a BRD outbreak include stress through weaning and changes in diet or weather. But also, long distance transport and mixture with members of different herds, as it is common on US feedlot markets, can cause the shift from commensal to pathogen (EDWARDS 2010).

It is common practice to vaccinate cattle against the viral agents involved in the BRD complex, but there are also vaccinations against M. haemolytica available. Furthermore, US farmers are known to practice metaphylaxis and use antimicrobial agents, preferentially macrolides, prophylactically on high risk livestock to prevent the onset of disease. However, if preventive measures failed and a BRD-associated M. haemolytica infection is identified, the diseased animal is treated with antimicrobial agents (EDWARDS 2010). This practice, paired with the subtherapeutic use of antimicrobial agents as growth promoter in North America, might have resulted in increasing resistance rates for M. haemolytica over the years, especially in the USA and Canada.
In a ten year study, PORTIS et al. (2012) investigated the resistance rates of bacteria involved in the BRD complex in North America. In 2009, the last year of the study, 304 *M. haemolytica* isolates were tested. The tested isolates proved to be resistant to danofloxacin (18.7%), enrofloxacin (20.1%), florfenicol (8.6%), penicillin (39.1%), tetracycline (51.3%), tilmicosin (40.5%) and the triamilide tulathromycin (12.2%), but all isolates were susceptible to ceftiofur. In Germany, data on the resistance rates of pathogenic bacteria is collected by the monitoring program GERM-Vet since 2001. The latest publicly available data for *M. haemolytica* isolates is from 2009 (n=45) and revealed low resistance rates for amoxicillin/clavulanic acid (0%), ceftiofur (6.7%), cephalothin (0%), chloramphenicol (2.2%), enrofloxacin (0%), florfenicol (0%), gentamicin (0%), penicillin (28.9%), spectinomycin (0%), tetracycline (6.7%), tilmicosin (4.4%) and the triamilide tulathromycin (2.2%). However, several isolates were considered intermediate for enrofloxacin (22.2%). Furthermore, MICs were considered to be low (≤1 mg/L) for cefoperazone, cefotaxime, cefquinome, colistin, doxycycline, trimethoprim and trimethoprim/sulfamethoxazole (BVL 2012).

3.3. Antimicrobial agents and molecular basis of antimicrobial resistance

The two latest macrolides, gamithromycin (Zactran®) and tildipirosin (Zuprevo®), were approved for the treatment of BRD in 2011. Gamithromycin is a 15-membered semisynthetic macrolide of the azalide subclass. Tildipirosin is a derivative of the 16-membered ring macrolide tylosin and was specifically designed to improve efficacy against Gram-negative pathogens. A few years earlier, in 2005, the macrolide tulathromycin (Draxxin®) had been approved for the treatment and control of BRD in the European Union and in the USA. It is a tribasic 15-membered macrolide, which was the first representative of a new subclass of macrolides named ‘triamilides’ (LETAVIC et al. 2002). As distributor of Draxxin®, Zoetis (formerly Pfizer Animal Health) has monitored the susceptibility status of the respective target pathogens. Most of the tulathromycin-resistant strains that could be identified were also resistant to several other antimicrobial agents used for farm animals. One of these strains, the multiresistant *P. multocida* isolate 36950, was analyzed in detail and shown to harbor the
integrative and conjugative element ICEPmu1, conferring resistance to several classes of antimicrobial agents (MICHAEL et al. 2012a; MICHAEL et al. 2012b).

Bacteria have developed many different ways to protect themselves from macrolide antibiotics. Resistance can occur due to rRNA methylation or mutations in domains II or V of the 23S rRNA genes or by mutations in the ribosomal protein genes L4 and L22. Furthermore, resistance can be mediated by efflux pumps or genes that modify the drug (DESMOLAIZE et al. 2011). The genes mediating macrolide resistance are well studied for many organisms and are catalogued in an online database “Nomenclature Center for MLS Genes” housed at the University of Washington (http://faculty.washington.edu/marilynr/). For Pasteurellaceae, very little information on the genes mediating macrolide resistance is currently available (WATTS and SWEENEY 2010; SCHWARZ 2008). The rRNA methylases erm(A), erm(B), erm(C) and erm(F) as well as the efflux pump mef(A) were described in Haemophilus influenzae isolates from patients with cystic fibrosis (ROBERTS et al. 2011). Additionally, msr(E)-mph(E) and erm(42) were identified in several P. multocida and M. haemolytica isolates (CHAPTER 4; KLIMA et al. 2014; MICHAEL et al. 2012a). Other than that, only the point mutations A2058G and A2059G in the 23S rRNA have been reported to confer resistance to macrolides for several species (NAKAJIMA 1999).

Within the EU, tetracyclines made up 48% of all of veterinary antimicrobials sold in 2007 per kg of the biomass of food-producing animals (GRAVE et al. 2010). They have been administered in subtherapeutic doses since the 1950s in some non-European countries (LEVY 1992). Resistance to tetracyclines is mediated by more than 40 different tetracycline resistance genes, nine of which can be found in the family Pasteurellaceae (http://faculty.washington.edu/marilynr/). Seven of these tetracycline resistance genes code for membrane-associated efflux proteins, while tet(M) and tet(O) encode ribosome protective proteins. HANSEN et al. (1993) first identified tet(H) and also found it to be the predominant tetracycline resistance gene among bovine P. multocida in 1996. This tetracycline resistance gene could be shown to be located in transposon Tn5706, making it highly mobile (KEHRENBERG et al. 1998). In Table 1, all genera of the family Pasteurellaceae are listed with all currently associated tetracycline resistance genes, according to http://faculty.washington.edu/marilynr/ (last accessed: 08/04/2014).
Table 1: Linking tetracycline resistance genes to genera of the family *Pasteurellaceae*.

<table>
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<th>Genera</th>
<th>Tetracycline resistance gene(s)</th>
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<tr>
<td><em>Actinobacillus</em></td>
<td>tet (B) (H) (L) (O)</td>
</tr>
<tr>
<td><em>Aggregatibacter</em></td>
<td>tet (B)</td>
</tr>
<tr>
<td><em>Gallibacterium</em></td>
<td>tet(31) (B) (H) (K) (L)</td>
</tr>
<tr>
<td><em>Haemophilus</em></td>
<td>tet (B) (K) (M)</td>
</tr>
<tr>
<td><em>Histophilus</em></td>
<td>tet (H)</td>
</tr>
<tr>
<td><em>Mannheimia</em></td>
<td>tet (B) (G) (H) (L)</td>
</tr>
<tr>
<td><em>Pasteurella</em></td>
<td>tet (B) (D) (G) (H) (L) (M) (O)</td>
</tr>
</tbody>
</table>

Aminoglycosides, like neomycin, kanamycin and streptomycin, are commonly used in veterinary medicine as therapeutic agents and, outside of the EU, as growth promoters (SHAIKH and ALLEN 1985). Resistance to this class of antibiotics in *Pasteurellaceae* is mainly mediated by inactivation of the drug, but mutations in rRNA have also been shown to confer resistance (SCHWARZ et al. 2006). The gene most commonly associated with streptomycin resistance is *strA*, which can often be found in close proximity to *strB*. These genes code for an aminoglycoside-3’’-phosphotranspherase and an aminoglycoside-6-phosphotranspherase, respectively. However, observations have shown that *strA* is more relevant to confer the streptomycin resistance. While differently truncated *strB* genes have been seen in the presence of intact *strA* genes in streptomycin-resistant isolates, those harboring intact *strB* genes with truncated *strA* genes express no resistance to streptomycin (OJO et al. 2002). Also, there are two adenylyltransferases, *aadA1* (WU et al. 2003) and *aadA14* (KEHRENBERG et al. 2005b), as well as mutations in *rpsL* known to confer resistance to streptomycin (STUY and WALTER 1992). Furthermore, the resistance genes *aadA1* and *aadA14*, as well as mutations in 16S rRNA (O’CONNOR and DAHLBERG 2002), and *rpsE* (DAVIES et al. 1998, KEHRENBERG and SCHWARZ 2007) confer resistance to spectinomycin. Moreover, there are two genes known in *Pasteurellaceae* that mediate resistance to neomycin and kanamycin. The aminoglycoside phosphotranspherasers *aphA1* (also known as *aph(3’)-Ia*), which is usually located on the chromosome (OKA et al. 1981), and *aphA3* (also known as *aph(3’)-III*), which was initially discovered on a small plasmid (KEHRENBERG and SCHWARZ 2005b).
Sulfonamides, like sulfamethoxazole, and trimethoprim made up 17% of all of veterinary antimicrobials sold per kg of the biomass of food-producing animals within the EU in 2007 (GRAVE et al. 2010). There are only two mechanisms of resistance to sulfonamides known in *Pasteurellaceae*, the type 2 dihydropteroate synthase *sul2* and a 15 bp insertion into the dihydropteroate synthase *folP*. Aside from being commonly found on small plasmids, *sul2* is also known to be linked to the *strA-strB* operon (KEHRENBERG and SCHWARZ 2001). While *sul2* can be found in nearly all members of the family *Pasteurellaceae* (SCHWARZ 2008), the 15 bp insertion could only be detected in *H. influenzae* (ENNE et al. 2002). But only *dfrA20*, originally located on a plasmid (KEHRENBERG and SCHWARZ 2005c) and *dfrA1*, found in a partially truncated class 2 integron (KEHRENBERG and SCHWARZ 2011) are known to confer resistance to trimethoprim in *Pasteurellaceae*.

Phenicols are divided into two major groups, the non-fluorinated phenicols, like chloramphenicol, and the fluorinated phenicols, like florfenicol. For more than thirty years, chloramphenicol was widely used, before it was restricted and later banned in the EU for veterinary therapy because of its potential side-effects (VASSORT-BRUNEAU et al. 1996). The fluorinated chloramphenicol derivative, florfenicol is exclusively licensed for veterinary therapy (KEHRENBERG and SCHWARZ 2005a). There are two mechanisms of resistance to phenicols, enzymatic inactivation and efflux. Resistance to non-fluorinated phenicols is commonly due to chloramphenicol acetyltransferases encoded by the genes *catA1* to *catA3* (VASSORT-BRUNEAU et al. 1996) or *catB2* (WU et al. 2003). So far, only one resistance gene is known, to confer resistance to fluorinated phenicols in *Pasteurellaceae*, the efflux transporter *floR* (KEHRENBERG and SCHWARZ 2005a).

