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Reflection paper on use of pleuromutilins in food-producing animals in the European Union: development of resistance and impact on human and animal health

Draft

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CVMP recommendations for action

Pleuromutilins (tiamulin and valnemulin) are used predominately in pigs but to some extent also in poultry and rabbits. It is an essential group of antimicrobial agents in veterinary medicine especially as it is the sole treatment option for enteritis in pigs caused by *Brachyspira hyodysenteriae* (swine dysenteria) resistant to macrolides. The negative consequences in case such a pathogen becomes pleuromutilin resistant would thus be considerable both from an economical and an animal welfare perspective.

There are several products available on the EU market and most of the use is for group medication in feed or water. In addition to the originator products there are a number of different tiamulin containing generics available on the market and the approved indications varies considerably. The overall use differs considerably between EU countries without known rational. Partly the difference might reflect difference in prevalence for the major diseases where pleuromutilins is used but there is likely additional explanation that would merit further exploration.

To contain resistance it is crucial to reduce unnecessary use and to avoid that antimicrobial agents are used preventively without applying adequate control programmes. For this reason it is important that responsible use principles are outlined in the SPCs for approved products.

The CVMP notes that medicinal products containing pleuromutilins are being developed for treatment against infections caused by meticillin resistant *Staphylococcus aureus* (MRSA) in humans. MRSA may be a zoonosis and thus the risk for spread of resistance from animals to humans is also to be considered. As detailed in the reflection paper on MRSA in food producing and companion animals in the EU (EMA, 2009), the CVMP believes the animal associated MRSA is best addressed by promotion of responsible use of antimicrobials in general and therefore all measures listed below would indirectly be effective reducing also the risk for MRSA although they are primarily intended to reduce the risk for resistance in target animal pathogens.

For veterinary medicinal products containing pleuromutilins the CVMP concluded that the following recommendations are for consideration by Competent Authorities:

Pleuromutilins should only be used for treatment of disease. The exception would be in well-defined eradication programme for swine dysenteria. Such eradication programmes should be restricted in time and include appropriate measures of effectiveness. This should be clearly stated in the SPCs for all products. Approved indications for unspecified prevention of disease should be withdrawn.

Indications should be worded to clearly express the intended use. General indications against infections in general should be avoided. Enhancement of productions (e.g. increase of feed efficiency or growth promotion) is not regarded as an acceptable indication and should be withdrawn.

Duration of treatment should be limited to the time needed for cure of diseases. Summary of Product Characteristics where the approved treatment duration is found unnecessarily long should be reviewed.

To the extent possible doses should be selected considering antimicrobial resistance related risks. In case data on dose selection are sparse doses should anyway be reviewed and in case they are too low (e.g. compared to other products containing the same active substance) this should be addressed. Notably there are often several different doses approved for different indications and thus there is an option to increase doses where relevant without asking for new tolerance or safety data.

Additional comments

Notwithstanding the list of recommendations above, the CVMP is of the opinion that antimicrobial resistance should not be considered in isolation but a global approach to the problem is needed. Implementation of prudent use principles remains a cornerstone to contain resistance together with biosecurity and other measures to promote animal health and thereby reduce the need for treatment.

Swine dysenteria is a major disease in pig production in EU. Eradication programme have successfully been applied in some countries in EU and CVMP would like to stress that such programme are crucial to reduce the need for pleuromutilins. CVMP would therefore recommend responsible bodies to take further action against this disease. Noteworthy, control of spread of disease is crucial as resistant bacteria will spread with transport and trade of animals.

There is today no system in place to provide harmonised monitoring data for pleuromutilin resistance in *Brachyspira hyodysenteriae*. CVMP would recommend responsible bodies to create such a monitoring system, first to allow baseline data to be collected and later to allow impact assessment of taken measures. Ideally, early warning systems should be created where treatment failures in case of swine dystenteria is systematically monitored. Furthermore, it is recommended to further explore the differences in pleuromutilin use between EU countries and investigate possible co-variability between use and resistance.

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1. Introduction

Pleuromutilin is a natural antimicrobial substance produced by the fungus (basidiomycete) *Pleurotus mutilus* (Kavanagh et al., 1951) now called *Clitopilus scyphoides* (Singer, 1986). Tiamulin and valnemulin are semi-synthetic derivatives of pleuromutilin. Both tiamulin and valnemulin are used exclusively in veterinary medicine. Tiamulin was approved for use in veterinary medicine in 1979, followed by valnemulin in 1999 (Sader et al., 2012a). Retapamulin was the first pleuromutilin approved for topical use in humans in 2007 (Novak, 2011). BC-3781, a pleuromutilin for systemic use in humans, is currently under development (Sader et al., 2012a; Sader et al., 2012b).

Pleuromutilins are antibacterial agents that inhibit protein synthesis by binding to the 50S subunits of ribosomes of bacteria. Pleuromutilins are active against Gram-positive bacteria such as streptococci and staphylococci, anaerobic bacteria and *Mycoplasmata* (Giguere, 2006; Islam et al., 2009; Jones et al., 2006) . They have been used for decades in veterinary medicine for the control of respiratory and intestinal infections in different animal species, especially in pigs and to a lesser extent in poultry and rabbits (Giguere, 2006; Islam et al., 2009). The objective of this reflection paper is to summarize current knowledge on resistance development and the potential impact of this resistance on animal and human health as detailed in a concept paper (http://www.ema.europa.eu/ema/pages/includes/document/open_document.jsp?webContentId=WC500116978).