Beta-lactam antibiotics accounted for 16% of the sales of veterinary antimicrobial agents within the EU in 2007 (GRAVE et al. 2010). In *Pasteurellaceae*, resistance to beta-lactam antibiotics is mediated by the presence of beta-lactamases, altered low-affinity penicillin-binding proteins (PBPs) or efflux proteins. Several substitutions in alternative PBPs, which have primarily been investigated in the human pathogen *H. influenzae*, were shown to confer resistance to ampicillin (TRISTRAM et al. 2007). The *acrAB* efflux transport system is known to mediate resistance to beta-lactam antibiotics and was shown to work at an increased efficacy if its regulator *acrR* had a frame-shift mutation causing a preliminary stop (KACZMAREK et al. 2004). So far, at least five beta-lactamase genes are known to confer resistance to beta-lactam antibiotics in *Pasteurellaceae*, *blaOXA-2* (MICHAEL et al. 2012a),
bla<sub>PSE-1</sub> (WU et al. 2003), bla<sub>ROB-1</sub> (SAN MILLAN et al. 2009), bla<sub>TEM-1</sub> (NAAS et al. 2001) and bla<sub>TEM-15</sub> (TRISTRAM et al. 2008).

3.4. Integrative and conjugative elements

Integrative and conjugative elements (ICEs) are mobile genetic elements that have been found in Gram-positive and Gram-negative bacteria. They are integrated into a specific site within the host’s chromosome, which is often one end of a tRNA or a conserved gene (TOLEMAN and WALSH 2011). These elements may be confused with conjugative transposons, which lack the site specificity of an ICE. While being integrated at a specific site within a chromosome, an ICE is able to induce its own excision from the chromosome to form a circular intermediate. Subsequently, a self-encoded mating pore facilitates the transfer to a new host in a single-stranded, replicative manner, followed by the (re-)integration of the double stranded intermediates into a specific site in the chromosome (SETH-SMITH and CROUCHER 2009). This transfer mechanism is mediated by a set of core genes which are supplemented by accessory genes, like antimicrobial or heavy metal resistance genes or metabolic genes (JUHAS et al. 2007).

Tn<sub>916</sub>, from Enterococcus faecalis, which was formerly described as a conjugative transposon (GAWRON-BURKE and CLEWELL 1982), is now regarded as the first identified ICE (SETH-SMITH and CROUCHER 2009; TOLEMAN and WALSH 2011). The first ICE in Gram-negative bacteria was described in 1995 by MURPHY and PEMBROKE in a South African Providencia rettgeri isolate. It was designated R391 and harbored the aminoglycoside phosphotransferase <i>aph(3')-Ic</i>, conferring resistance to kanamycin and the <i>merCRT</i> operon, conferring resistance to mercury. Furthermore, several proteins involved in DNA mutation and repair are encoded on the R391. The ICE’s stability is probably ensured through a host addiction system, as it was found in SXT elements of Vibrio cholerae (WOZNIAK and WALDOR 2009).

A total of 363 ICEs have been described so far, of which 173 were found in Gram-positive and 187 in Gram-negative bacteria (http://db-mml.sjtu.edu.cn/ICEberg/ last accessed: 06/30/2014). Most of the ICEs found in Gram-negative bacteria are members of 3 families
(SXT/R391, pKLC102/PAGI and Tn4371), the remaining ICEs could not be grouped yet (TOLEMAN and WALSH 2011). Preceding this work, ICEs have been identified in other members of the family Pasteurellaceae, for instance in P. multocida (MICHAEL et al. 2012a and MICHAEL et al. 2012b) and H. somni (MOHD-ZAIN 2004). One of these is ICEPmu1, identified in P. multocida 36950, which has a size of 82,214 bp. The element integrates into a tRNA\textsubscript{\textsuperscript{Leu}}, thereby disrupting it, but also harbors an intact copy of tRNA\textsubscript{\textsuperscript{Leu}} to substitute the disrupted one. ICEPmu1 carries 87 additional genes, including a core region and 12 antimicrobial resistance genes. Two resistance gene regions, which were likely shaped by gene cassettes, IS elements and plasmids, harbor the antimicrobial resistance genes. Furthermore, the activity of ICEPmu1 was verified by conjugation assays and detection of the circular intermediate (MICHAEL et al. 2012a and MICHAEL et al. 2012b).

### 3.5. Aims of this Ph.D. thesis

The aims of this Ph.D. thesis were:

1. to investigate the role of the genes \textit{erm}(42), \textit{msr}(E) and \textit{mph}(E) in resistance to the most recently approved macrolides gamithromycin and tildipirosin among bovine Pasteurellaceae isolates (CHAPTER 4)

2. The molecular analysis of multiresistant \textit{M. haemolytica} isolates for conserved components of an ICE structure, followed by whole genome sequencing and gap closure to provide one of the first complete genome sequences for \textit{M. haemolytica} (CHAPTER 5)

3. to analyze the multiresistance ICEMh1, detected in the plasmid-free \textit{M. haemolytica} strain 42548, for its structure and transfer abilities (CHAPTER 6)
4. First publication

Title:

Increased MICs of gamithromycin and tildipirosin in the presence of the genes \textit{erm}(42) and \textit{msr}(E)-\textit{mph}(E) for bovine \textit{Pasteurella multocida} and \textit{Mannheimia haemolytica}.

Authors:


* both authors contributed equally to this study

Journal and Doi-no.:


10.1093/jac/dks076.

Link:

http://jac.oxfordjournals.org/content/67/6/1555.long

Abstract:

Background: Macrolides play an important role in the treatment of bovine respiratory disease (BRD). Two new macrolides have been approved for the treatment of BRD in 2011: the 15-membered macrolide gamithromycin and the 16-membered macrolide tildipirosin. The aim of this study was to determine whether the recently identified ICE\textit{Pmu1}-associated macrolide resistance genes \textit{erm}(42) and \textit{msr}(E)-\textit{mph}(E) have an effect on minimum inhibitory concentrations (MICs) of gamithromycin and tildipirosin.
Material and Methods: The genes \textit{erm}(42) and \textit{msr}(E)-\textit{mph}(E) were cloned separately and expressed in the \textit{P. multocida} recipient strain B130. These clones and the recipient strain were tested comparatively for their MICs. In addition, naturally occurring \textit{P. multocida} (n=32) and \textit{M. haemolytica} isolates (n=22) from BRD cases which carry the genes \textit{erm}(42) and/or \textit{msr}(E)-\textit{mph}(E) were tested for their MIC values of gamithromycin and tildipirosin.

Results: In the \textit{P. multocida} B130 clone carrying \textit{erm}(42), the MIC of tildipirosin increased 128-fold to 32 mg/L while that of gamithromycin increased only 16-fold to 4 mg/L. In the \textit{P. multocida} B130 clone carrying \textit{msr}(E)-\textit{mph}(E), an opposite observation was made: the MIC of tildipirosin increased only 8-fold to 2 mg/L while that of gamithromycin increased 256-fold to 64 mg/L. \textit{P. multocida} field isolates that carried all three genes showed MIC values of 16–64 mg/L for gamithromycin and 16–32 mg/L for tildipirosin while similar MIC values of 32–64 mg/L for both macrolides were seen among the \textit{M. haemolytica} field isolates. The ten \textit{P. multocida} isolates that carried only \textit{erm}(42) exhibited low MICs of 2–4 mg/L for gamithromycin but had higher MICs of 16–32 mg/L for tildipirosin. The single \textit{M. haemolytica} that harbored only \textit{erm}(42) showed MIC values of 4 mg/L and 32 mg/L for gamithromycin and tildipirosin, respectively. The two \textit{P. multocida} isolates that carried only \textit{msr}(E)-\textit{mph}(E) exhibited a high MIC of 32 mg/L for gamithromycin and a low MIC of 2 mg/L for tildipirosin.

Conclusions: The analysis of \textit{P. multocida} and \textit{M. haemolytica} field isolates from BRD cases confirmed the results obtained with the cloned \textit{erm}(42) and \textit{msr}(E)-\textit{mph}(E) amplicons. Pronounced increases in the gamithromycin MIC values were seen in the presence of \textit{msr}(E)-\textit{mph}(E) whereas distinct increases in the tildipirosin MICs were detected in the presence of \textit{erm}(42). Isolates that carry all three genes showed elevated MICs to both new macrolides.

The contribution of Christopher Eidam to the article is evaluated according to the following scale:

- A. Has contributed to collaboration (0-33%)
- B. Has contributed significantly (34-66%).
- C. Has essentially performed this part of the study independently (67-100%)
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5. Second publication

Title:
Complete genome sequence of *Mannheimia haemolytica* strain 42548 from a case of bovine respiratory disease.

Authors:

Journal and Doi-no.:
Genome announcements 2013 May 30;1(3).
10.1128/genomeA.00318-13

Link:
http://genomea.asm.org/content/1/3/e00318-13.long

Abstract:
*Mannheimia haemolytica* is the major bacterial component in the bovine respiratory disease complex, which accounts for considerable economic losses to the cattle industry worldwide. The complete genome sequence of *M. haemolytica* strain 42548 was determined. It has a size of 2.73 Mb and contains 2,888 genes, including several antibiotic resistance genes.
The contribution of Christopher Eidam to the article is evaluated according to the following scale:

A. Has contributed to collaboration (0-33%)
B. Has contributed significantly (34-66%).
C. Has essentially performed this part of the study independently (67-100%)

Design of the project including design of individual experiments: B
Performing the experimental part of the study: C
Analysis of the experiments: C
Presentation and discussion of the study in article form: C
Abstract:

Objectives: The aim of this study was to identify and analyze the first integrative and conjugative element (ICE) from *Mannheimia haemolytica*, the major bacterial component of the bovine respiratory disease (BRD) complex.

Methods: The novel ICE*Mh1* was discovered in the whole-genome sequence of *M. haemolytica* 42548 by sequence analysis and comparative genomics. Transfer of ICE*Mh1* was confirmed by conjugation into *Pasteurella multocida* recipient cells.
Results: ICE\textsubscript{Mh1} has a size of 92345 bp and harbors 107 genes. It integrates into a chromosomal tRNA\textsubscript{Leu} copy. Within two resistance gene regions of ~7.4 and 3.3 kb, ICE\textsubscript{Mh1} harbors five genes, which confer resistance to streptomycin (\textit{strA} and \textit{strB}), kanamycin/neomycin (\textit{aphA1}), tetracycline [\textit{tetR-tet(H)}] and sulphonamides (\textit{sul2}). ICE\textsubscript{Mh1} is related to the recently described ICE\textsubscript{Pmu1} and both ICEs seem to have evolved from a common ancestor. A region of ICE\textsubscript{Mh1} that is absent in ICE\textsubscript{Pmu1} was found in putative ICE regions of other \textit{M. haemolytica} genomes, suggesting a recombination event between two ICEs. ICE\textsubscript{Mh1} transfers to \textit{P. multocida} by conjugation, in which it also uses a tRNA\textsubscript{Leu} as the integration site. PCR assays and susceptibility testing confirmed the presence and activity of the ICE\textsubscript{Mh1}-associated resistance genes in the \textit{P. multocida} recipient.

Conclusions: These findings showed that ICEs, with structurally variable resistance gene regions, are present in BRD-associated \textit{Pasteurellaceae}, can easily spread across genus borders and enable the acquisition of multidrug resistance via a single horizontal gene transfer event. This poses a threat to efficient antimicrobial chemotherapy of BRD-associated bacterial pathogens.

The contribution of Christopher Eidam to the article is evaluated according to the following scale:

A. Has contributed to collaboration (0-33%)
B. Has contributed significantly (34-66%).
C. Has essentially performed this part of the study independently (67-100%)

Design of the project including design of individual experiments: C
Performing the experimental part of the study: C
Analysis of the experiments: C
Presentation and discussion of the study in article form: B
7. Forth publication

Title:
Emerging issues in antimicrobial resistance of bacteria from food-producing animals.

Authors:
Geovana Brenner Michael, Christin Freitag, Sarah Wendlandt, Christopher Eidam, Andrea T. Feßler, Graciela Volz Lopes, Kristina Kadlec and Stefan Schwarz

Journal and Doi-no.:
Future Microbiology 2015 March
10.2217/FMB.14.93

The contribution of Christopher Eidam to the article is evaluated according to the following scale:

A. Has contributed to collaboration (0-33%)
B. Has contributed significantly (34-66%).
C. Has essentially performed this part of the study independently (67-100%)

Design of the project including design of individual experiments: A
Performing the experimental part of the study: A
Analysis of the experiments: A
Presentation and discussion of the study in article form: A
Emerging issues in antimicrobial resistance of bacteria from food-producing animals

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**Key words:** ESBL, carbapenemase producers, MRSA, methicillin resistance, multi-drug resistance, ICE, new resistance genes, horizontal gene transfer

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Abstract

During the last decade, antimicrobial resistance in bacteria from food-producing animals has become a major research topic. In the present review, different emerging resistance properties related to bacteria of food-producing animals are highlighted. These include (i) extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae, (ii) carbapenemase-producing bacteria, (iii) bovine respiratory tract pathogens, such as *Pasteurella multocida* and *Mannheimia haemolytica*, which harbor the multiresistance mediating integrative and conjugative element ICEPmu1, (iv) Gram-positive and Gram-negative bacteria which carry the multiresistance gene *cfr*, and (v) the occurrence of numerous novel antimicrobial resistance genes in livestock-associated methicillin-resistant *Staphylococcus aureus*. The emergence of the aforementioned resistance properties is mainly based on the exchange of mobile genetic elements that carry the respective resistance genes.
Concern about the development and spreading of antimicrobial (multi)resistance started as early as in the 1960’s. In 1965, Anderson and collaborators [1] followed the development of multi-resistance in bovine *Salmonella* isolates and assumed that the therapeutic and prophylactic use of antimicrobial agents are “determining factors” for the emergence of (multi)resistant isolates. They also emphasized the role of antimicrobial agents in the maintenance, co-selection, vertical and horizontal spread of antimicrobial resistance among commensal and pathogenic bacteria as well as in the transfer of antimicrobial resistance genes or resistant isolates from animals to humans through the food chain. In 1969, the Swann Report on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine was released [2] and presented a first landmark in a series of recommendations on prudent use of antimicrobial agents. During the next decades, Anderson et al.’s assumptions proved to be true and almost 50 years later, the discussion about the use of antimicrobial agents in food-producing animals is still an actual and controversially debated topic [3-6].

Nowadays, it is an accepted fact that antimicrobial resistance is an ever evolving field in which the development and the use of new antimicrobial agents is usually followed sooner or later by the occurrence of bacteria that exhibit resistance to these antimicrobial agents. This applies not only to antimicrobial agents that are used in human or veterinary medicine, but also to those used in horticulture and aquaculture. The spread of antimicrobial resistant bacteria and antimicrobial resistance genes clearly illustrates how humans, animals and the environment are connected, a fact that has also been highlighted in the ‘One Health’ principle (discussed in [7]).

Bacteria present in food-producing animals play an important role as they are at the interface between human and animal health as well as human nutrition. Along the food chain, antimicrobial agents are used at the farm level to control bacterial infections. Even if used in a prudent and judicious way, every antimicrobial use provides a selection pressure under which resistant bacteria can develop and/or spread. Resistant bacteria that are present in or on food-producing animals can contaminate their carcasses during slaughter and find their way into the food chain. In this regard, bacteria resistant to ‘last resort’ antimicrobial agents and multi-to pan-resistant bacteria, that can also cause infections in humans, are of particular importance.

In the present review, five different emerging antimicrobial resistance properties are presented, which have been seen during recent years in bacteria of food-producing animals.
Although this selection mainly represents the authors’ own view on what is or may become relevant in terms of emerging resistance properties, some of these topics, e.g. extended-spectrum β-lactamase- and carbapenemase-producing Enterobacteriaceae or multiresistant livestock-associated methicillin-resistant *Staphylococcus aureus*, already form the body of national or international research consortia.
**Extended-spectrum β-lactamase (ESBL)-producing isolates**

The production of β-lactamases is one of the most important resistance mechanisms against β-lactam antibiotics in Gram-negative bacteria. Since β-lactam antibiotics play an important role in the control of bacterial infections in humans and animals, extended-spectrum β-lactamase (ESBL)-producing isolates represent risks for public health and have been associated with economic losses in livestock production. ESBLs exhibit an expanded spectrum of activity and confer resistance to important sub-classes of β-lactam antibiotics, such as penicillins (e.g. ampicillin, amoxicillin), cephalosporins (including 3rd- and 4th-generation) and monobactams by the hydrolysis of the β-lactam ring. However, they are not able to hydrolyse cephemycins or carbapenems and are inhibited by β-lactamase inhibitors such as clavulanic acid [8].

**Origins and diversity of ESBLs.** The most frequently found ESBLs belong to the families TEM, SHV and CTX-M (nomenclature of β-lactamases is reviewed in [9]), the genes of which are commonly located on plasmids. The TEM- and SHV-ESBL families arose by point mutations in the sequences of narrow-spectrum β-lactamase genes (e.g. *bla*_{TEM-1} and *bla*_{SHV-1} genes). A wide variety of β-lactamases is known and their actual nomenclature and structural relationships are available at the website of the Lahey Clinic [10]. The first ESBL, a SHV-2 variant (encoded by the gene *bla*_{SHV-2}), was found in 1983 in a human clinical *Klebsiella ozaenae* in Germany [11]. The TEM- and SHV-ESBLs were predominant until the early 2000s and were mainly associated with *K. pneumoniae* isolates of nosocomial infections. Since the first decade of the 2000s, CTX-M enzymes became the most prevalent ESBLs and *Escherichia coli* the major source of ESBLs with an additional increase in community-acquired infections. In contrast to TEM- and SHV-ESBLs, CTX-M enzymes often show a higher hydrolytic activity against cefotaxime than against ceftazidime and their origin has not been associated to point mutations in the sequences of narrow-spectrum β-lactamase genes, but to a mobilization process of chromosome-encoded *bla* genes from *Kluyvera* spp. (reviewed in [12, 13]). It has been proposed that the worldwide dissemination and the persistence of *bla*_{CTX-M} genes is due to their location on mobile genetic elements (e.g. plasmids and transposons) and their association to clones with high dissemination potential (e.g. *bla*_{CTX-M-15} gene found in the *E. coli* O25:H4-ST131 clone) or to other antimicrobial resistance genes, which confer resistance to commonly used antimicrobial agents, such as fluoroquinolones, aminoglycosides, trimethoprim and sulfonamides. In this way, the co-location of different resistance genes on the same genetic element ensures the co-selection of
ESBL genes as well as their persistence even in the absence of a direct selection pressure. It is noteworthy that multiresistance associated with ESBL-producing isolates may compromise the success of an antimicrobial therapy [13].

**Distribution of common ESBLs in isolates from food-producing animals.** Although the occurrence of ESBL genes may vary according to the geographical area [14] (Figure 1), the European Food Safety Authority (EFSA) has ranked the \( \text{bla}_{\text{CTX-M-1}} \), \( \text{bla}_{\text{CTX-M-14}} \), \( \text{bla}_{\text{TEM-52}} \) and \( \text{bla}_{\text{SHV-12}} \) as the most common ESBL genes found in food-producing animals [8]. The dissemination of these genes is supposed to be more related to a horizontal transmission of these genes than to the spread of clones [8, 13, 14]. These genes have been mainly associated with ESBL-producing \( E. \) coli and non-typhoidal Salmonella (e.g. \( S. \) Typhimurium, \( S. \) Newport and \( S. \) Heidelberg) found in apparently healthy and diseased food-producing animals (e.g. calves, cattle, broilers, poultry and pigs) [8, 15]. Such \( \text{bla}_{\text{CTX-M-1}} \) genes have been commonly located on plasmids of incompatibility group IncN or IncI1 (broad host-range replicon plasmids) [16], the \( \text{bla}_{\text{CTX-M-14}} \) genes on IncF or IncK plasmids, and the \( \text{bla}_{\text{SHV-12}} \) and the \( \text{bla}_{\text{TEM-52}} \) genes on IncI1 plasmids. These last two ESBL genes have mainly been found in poultry and/or poultry meat samples [17, 18]. In contrast, \( \text{bla}_{\text{CTX-M-14}} \) is widely spread among isolates of food-producing animals (Figure 1) and also humans. According to Leverstein-van Hall and collaborators [19], the genetic correlation among \( \text{bla}_{\text{CTX-M-1}} \)-producing isolates from human, poultry and poultry meat and the sequence types of IncI1 plasmids revealed that the transmission of CTX-M-1-producing isolates between food-producing animals and humans may occur through the food chain. Kluytmans et al. [20] investigated ESBL-producing \( E. \) coli isolates from chicken meat and humans in the southern part of The Netherlands. They found significant genetic similarities - based on the analysis of mobile resistance elements, virulence genes and genomic backbone - and concluded that chicken meat is a likely contributor to the recent emergence of ESBL-producing \( E. \) coli in human infections in the study region. Nevertheless, results obtained in another study showed a higher similarity among ESBL-producing isolates of human origin than between isolates of human and animal origin, which might suggest a direct transmission of ESBL-producing \( E. \) coli from human-to-human [21]. The \( \text{bla}_{\text{CTX-M-14}} \) genes (besides the \( \text{bla}_{\text{CTX-M-15}} \) genes) are the most successfully disseminated \( \text{bla}_{\text{CTX-M}} \) genes; they have been isolated from the environment, humans, food and animals [13, 15, 22].

In contrast to the situation in Europe, ESBL genes have not been so commonly reported in isolates of animal origin in North America. Instead, plasmid-encoded AmpC β-lactamase
genes (e.g. \textit{bla}_{CMY-2}) are more frequently found in North America. In a study on clinical human isolates [23], \textit{bla}_{CTX-M-15} genes were found in only 3/109 isolates and two of them also carried \textit{bla}_{SHV-12} genes. In another study conducted in the USA and involving 2,034 clinical isolates, \textit{bla}_{CTX-M-1} genes located on IncI1 plasmids were found in 6/88 isolates from turkeys and in 1/940 isolates from pigs, but in none of the cattle (n=581) or chicken (n=83) isolates [24]. In Brazil, the \textit{bla}_{CTX-M-2} gene has been found on IncP plasmids from poultry and clinical human isolates [25] or chromosomally located in non-related isolates from healthy broiler chickens [26]. A high dissemination of \textit{bla}_{CTX-M-2} genes has been reported in South America since 1989 [27]. These geographical distributions and the prevalence of \textit{bla}_{CTX-M} genes in specific regions may be associated with the presence of specific selective driving forces (e.g. antimicrobial usage), geographical factors, such as the presence of specific clones or organisms, and may be altered by the production system of food-producing animals (e.g. international trade of live animals) or even by the import of contaminated food [13, 18, 22].

\textbf{Harmonization of detection methods and surveillance/monitoring programs.} The data concerning the prevalence of ESBLs isolates cannot be easily compared due to the lack of a harmonized methodology for the detection and characterization of the isolates. In addition, surveillance and monitoring programs usually determine the resistance rates to third-generation cephalosporins, but many of them do not perform ESBL phenotypic confirmatory tests or the molecular identification of the ESBL genes. In general, the prevalence may vary also if: (i) clinical or non-clinical isolates were investigated, (ii) supplementary surveillance with selective enrichment for the detection of isolates was performed, (iii) the programs used different cephalosporins in the antimicrobial susceptibility tests, and (iv) different methods for the confirmation of ESBL resistance phenotype and characterization of the ESBL-producing isolates were used [18, 28, 29]. According to EFSA, the harmonization of the methodology should include protocols on (i) sampling strategies, (ii) preferential method of susceptibility testing, (iii) antibiotics to be tested and (iv) criteria for categorizing isolates as susceptible or resistant, as well as quality control and reporting [8]. Extremely important is also the classification of the ESBL genes. To properly identify an ESBL gene, the sequence of the entire ESBL gene (i.e. from start to stop codon) must be analyzed and the sequence must be compared with references (accession numbers can be obtained from [10]) to identify already existing or novel ESBL gene/protein variants.

\textbf{Perspectives on ESBLs persistence, evolution and dissemination.} It has been thought that evolution and dissemination of CTX-M enzymes might be assured by (i) further
mutations in the respective genes and/or (ii) further recombination events between \( \text{bla}_{\text{CTX-M}} \) genes, (iii) selection pressure, (iv) location on broad host-range replicon plasmids such as IncN, IncI1 and IncL/M (with \textit{Salmonella} and \textit{E. coli} isolates from animals as reservoirs) and/or association with clones that have high dissemination potential and (v) co-selection processes, since \( \text{bla}_{\text{CTX-M}} \) genes are commonly associated with other resistance genes including genes encoding carbapenemases. Such carbapenemase genes have also been found in CTX-M producing isolates [13]. Although the control of the dissemination of ESBLs or β-lactamases in general is a task plenty of challenges, the reduced use of antimicrobial agents in food-producing animals may be a good first step into the right direction [8, 29].

**Carbapenemase-producing bacteria from livestock**

In addition to ESBL-producing Enterobacteriaceae, carbapenemase-producing bacteria present another major challenge to antimicrobial chemotherapy [30, 31]. This mainly applies to human medicine as carbapenems are not approved for use in veterinary medicine worldwide. However, carbapenems may be used for pet and companion animals via the Animal Medicinal Drug Use Clarification Act (AMDUCA), which allows veterinarians to prescribe certain approved animal drugs and approved human drugs as “extralabel use” for animals under certain conditions [32]. For livestock animals, carbapenems must not be used at any time and situation. Nevertheless, carbapenem-resistant bacteria have also been isolated from livestock animals during recent years. Three recent review articles dealt with this topic [33-35]. Woodford and co-workers [34] listed the carbapenem-resistant bacteria from non-human sources (environment including water, sewage and effluents, livestock animals, companion animals and wildlife) and the corresponding carbapenemase genes.

**Carbapenem resistant bacteria in livestock animals.** There are only limited and sporadical findings of carbapenemase-producing bacteria in livestock. The first report was published in mid-2012 when VIM-1 carbapenemase-producing \textit{E. coli} was detected on a swine farm in Germany [36]. Soon thereafter, the same authors identified also VIM-1-producing \textit{Salmonella enterica} serovar Infantis in swine and poultry farms [37]. However, it should be noted that the respective samples were taken from the farm environment (and in one case even outside the farm), but not from colonized or infected swine or poultry. Other reports identified \textit{Acinetobacter} spp. isolates producing the OXA-23 carbapenemase that were found in horses from Belgium [38] and in dairy cattle from France [39]. Two reports from
China identified *bla*NDM-1 carrying *Acinetobacter lwoffii* from poultry [40] and *Acinetobacter baumannii* from a pig [41]. In the latter case, the *bla*NDM-1 gene was located on a conjugative plasmid of 47 kb that also carried other resistance genes, such as *aphA6*, *ble* and *msr*(E)-*mph*(E).

This relatively limited number of reports about carbapenem-resistant bacteria from livestock may suggest that such bacteria are currently present in livestock at a very low prevalence. This may reflect the lack of a direct selection pressure as carbapenems are not approved for use in livestock. However, Poirel and colleagues [39] also pointed towards co-selection of carbapenemase genes under the selection pressure imposed by the use of aminopenicillins and aminopenicillin–β-lactamase inhibitor combinations in livestock. While some authors see carbapenem-resistant bacteria from livestock and companion animals as a public health risk of currently undefinable magnitude [33-35, 38], a recent study considered food-producing animals as a marginal risk factor for the public health [42]. The authors stated that there is little if no evidence that animals – in particular food-producing animals – pose a risk for humans to become infected by carbapenemase-producing bacteria. More likely is a human-to-human transfer of carbapenemase-producers based on an increased consumption of carbapenems among humans worldwide and increased mobility which also includes travels into and migration from geographical regions where carbapenemase-producers are endemic [42].

Rapid identification of carbapenemase producers using a harmonized methodology and implementation of surveillance studies will be an important step to determine how frequent carbapenemase-producers are truly found among livestock and companion animals. Detailed molecular typing of the respective isolates will also allow to trace back the origin of the isolates and thereby see whether the occurrence of carbapenemase producers in animals originates from human sources or from human contaminations of the environment [42].

**ICEPmu1-mediated multidrug resistance in bovine *Pasteurella multocida* and *Mannheimia haemolytica***

*Pasteurella multocida* and *Mannheimia haemolytica* are – together with *Histophilus somni* – the major bacterial components in the bovine respiratory disease (BRD) complex. BRD is one
of the economically most important diseases of cattle. Global losses of the feedlot industry are estimated to be over $3 billion per year [43].

**Antimicrobial resistance in BRD pathogens.** Antimicrobial agents are commonly used to control the bacterial pathogens involved in BRD. Previous studies on antimicrobial resistance in *P. multocida* and *M. haemolytica* showed an overall favourable situation [44, 45]. In Europe, low percentages of isolates that were resistant to newer antimicrobial agents such as florfenicol (0-2.0% resistance for both *M. haemolytica* and *P. multocida*) or fluoroquinolones (0-5.8% and 0-6.3% resistance for *M. haemolytica* and *P. multocida*, respectively) were identified [46]. During a 10-years (2000-2009) survey of antimicrobial resistance in BRD pathogens in the USA and Canada, increasing percentages of isolates that were resistant to florfenicol, enrofloxacin but also to tulathromycin were detected. In 2009, resistance to florfenicol, tulathromycin and enrofloxacin was seen in 11.6%, 4.6% and 2.1% of the *P. multocida* and in 8.6%, 8.9% and 6.6% of the *M. haemolytica* isolates [47]. Especially the detection of tulathromycin-resistant BRD pathogens, which were often also resistant to multiple other agents, was alarming as resistance to triamilides has not been detected before in *P. multocida* and *M. haemolytica*.

Whole genome sequencing of the representative multiresistant *P. multocida* isolate 36950 was conducted [48] and led to the identification of three novel macrolide/triamilide resistance genes. These comprised the rRNA methylase gene *erm*(42) [48, 49], the ABC transporter gene *msr*(E) and the macrolide phosphotransferase gene *mph*(E) [48]. Cloning and expression of these resistance genes showed that *erm*(42) conferred resistance to macrolides and lincosamides whereas the genes *msr*(E)-*mph*(E), which were organized in an operon-like structure, mediated resistance to macrolides and triamilides [48]. Further studies showed that these genes also increased the minimal inhibitory concentrations for the two most recently approved macrolides tildipirosin and gamithromycin [50]. It has been observed that *erm*(42) and *msr*(E)-*mph*(E) can occur separately or together in bovine *P. multocida* and *M. haemolytica* isolates [48, 51, 52].

**Structure and transferability of ICEPmu1.** Analysis of the whole genome sequence of *P. multocida* isolate 36950 showed that the three aforementioned genes were part of a novel integrative and conjugative element, designated ICEPmu1 [52, 53]. ICEPmu1 was the first ICE identified in *P. multocida*. ICEPmu1 is 82,214-bp in size and harbors 88 genes [52]. The core genes of ICEPmu1 are involved in the excision/integration and the conjugative
transfer of the element. These core genes resemble those found in a 66,641-bp ICE from *H. somni* [54]. ICEPmu1 integrates into a tRNA\textsubscript{Leu} and – when integrated – is flanked by 13-bp direct repeats [52] (Figure 2). Transfer experiments confirmed that ICEPmu1 is able to transfer by conjugation to *P. multocida*, *M. haemolytica* and *E. coli* where it also uses a tRNA\textsubscript{Leu} for integration and produces closely related 13-bp direct repeats [52]. After excision and before integration into a new vector molecule, ICEPmu1 forms an intermediate circular form whose presence has also been confirmed by PCR [52]. Besides the core genes, ICEPmu1 harbors two accessory gene regions of approximately 15.7 and 9.8 kb. These two regions are located 42,526 bp apart from each other and contain a total of 12 antimicrobial resistance genes [53] (Figure 2). Resistance gene region 1 comprises the genes *aphA1* (kanamycin/neomycin resistance), *strA* and *strB* (streptomycin resistance), *sul2* (sulfonamide resistance) *floR* (chloramphenicol/florfenicol), and *erm(42)* (tilmicosin/clindamycin resistance). The following resistance genes are located in the resistance gene region 2: *aadA25* (streptomycin/spectinomycin resistance), *aadB* (gentamicin resistance), *bla\textsubscript{OXA-2}* (ampicillin resistance), *msr(E)-mph(E)* (tilmicosin/tulathromycin resistance), and *tetR-tet(H)* (tetracycline resistance) [53]. It should be noted that the *bla\textsubscript{OXA-2}* gene is functionally inactive in *P. multocida* and *M. haemolytica* hosts, but functionally active in *E. coli*. A detailed analysis of the structure of these two resistance gene regions suggested that plasmids, gene cassettes and insertion sequences might have played a role in the development of the resistance gene regions within ICEPmu1 [53]. A related ICE, designated ICEMh1, was detected during whole genome sequencing of *M. haemolytica 42548* and carried the resistance genes *aphA1*, *strA*, *strB*, *sul2*, and *tetR-tet(H)*, which accounted for the multiresistance phenotype of this isolate [55]. In another study, *M. haemolytica*, *P. multocida*, and *H. somni* from the USA were shown by PCR to possess ICEs that conferred resistance for up to seven different antimicrobial classes. The ICEs proved to transfer via conjugation from *P. multocida* to *E. coli* and from *M. haemolytica* and *H. somni* to *P. multocida* [56].

Bearing in mind that *P. multocida* and *M. haemolytica* represent the major bacterial pathogens involved in BRD and that horizontal intergenus transfer of closely related ICEs has obviously already happened *in vivo*, the spreading of multi-resistance ICEs, such as ICEPmu1 and ICEMh1, may distinctly limit the therapeutic options for bovine respiratory disease. Moreover, the particular structure of the resistance gene regions, e.g. the presence of numerous insertion sequences as well as the presence of part of a class 1 integron, may allow
for the acquisition of resistance genes via insertion sequence-mediated recombination processes, but also for the incorporation of additional cassette-borne resistance genes [53].

**Cfr-mediated antimicrobial multi-resistance in Gram-positive and Gram-negative bacteria**

The gene *cfr* is the only transferable oxazolidinone resistance gene known to date [57, 58]. This gene was initially described as a chloramphenicol/florfenicol resistance gene [59]. However, with the elucidation of the *cfr*-mediated resistance mechanism, it became obvious that this gene confers resistance to several other protein biosynthesis inhibitors [60, 61]. The gene *cfr* encodes an RNA methyltransferase that modifies the adenine residue at position 2503 of the 23S rRNA gene. This position is located within the overlapping binding sites of phenicols, lincosamides, oxazolidinones, pleuromutilins, streptogramin A antibiotics and certain 16-membered macrolides, such as josamycin and spiramycin, at the peptidyltransferase centre of the ribosome [58, 61]. It is believed that the additional Cfr-mediated methylation of A2503 interferes with the correct positioning and binding of the aforementioned classes of antimicrobial agents to the ribosome and thereby confers combined resistance to them.

**Presence of *cfr* in staphylococci.** Although initially identified in coagulase-negative staphylococci from cattle [59], this gene was detected during the last decade in various staphylococcal species from humans and livestock (reviewed in [57, 58]). In most cases, the *cfr* gene was located on plasmids. Sequence analysis of these plasmids revealed the colocation of other resistance genes, such as the macrolide-lincosamide-streptogramin B resistance genes *erm*(A), *erm*(B), *erm*(C) or *erm*(33), the aminoglycoside resistance genes *aacA-aphD* or *aadD*, the tetracycline resistance gene *tet*(L) linked to the trimethoprim resistance gene *draK*, the phenicol exporter gene *fexA*, or the ABC transporter gene *lsu*(B) [62-67] (Figure 3). A recent review [58] showed a comparison of the known *cfr* resistance gene regions on plasmids and in the chromosomal DNA. This comparison also revealed the presence of insertion sequences, such as IS21-558 or IS256-like, in the close proximity of the *cfr* gene. For IS21-558, the involvement in mobility of *cfr* was shown and a model of transfer, that included circular intermediate forms, was proposed [68].
**Presence of cfr in other bacteria.** First hints towards the presence of the gene cfr in bacteria other than staphylococci were published in 2010 when Dai and co-workers found cfr together with fexA in a Bacillus spp. from swine [69]. Soon thereafter, structurally diverse plasmids that harboured the cfr gene in different genetic contexts were identified in Bacillus spp. [70, 71]. Besides Bacillus, the cfr gene was also detected on a plasmid in Enterococcus faecalis of swine [72-74] and human origin [75, 76]. Searching for cfr-carrying bacteria from swine also identified this gene in isolates of Macrococcus caseolyticus and Jeotgalicoccus pinnipedialis [77] and in Streptococcus suis [78]. In virtually all these cases, the cfr gene was located on plasmids, which explains its dissemination into other Gram-positive bacteria via horizontal gene transfer.

In addition to Gram-positive bacteria, the gene cfr has also been found in the chromosomal DNA of the Gram-negative Proteus vulgaris [79]. The respective isolate was also from swine and the cfr segment, which apart from the cfr gene resembled part of a staphylococcal plasmid, was bracketed by insertion sequences of the type IS26. Two more studies identified the cfr gene in another Gram-negative bacterial species, namely E. coli [80, 81]. In both cases, the cfr gene was located on plasmids. On the ca. 110-kb plasmid pEC-01, the cfr gene was bracketed again by IS26 elements [70]. On the completely sequenced 135,615-bp IncA/C plasmid pSCEC2, two resistance gene segments were identified [81]. One segment contained the cfr gene flanked by two IS256 elements whereas the other segment harbored the resistance genes floR, tet(A)-tetR, strA/strB and sul2. Except for these two resistance gene regions, the pSCEC2 backbone displayed >99% nucleotide sequence identity to that of other IncA/C family plasmids isolated in Europe as well as in North and South America [81].

It should be noted that oxazolidinones are considered as ‘last-resort’ antimicrobial agents in human medicine. Oxazolidinones are not approved for use in animals worldwide. The fact that the cfr gene is nevertheless found in bacteria of livestock is most likely the effect of selection of this multiresistance gene under the selective pressure imposed by the use of other antimicrobial agents to which it confers resistance. As such, phenicols (e.g. florfenicol), lincosamides (e.g. lincomycin, pirlimycin), and pleuromutilins (e.g. tiamulin, valnemulin) are approved for use in food-producing animals. Analysis of the antimicrobial usage patterns on livestock farms from which cfr-positive bacteria have been obtained showed an excellent correlation to the resistance patterns of the cfr-positive isolates [82, 83].
Novel antimicrobial resistance genes in livestock-associated MRSA

Since the mid 2000s, a new group of methicillin-resistant *Staphylococcus aureus* (MRSA) has gained particular public attention (reviewed in [84-87]). These bacteria were termed livestock-associated (LA-) MRSA since they were first identified in swine and people with occupational contact to swine. Initially, only isolates of the clonal complex (CC) 398 were thought to represent LA-MRSA. However during recent years, also isolates of other CCs have been identified among MRSA from livestock, including CC9, CC5 among others [88]. Numerous studies have been conducted which identified LA-MRSA not only in pigs, but also in other animal species, such as cattle [89, 90], horses [91], poultry [92], dogs [93, 94], cats [94, 95], sheep [90] and rabbits [96].

**Novel resistance genes detected in LA-MRSA 2009-2012.** The increased interest in LA-MRSA also prompted researchers to have a closer look at the virulence and antimicrobial resistance properties of these isolates. While LA-MRSA commonly do not carry virulence genes except the enterotoxin gene cluster in CC9 and CC5 isolates [97], LA-MRSA vary distinctly in their antimicrobial resistance pheno- and genotypes. A large number of the antimicrobial resistance genes so far detected in staphylococci of animal origin [98] has also been detected in LA-MRSA. In addition, several novel and uncommon resistance genes were described for the first time in LA-MRSA (reviewed in [99]). These include the trimethoprim resistance gene *dfrK*, which was either linked to the tetracycline resistance gene *tet* (L) [100] or part of the non-conjugative transposon Tn559 [101] and the MLSB resistance gene *erm*(T) [102]. Further new resistance genes are the ABC transporter genes *vga*(C) [103, 104] and *vga*(E) [105, 106], both of which confer combined resistance to pleuromutilins, lincosamides and streptogramin A antibiotics. While the gene *vga*(C) is located as the only resistance gene on small plasmids [104] or together with other resistance genes on larger multiresistance plasmids [103], the gene *vga*(E) is located on a non-conjugative transposon that preferentially integrates at a specific site into the chromosomal DNA [105]. Another new resistance gene is the apramycin resistance gene *apmA*, which may also be located either on large multiresistance plasmids [107] or on small plasmids [108]. In 2011, a new *mec* homologue, initially referred to as *meca*<sub>LG4251</sub> but nowadays known as *mecC*, was detected in *S. aureus* from humans and dairy cattle [109, 110]. During the following years, *mecC* has been identified also in pets [111, 112] and wildlife animals [113, 114]. A comprehensive review on
the emergence of mecC-carrying *S. aureus* including the geographical and host species distribution as well as the MLST and *spa* types of the respective isolates has recently been published [115]. Screening of strain collections revealed that the oldest mecC-carrying *S. aureus* isolate dates back to 1975 [115]. During recent years, two new mecC allotypes have been identified: mecC1 in *Staphylococcus xylosus* [116] and mecC2 in *Staphylococcus saprophyticus* [117].

**Novel resistance genes detected in LA-MRSA 2013-now.** Since then, another three novel resistance genes have been identified in LA-MRSA. Two of them, *lsa*(E) [118] and *spw* [119], are part of multiresistance gene clusters of most likely enterococcal origin which have recently been identified in LA-MSSA CC9, LA-MRSA CC9 and LA-MRSA CC398 from swine and humans [120-122]. At least three different types of these clusters have been identified so far and have been described in detail [120-122]. The gene *lsa*(E) codes for an ABC transporter which confers combined resistance to pleuromutilins, lincosamides and streptogramin A antibiotics [118] whereas the gene *spw* confers high-level spectinomycin resistance [119]. While these clusters are commonly located in the chromosomal DNA, the most expanded multiresistance gene cluster was found on plasmid pV7037 from a porcine LA-MRSA ST9 isolate [123]. This cluster comprised – besides *lsa*(E) and *spw* – the resistance genes *aacA-aphD* (gentamicin-kanamycin-tobramycin resistance), *erm*(B) (MLS\(_B\) resistance), *aadE* (streptomycin resistance) and *lnu*(B) (lincosamide resistance) [123]. It should be noted that plasmid pV7037 also carried a functionally active *tet*(L) gene for tetracycline resistance and a *cadDX* cadmium resistance operon [123]. The third and most recently detected novel resistance gene in LA-MRSA is the spectinomycin resistance gene *spd* [124]. This gene also confers high-level spectinomycin resistance and was initially found on a small plasmid of 3,928 bp in LA-MRSA CC398 from various human and animal sources in Belgium. Most recently, the same gene was detected on a different type of plasmid in MRSA ST398 and MSSA ST433 isolates from humans, animals and food of animal origin in The Netherlands, Germany and Austria [125].

These findings confirm the role of LA-MRSA as recipient and donor of antimicrobial resistance genes. The detection of a multiresistance gene cluster that was previously seen on different enterococcal plasmids also underlines the presence of a gene flux between staphylococci and enterococci. Continuous surveillance of LA-MRSA for acquisition of new antimicrobial resistance genes but also virulence genes is warranted.
Conclusion & future perspective

Since no new classes of antimicrobial agents for use in livestock animals are to be expected in the near future, our aim must be to preserve the efficacy of the currently available antimicrobial agents for as long as possible. This includes measures to counteract the emergence of antimicrobial (multi)resistance among bacteria from livestock animals. There is no fast and easy solution to the problem. More likely, it will be a joint approach that includes on one side (i) improved preventive measures such as vaccination, (ii) improved farm management accompanied by a tendency to implement integrated farming systems, (iii) improved hygiene on farms, and (iv) prudent and judicious use of antimicrobial agents. On the other side, more emphasis must be put on research to identify emerging resistance genes, the mobile genetic elements with which they are associated and the modes of spreading of these elements. Understanding the mechanism(s) of resistance and knowing the conditions of optimized horizontal gene transfer are important first steps to develop means and ways to inhibit the resistance mechanism [126] and to counteract resistance gene dissemination. Especially the knowledge about co-located resistance genes, which allow co-selection and persistence of resistance genes even in the absence of a direct selection pressure, is indispensable to predict the success or failure of measures such as the ban or the limitation of use of a certain antimicrobial agent in order to reduce resistance rates. Another issue is the non-therapeutic use of antimicrobial agents for growth promotion (reviewed in [127]). Although antimicrobial growth promoters have been banned in 2006 from use in food-producing animals in the European Union, they are still used in many non-EU countries. The amount of antimicrobial agents used for growth promotion may be equal or even superior to the amount used in therapy [127]. It would be an option to consider a global ban of antimicrobial growth promoters in food animal production, especially since there are examples which showed that the ban of antimicrobial growth promoters had no negative impact on health and productivity of food-producing animals [128, 129].

Since bacteria do not live in genetic isolation on the skin and the mucosal surfaces of humans and animals, but in polymicrobial environments, there will always be partners for the exchange of genetic material. Therefore, it is impossible to prevent the dissemination of plasmids, transposons or ICEs within bacterial populations. However, using correct dosage schemes and choosing the most promising antimicrobial agent based on the results of in-vitro
susceptibility testing will minimize the spread of resistant bacteria and resistance genes. Commercial rearing of livestock without using antimicrobial agents is not possible to date. Although the use of antimicrobial agents is considered an important factor driving antimicrobial resistance, very limited detailed information on the use of antimicrobial agents in animals is currently available [28, 130]. However, it is necessary to understand which antimicrobial agents are used at which quantities for which purpose in which animal species. Currently, attempts are made in various countries to determine valid consumption figures at the farm level. One aim of these approaches is to identify farms which use below average amounts of antimicrobial agents and to learn from them how to change the husbandry and farm management conditions in order to efficiently reduce the amount of antimicrobial agents used. Even though the voluntary ban of certain antimicrobial agents, such as cephalosporin use in Danish pig production, has effectively reduced extended-spectrum cephalosporinase-producing *Escherichia coli* in slaughter pigs [131], it is important to understand that there is no direct correlation between antimicrobial consumption and the presence and dissemination of antimicrobial resistant bacteria/antimicrobial resistance genes [132, 133]. Factors like co-location of resistance genes on the same mobile genetic element, co-transfer of these resistance genes during spread of the element as well as co-selection and persistence of resistance genes during direct or indirect selection pressure play an important role in the interplay between antimicrobial agents and bacteria. It is important to understand that antimicrobial resistance is an evolutionary principle by which bacteria try to adapt to changed environmental conditions, i.e. survival in the presence of antimicrobial agents. As such, it is impossible to stop antimicrobial resistance. However, it is possible to slow down the development and dissemination of antimicrobial resistance by reduction of the selection pressure and prudent and judicious therapeutic use of the available antimicrobial agents.
Executive summary

Extended-spectrum β-lactamase (ESBL)-producing isolates

- Represent risks for public health and have been associated with economic losses in food-animal production
- CTX-M enzymes are the most prevalent ESBLs nowadays
- blaCTX-M genes are commonly associated with mobile genetic elements
- blaCTX-M-1, blaCTX-M-14, blaTEM-52 and blasHV-12 are the most common ESBL genes found in food-producing animals
- ESBL genes may be located on broad host-range plasmids
- Persistence and dissemination of ESBL genes may occur by co-selection processes (association with other resistance genes including carbapenemase genes)

Carbapenemase-producing isolates

- Rarely found among food-producing animals (carbapenems are not approved for the treatment of animals)
- Reports about blavIM-1-carrying E. coli and S. enterica serovar Infantis from pig and poultry farms
- Reports about blaoXA-23-carrying Acinetobacter from horses and dairy cows
- Reports about blanDM-1-carrying A. lwoffii from poultry and A. baumannii from pigs
- ‘Tip of the iceberg’ or rare accidental findings?

Integrative and conjugative element ICEPmu1

- Found in P. multocida and M. haemolytica from cases of bovine respiratory disease (BRD)
- Highly mobile self-transferable genetic element that moves across species and genus
boundaries

- Carries 12 resistance genes, some of which also confer resistance to the most recently approved antimicrobial agents for treatment of BRD
- Its dissemination drastically limits the treatment options

**Cfr-mediated multiresistance**

- The gene *cfr* encodes a rRNA methylase that confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A
- The only so far known transferable oxazolidinone resistance gene
- Often located on plasmids that carry additional resistance genes
- Initially found in coagulase-negative staphylococci
- Recent dissemination into other Gram-positive bacterial genera, such as *Bacillus, Enterococcus, Macrococcus, Jeotgallicoccus* and *Streptococcus*
- Recent dissemination into Gram-negative bacterial genera, such as *Escherichia* and *Proteus*

**New resistance genes in LA-MRSA**

- LA-MRSA can acquire new resistance genes from other bacteria
- Novel resistance genes identified during the last years include *apmA* (apramycin resistance), *dfrK* (trimethoprim resistance), *erm(T)* (MLS$_B$ resistance), and *vga(C)* and *vga(E)* (resistance to lincosamides, pleuromutilins and streptogramin A), *mecC* (resistance to β-lactams)
- Recent acquisition of a multiresistance gene cluster from enterococci which includes the novel genes *lsa(E)* (resistance to lincosamides, pleuromutilins and streptogramin A) and *spw* (spectinomycin resistance)
- Latest new resistance gene: *spd* (spectinomycin resistance)
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Papers of special note have been highlighted as:

** of considerable interest


** Comprehensive review of ESBL-producing isolates in food-producing animals.


** Comprehensive review on ESBL genes in Enterobacteriaceae from animals.


** Comprehensive review of carbapenemase-producing isolates from non-human sources.


** A different view on the role of animals in the dissemination of carbapenemase-producing bacteria.


** Comprehensive review of the genetic environment of the multi-resistance gene cfr in Gram-positive and Gram-negative bacteria.


** First description of the Cfr-mediated multi-resistance phenotype.


** Comprehensive review of MRSA of different sequence types and clonal complexes and their virulence and resistance properties.

** Comprehensive review of antimicrobial resistance genes in staphylococci from animals.

** Comprehensive review of novel resistance genes in LA-MRSA.


Figure 1: Geographical distribution of extended-spectrum $\beta$-lactamases (CTX-M, TEM and SHV) found in Enterobacteriaceae (especially E. coli and Salmonella spp.) isolated from food-producing animals (poultry, cattle and pigs) or poultry meat. The majority of these data is based on the EFSA report [8] and additional recently published studies [16, 18, 19, 24-26]. The data presented are examples for the worldwide distribution of ESBLs; however, they do not show the prevalence of ESBLs or the actual occurrence of ESBLs in the different countries. Countries, for which no ESBL data are presented, are not necessarily free of ESBLs among bacteria from livestock animals.
Figure 2: Schematic presentation of ICEPmu1 and detailed structure of the resistance gene regions 1 and 2. Genes are presented as arrows with the arrowhead indicating the direction of transcription. Insertion sequences and ISCR elements are shown as boxes with the arrows inside the boxes indicating the transposase genes. For the function of the various resistance genes, please refer to the respective part of the text. This figure was modified from Figure 1 in [53].
Figure 3: Comparative analysis of the genetic environment of the cfr gene in plasmids from *Staphylococcus* spp., *Bacillus* spp., *Enterococcus faecalis*, *Streptococcus suis* and *Jeotgalicoccus pinnipedialis*. The arrows indicate the positions of the genes and their directions of transcription. The cfr gene is presented as a checkered arrow while other resistance genes are shown as pink arrows. Genes whose products are involved in replication, transposition or plasmid recombination/mobilization are shown as dark violet, orange or green arrows, respectively. IS elements are presented as black boxes with the white arrows therein representing the transposase genes. Δ indicates a truncated gene. A 1 kb distance scale is displayed in the upper right or left corner. Some of the maps were from Fig. 1a and 1b in [58].
8. Discussion

8.1. General discussion

Due to the growing demand of food, especially beef, worldwide, the feedlot industry has to adapt rapidly. Herd sizes, as well as production efficiency in all sectors need to be increased, while maintaining or improving health and welfare of the animals. Unfortunately, the increase in meat production is easy to achieve, while maintaining the welfare standard at the same time is much more difficult and work intensive. On U.S. feedlots, it is more common to use antibiotics, especially macrolides prophylactically instead of reducing environmental and handling stress (RICE et al. 2007). However, this practice might lead to a higher incidence of antimicrobial resistance in pathogenic bacteria (CALL et al. 2008).

The losses to the global cattle industry through BRD were discussed by several authors and organizations. Two articles with a high recognition in the scientific community were published by YATES (1982) and SNOWDER et al. (2006), and were cited according to Scopus (http://www.scopus.com/) since 1996 in 219 and 98 documents, respectively. Other articles discussing the losses due to BRD are by WHITELY et al. (1992), 108 citations, GRIFFIN (1997), 88 citations, and WATTS and SWEENEY (2010), 18 citations. The authors state the approximate costs of shipping fever in the USA. YATES (1982) in 1972 states the costs to be 300 million dollars, followed by WHITELY et al. (1992) and GRIFFIN (1997) who predict costs of more than one billion dollars for Northern America or just the USA, respectively. Thus, the costs for BRD seem to have tripled over the course of 10 to 15 years. After a similar timeframe, SNOWDER et al. (2006) assessed a price of $13,895 per 1,000 animals. Considering the total count for cattle and calves is currently 95 million animals, this equals total costs of 1.32 billion dollars (NASS and USDA 2014). Whereas the latest of the before mentioned, publications by WATTS and SWEENEY (2010) estimates losses of 3 billion dollars to the global cattle industry.

BRD is considered to have a similar or even higher economic impact on US feedlots than the costs of other cattle diseases combined (HIGHLANDER 2001; YATES 1982). M. haemolytica, P. multocida, and H. somni are considered to be the major bacterial etiologic agents for BRD, making them organisms of high economic importance (PORTIS et al. 2012). Of these three organisms, only P. multocida has been reported to be transmittable to humans.
with certainty. There have been reports of *M. haemolytica* infecting humans, but most likely it was confused with *P. multocida* (HIGHLANDER 2001). Even aside from the cattle industry, *M. haemolytica* has a considerable economic impact. For instance, it could be shown, that domestic sheep and bighorn sheep on one range share *M. haemolytica* strains (WARD et al. 1997) and that bighorn sheep are very likely to die, if exposed to *M. haemolytica* strains of serotype A2 (FOREYT et al. 1994). Also, most of the data available for *Pasteurellaceae* in general has been obtained from diseased or deceased farm animals. Thus, there is very little information on the dissemination and impact of *Pasteurellaceae* in wild animals (SCHWARZ 2008).

The traditional therapy of BRD involves the extensive use of antimicrobial agents. This can be hindered by antimicrobial resistance expressed by the pathogens, and can increase the resistance rates among involved pathogens (WATTS et al. 1994). To prevent a delay or even failure of therapy, it is more desirable to use non-antibiotic prophylaxis, like vaccination or reduction of stress. However, many papers which emphasize the efficacy of commercially available vaccines against BRD present disputable data and some of them are suffering from experimental design flaws (PERINO and HUNSAKER 1997). Furthermore, some farmers could be hampered to use vaccination due to economic reasons, like the price of the vaccine itself, the costs of the applying veterinarian(s) and the potential need to repeat the procedure (ROIER et al. 2013). Consequently, farmers might calculate that it is cheaper to risk a BRD outbreak and to treat some animals with antimicrobial agents, rather than vaccinating the entire herd. However, this practice bears the risks mentioned above.

Resistance rates of *M. haemolytica* and other *Pasteurellaceae* can be considered generally low in Germany (BVL 2012). However in Northern America, one is able to compare different resistance rates of the three major bacterial etiologic agents of BRD. *M. haemolytica* has a considerable higher resistance rate to penicillin, danofloxacin and enrofloxacin than *P. multocida*, and *H. somni* (PORTIS et al. 2012). All three major agents have been shown to carry different combinations of the antimicrobial resistance genes harbored by ICE*Pmu1* (KLIMA et al. 2014). These resistance genes could account for all resistance phenotypes, with the exception of the resistance to danofloxacin.

(beta-lactam antibiotics), msr(E)-mph(E) (macrolides/tulathromycin), and tetR-tet(H) (tetracycline). Some of these genes can also be found on plasmids of M. haemolytica. The NCBI genome database lists a total of only three plasmid sequences, but five additional plasmids could be found in the literature. The plasmids pMHSCS1, pPMSS1 (KEHRENBERG and SCHWARZ 2001) and pYFC1 (CHANG et al. 1992) carry strA and sul2, pMh1405 floR (KATSUDA et al. 2012) and pMHT1 the tetR-tet(H) operon (KEHRENBERG et al. 2001). Other resistance genes that mediate resistance to the same classes of antimicrobial agents as the ones carried by ICEPmu1 could also be found. pMHSCS1 additionally harbors a catA3, which confers resistance to chloramphenicol. pCCK3259 carries the tetracycline resistance gene tet(L) (KEHRENBERG et al. 2005a), and pAB2 (CRAIG et al. 1989) as well as pYFC2 (CHANG et al. 1992) each harbor a beta-lactam resistance gene, blaROB-1 and amp respectively. This limits resistance to aminoglycosides, MLSB, and spectinomycin in M. haemolytica up to today exclusively to ICEs.

8.2. Role of the genes ermA and msr(E)-mph(E) on the resistance to recently approved macrolides

In addition to results of the first listed publication (CHAPTER 4), it could be shown, that P. multocida and M. haemolytica isolates harboring only ermA exhibited high MICs to the lincosamide clindamycin, while isolates harboring msr(E)-mph(E) show higher MICs to the triamilide tulathromycin (KADLEC et al. 2011). Furthermore, these MIC changes were proven to be different in E. coli isolates. Regarding tilmicosin, ermA confers protection with a reduced efficiency, while the tulathromycin MIC value is slightly elevated. In contrast, isolates carrying msr(E)-mph(E) confers distinctly lower MICs for gamithromycin and tilmicosin as well as a slightly lower MIC to tulathromycin (DESMOLAIZE et al. 2011).

A total of 115 different isolates have been screened so far for ermA and/or msr(E)-mph(E) while being checked for their respective MICs to gamithromycin and tildipirosin. Sixty-nine of these tested isolates were discussed in CHAPTER 4, the remaining 46 isolates were analyzed by DESMOLAIZE et al. (2011) and ROSE et al. (2012). The data collected in these latter two studies correspond well with the data published in the course of this Ph.D. project.
(Table 2), except for four MIC ranges that are more than one step higher. The gamithromycin MIC range for *P. multocida* isolates carrying only *erm*(42) goes up to 16 mg/L, while the data in CHAPTER 4, with 10 tested isolates ranges between 2-4 mg/L. Furthermore, the tildipirosin MIC for *P. multocida* harboring all three resistance genes and only *erm*(42) as well as *M. haemolytica* isolates carrying only *erm*(42) is ≥128 mg/L, while the data presented in this project had an upper limit of 32 mg/L. The higher MIC values might be due to the extraction and purification of tildipirosin from Zactran® by DESMOLAIZE *et al.* (2011) and ROSE *et al.* (2012), while in this project the solution for injection was used directly.

The isolates by DESMOLAIZE *et al.* (2011) and ROSE *et al.* (2012) have been obtained from nasal swabs of cattle in the United States, with the exception of one *P. multocida* without any macrolide resistance gene, which has been collected in France. There is no information available, whether the sampled cattle suffered from BRD or not, while the isolates used in this project were all obtained from BRD cases in the USA. The possible difference regarding the health status of the cattle might have had an impact on the composition of isolates obtained in the respective studies. This is also reflected by the fact, that most of the *P. multocida* isolates from BRD cases harbored all three resistance genes and the *M. haemolytica* isolates mainly harbored only the *erm*(42). The isolates collected by DESMOLAIZE *et al.* (2011) and ROSE *et al.* (2012) showed a very homogeneous distribution of the genes *erm*(42) and/or *msr*(E)-*mph*(E). A large scale screening study, comparing the genotypes of *P. multocida* and *M. haemolytica* isolates from healthy cattle and from BRD cases, would be highly interesting.
**Table 2.** Comparison of the data from the study found in CHAPTER 4 and the combined data of DESMOLAIZE et al. 2011 and ROSE et al. 2012.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Macrolide resistance gene(s) present</th>
<th>No. of isolates</th>
<th>MIC [mg/L] range of</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. multocida</td>
<td>—</td>
<td>6</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>8</td>
<td>0.25-0.5</td>
<td>0.25-0.5</td>
</tr>
<tr>
<td></td>
<td><strong>erm(42)</strong></td>
<td>8</td>
<td>4-16</td>
<td>≥128</td>
</tr>
<tr>
<td></td>
<td><strong>erm(42)</strong></td>
<td>10</td>
<td>2-4</td>
<td>16-32</td>
</tr>
<tr>
<td></td>
<td><strong>msr(E)-mph(E)</strong></td>
<td>6</td>
<td>32-64</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><strong>msr(E)-mph(E)</strong></td>
<td>2</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><strong>erm(42)+msr(E)-mph(E)</strong></td>
<td>4</td>
<td>64-128</td>
<td>≥128</td>
</tr>
<tr>
<td></td>
<td><strong>erm(42)+msr(E)-mph(E)</strong></td>
<td>20</td>
<td>16-64</td>
<td>16-32</td>
</tr>
<tr>
<td>M. haemolytica</td>
<td>—</td>
<td>5</td>
<td>0.25-1</td>
<td>0.5-2</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>7</td>
<td>0.5-1</td>
<td>0.5-2</td>
</tr>
<tr>
<td></td>
<td><strong>erm(42)</strong></td>
<td>6</td>
<td>4-8</td>
<td>≥128</td>
</tr>
<tr>
<td></td>
<td><strong>erm(42)</strong></td>
<td>1</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td><strong>msr(E)-mph(E)</strong></td>
<td>5</td>
<td>64-128</td>
<td>0.5-2</td>
</tr>
<tr>
<td></td>
<td><strong>msr(E)-mph(E)</strong></td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><strong>erm(42)+msr(E)-mph(E)</strong></td>
<td>6</td>
<td>64-128</td>
<td>≥128</td>
</tr>
<tr>
<td></td>
<td><strong>erm(42)+msr(E)-mph(E)</strong></td>
<td>21</td>
<td>32-64</td>
<td>32-64</td>
</tr>
</tbody>
</table>
8.3. Analysis of the complete genomic sequence of *Mannheimia haemolytica* strain 42548

Despite the great economical and veterinary importance of *M. haemolytica* for the global cattle industry, there was no complete genome sequence available when this Ph.D. project started. A complete and closed genome sequence is imperative for founded, scientific work. Announced shortly after the first two published complete genomic sequences of *M. haemolytica* (HARHAY et al. 2013), the sequence of strain 42548 is the third published complete sequence of an isolate belonging to this bacterial species and has great scientific value.

Today, seven complete genome sequences, including the three mentioned above, are available for *M. haemolytica*, but it is important to keep in mind, that every complete genome sequence is only a snapshot of the genotype of a species (HALL 2007). The genome of every species varies between its strains. In the case of *M. haemolytica*, the GC content ranges from 40.83% to 41.14% and the genome size is between 2.50 and 2.73 Mb (Table 3). However, there are more draft sequences available than complete sequences. The main disadvantage of draft sequences lies within the missing information. This information might include the orientation of and distance between two or more genes, as well as the presence and total count of all CDS harbored by the organism.

All complete genomes of *M. haemolytica* strains have been sequenced using different sequencing methods and annotation tools. It is very likely, that there is wrong or missing information in every single sequence, but used as a whole, these errors are reduced. The four methods that were used to close the seven genomes of *M. haemolytica* are 454 pyrosequencing, Illumina, single-molecule real-time (SMRT) sequencing and the chain termination method, also called Sanger sequencing (Table 3). These methods produce reads of different lengths. SMRT produces the longest reads (avg. 7,000 bp), followed by Sanger and 454 sequencing (avg. 700 bp), while Illumina produces the smallest reads (avg. 250 bp) (ROBERTS 2013; LIU 2012). Longer reads usually mean longer contigs and a lower number of gaps between contigs after sequence assembly. Closing gaps is a laborious process that bears the risk of losing information. This might happen, if two contigs that harbor repeat regions at their respective ends are connected directly, while actually a small contig might be located between them. Two other important factors to consider are the number of reads
produced in a single run and their accuracy. While Sanger does have the highest accuracy (99.99%) of the methods, it produces only one, single read. The next most accurate method is 454 pyrosequencing (99.9%), which produces one million reads. Illumina has an accuracy of 98%, but it produces 3 billion sequences to compensate for it (LIU 2012). SMRT has the lowest accuracy (87%) of its single reads and it produces only 400 million sequences. However, using the technology to its fullest, PacBio developed a sequence assembler that uses small SMRT sequences to polish the contigs, thereby pushing the accuracy to 99.999%. (ROBERTS 2013)

Problems may also arise in using 454 pyrosequencing, since it is known to be prone to mistakes in homopolymer stretches (WIRAWAN 2014). This is especially problematic within CDS, since an additional or missed base will shift the reading frame. With the exception of strain USMARC_2286, all other genomes were closed using more than one sequencing method, decreasing the possibilities for mistakes (Table 3). Both the genomes USMARC-183 and USMARC-185 had no gaps after they were assembled, leaving the possibility of assembly mistakes to the used assembly program Celera. The best sequencing result can be expected from sequence D153, which combines 454, Illumina and SMRT sequencing, combined with the usage of optical mapping (HAUGLUND 2013).

Table 3.: All M. haemolytica sequences available in the NCBI database with a complete genome (accessed: 02.07.2014).

<table>
<thead>
<tr>
<th>Name</th>
<th>Size (Mb)</th>
<th>GC%</th>
<th>RefSeq</th>
<th>Genes</th>
<th>Serotype</th>
<th>Sequencing method</th>
</tr>
</thead>
<tbody>
<tr>
<td>USMARC-185</td>
<td>2.54</td>
<td>40.83</td>
<td>NC_020834.1</td>
<td>2617</td>
<td>A6</td>
<td>454 &amp; SMRT</td>
</tr>
<tr>
<td>USMARC-183</td>
<td>2.66</td>
<td>40.91</td>
<td>NC_020833.1</td>
<td>2815</td>
<td>A1</td>
<td>454 &amp; SMRT</td>
</tr>
<tr>
<td>M42548</td>
<td>2.73</td>
<td>41.05</td>
<td>NC_021082.1</td>
<td>2888</td>
<td>?</td>
<td>454 &amp; Illumina</td>
</tr>
<tr>
<td>D174</td>
<td>2.70</td>
<td>41.07</td>
<td>NC_021739.1</td>
<td>2814</td>
<td>A6</td>
<td>454, Illumina &amp; SMRT</td>
</tr>
<tr>
<td>D153</td>
<td>2.68</td>
<td>41.08</td>
<td>NC_021743.1</td>
<td>2766</td>
<td>A1</td>
<td>454, Illumina &amp; SMRT</td>
</tr>
<tr>
<td>D171</td>
<td>2.50</td>
<td>41.14</td>
<td>NC_021738.1</td>
<td>2487</td>
<td>A2</td>
<td>454, Illumina &amp; SMRT</td>
</tr>
<tr>
<td>USMARC_2286</td>
<td>2.66</td>
<td>41.06</td>
<td>NC_021883.1</td>
<td>2791</td>
<td>?</td>
<td>SMRT</td>
</tr>
</tbody>
</table>

Additionally, the sequence of strain 42548 sets itself apart from the other M. haemolytica sequences by being the only strain harboring antimicrobial resistance genes. Strain 42548 can be considered multiresistant, as it is resistant to at least three classes of antimicrobial agents.
Even though strain 42548 has not been typed, it is very likely that it belongs to serotype A1, since it shares the highest sequence identity with the complete genome sequences of USMARC-183 and D153, both of which belong to serotype A1.

8.4. Analysis of the multiresistance mediating mobile element ICEMh1

Integrative and conjugative elements have been identified in several different species, including Pasteurellaceae. ICEMh1 is the first documented ICE within M. haemolytica, but is closely related to ICEPmu1.

A recent study obtained several isolates of different Pasteurellaceae from samples collected from 68 BRD cases with fatal outcome. They identified ICEs by PCR mapping approaches in M. haemolytica (32.7%), P. multocida (37.5%) and H. somni (30%), all of which confer resistance to at least three classes of antimicrobial agents, and can therefore be considered multiresistant. Except for the danofloxacin resistance determinant, all resistance genes could be associated with the harbored ICE (KLIMA et al. 2014). Within strain 42548, ICEMh1 harbors all identified antimicrobial resistance genes, encoding resistance to kanamycin/neomycin (aphA1), streptomycin (strA and strB), sulfonamides (sul2) and tetracycline [tetR-tet(H)]. These resistance genes are arranged in a structure very similar to the resistance gene regions 1 and 2 in ICEPmu1 (MICHAEL et al. 2012a). However, several resistance genes that can be found in ICEPmu1 are not present in ICEMh1 (Figure 1). While resistance region 1 in ICEPmu1 has IS elements flanking the region that is missing in ICEMh1, this is not the case in resistance region 2. One possible explanation would be the development of ICEMh1 and ICEPmu1 from a common ancestor, before the missing resistance genes were acquired. Another possibility would be that these genes were deleted. In resistance region 1, the IS elements might have recombined in a faulty manner, leaving a disrupted ISAp1l, while in resistance region 2, the tetR genes could have recombined excising the sequence between them.

ICEMh1 has a size of 92,345 bp, of which the first 28,497 bp (30.86%) match with a putative ICE region found in M. haemolytica USDA-ARS-USMARC-183 (NC_020833.1), while the remaining 63,848 bp (69.14%) correspond to sequences found in ICEPmu1. The observation,
that no IS elements or overlapping base homologies are present in the vicinity of the point of transition, rules out the most common explanations for this apparent recombination. The genetic setup for different bacterial homologous recombination systems is present, but without any overlap larger than 1 bp, it is very unlikely that this recombination was facilitated by them. The hypothetical protein MHH_c22530, that is close to the point of recombination, is classified as MobA in *Bibersteinia trehalosi* USDA-ARS-USMARC-190 (CP006956.1), but has only 15 aa in common with the referenced MobA in the plasmid pMbo4.6 of *Moraxella bovis* (NC_013500.1).

ICE*Mh1* shares high identity with ICE*Pmul* and putative ICE regions found in *M. haemolytica* USDA-ARS-USMARC-183 and *B. trehalosi* USDA-ARS-USMARC-190. Moreover, all mentioned isolates were collected in different parts of North America and years apart from each other. *M. haemolytica* USDA-ARS-USMARC-183 is the oldest isolate and was collected in Kansas in 1991, followed by *P. multocida* 36950 in 2005 in Nebraska. *M. haemolytica* 42548 in 2007 from a feedlot in Pennsylvania and *B. trehalosi* USDA-ARS-USMARC-190 was collected in Nebraska in 2010. It has been shown, that *B. trehalosi* and *P. multocida* inhibit the growth of *M. haemolytica* in a contact-dependent mechanism (BAVANANTHASIVAM et al. 2012; DASSANAYAKE et al. 2009). This stress might increase the transfer frequency of ICEs and thereby promote the spread and the recombination of the elements.

Finding ICEs that carry multiple, different antimicrobial resistance genes in *M. haemolytica* field isolates from BRD cases that can be passed on to other BRD-associated pathogens proves the threat to therapeutic antimicrobial strategies in all fields, not only for the treatment of BRD.
Figure 1: Schematic comparison of the resistance gene regions 1 (upper left) and 2 (upper right) of ICE\textit{Mh1} and ICE\textit{Pmu1}, as well as a schematic comparison of the entire ICE\textit{Mh1} with ICE\textit{Pmu1} and the putative ICE region of \textit{M. haemolytica} USDA-ARS-USMARC-183. Genes are presented as arrows, with the arrowhead indicating the direction of transcription. Integrase genes are represented by blue arrows, green arrows depict relaxase genes, while red arrows indicate resistance genes. Insertion sequences and IS elements are shown as boxes, with the yellow arrows inside the boxes indicating the transposase genes. A dark grey background behind the arrows indicates a core region. Areas between the ICEs shaded in a light grey indicate regions of $\geq 67\%$ sequence identity between ICE\textit{Mh1}, ICE\textit{Pmu1} and the putative ICE region of \textit{M. haemolytica} USDA-ARS-USMARC-183, while dark grey shaded areas indicate $\geq 99\%$ sequence identity.
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