2. The use of pleuromutilins in veterinary medicine

In the Member States of the European Union tiamulin is authorised nationally and available in most of the EU Member States. Following a recent referral, the indications for tiamulin (EMA, 2010a) in pigs are treatment and prevention of swine dysentery (*Brachyspira hyodysenteriae*), treatment of colitis (*Brachyspira pilosicoli*), treatment of ileitis (*Lawsonia intracellularis*) and treatment of enzootic pneumonia (*Mycoplasma hyopneumoniae*). However as different products containing tiamulin are nationally approved other indications might still be listed. Tiamulin is also authorised for *Gallus gallus* (chickens) for the treatment and prevention of chronic respiratory disease (CRD) and airsacculitis caused by *Mycoplasma gallisepticum* and *Mycoplasma synoviae* and for turkeys for treatment and prevention of infectious sinusitis and airsacculitis caused by *Mycoplasma gallisepticum*, *Mycoplasma meleagridis* and *Mycoplasma synoviae* and for rabbits for treatment of epizootic rabbit enterocolitis. Valnemulin is authorised centrally for treatment and prevention of swine dysentery, spirocheatal diarrhoea and enzootic pneumonia and for treatment of clinical sings of porcine proliferative enteropathy[1]. Tiamulin is available as medicated feed premix and as a solution for medication in drinking water (Islam et al., 2009). In addition it is available as injectable for pigs. Valnemulin is available as premix for feed and oral powder[2]. Pleuromutilins are also used off-label to treat the polyetiological disease porcine respiratory disease complex (PRDC), and more rarely, leptospirosis (Giguere, 2006).

Pleuromutilins have become frequently used drugs in the treatment of swine, especially in weaner pigs and finisher pigs (Jensen et al., 2012).

In pigs the dose of valnemulin varies between 1 and 12 mg/kg bw according to the indication and the duration of treatment can be related to clinical response and varies between 7 and 28 days (EMA,

[1] See http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/veterinary/medicines/000042/vet_med_000116.jsp&mid=WC0b01ac058001fa1c

[2] http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Assessment_Report_-_Variation/veterinary/000042/WC500100721.pdf

2011). According to the authorisation for valnemulin, long term preventative use of valnemulin should be avoided by improving management practice and thorough cleansing and disinfection and consideration should be given to the eradication of infection from the farm.

In pigs the dose following the article 34 referral for tiamutin premix (EMA, 2010b) varies between 2 and 10 mg/kg bw according to the indication and the duration of treatment can be related to clinical response and varies between 7 and 28 days. According to the outcome of the referral (EMA, 2010b) preventive treatment with tiamulin should only be initiated after confirmed infection with *B. hyodysenteriae* and then as part of a program including measures aiming to eradicate or control the infection in the herd. It is not known if such recommendation in included in the SPC for other products containing tiamulin.

There is limited data on the extent to which this advice on preventive use is followed; it is very important that such use is not undertaken without appropriate accompanying measures in order to minimise the emergence of resistance.

Data on trends in sales of antimicrobials 2005-2009 in nine countries were used to assess trends over time (ESVAC, 2011). Data for one country (Switzerland) was excluded as no data was available for 2005. The total sales of pleuromutilins expressed in tonnes active substance was divided by an estimate of the live weight of pigs expressed as mg per population correction unit (PCU). The PCU takes into account the estimated weight of livestock, slaughtered animals and transport of animals for fattening and slaughter in another Member State. It is probable that in most countries, most of the sales are for pigs but as pleuromutilins are also authorised for poultry, data are expressed both as mg/PCU pigs and as mg/PCU of pigs and poultry. The results shown in Figure 1 indicate an overall increasing trend in overall sales from 2005 to 2008 followed by a slight decrease in 2009.

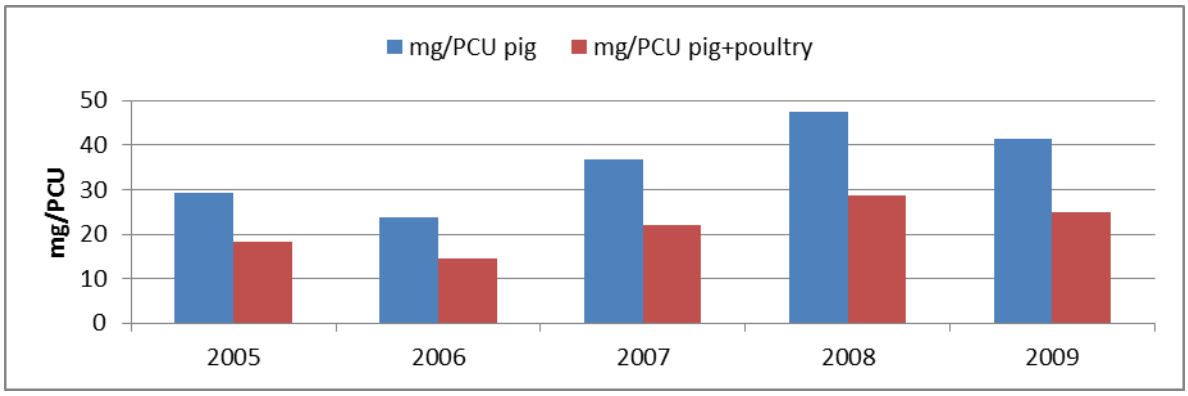

Figure 1. Total sales of pleuromutilins in eight countries expressed as mg/population correction unit (PCU) pigs and as mg/ PCU pig+poultry.

Data on sales of pleuromutilins in 19 countries in 2010 are shown in Figure 2 (based on data from (ESVAC, 2012)). One Member State, Lithuania had no sales of pleuromutilins in 2010. As above, the sales in tonnes were calculated to mg/PCU pig and mg/PCU pig and poultry to represent an approximation of the exposure of the pig or pig and poultry population. Acknowledging that in some countries pleuromutilins will be used only for pigs while in others they are also used in poultry, the data still indicate that the population exposure varies widely between countries.

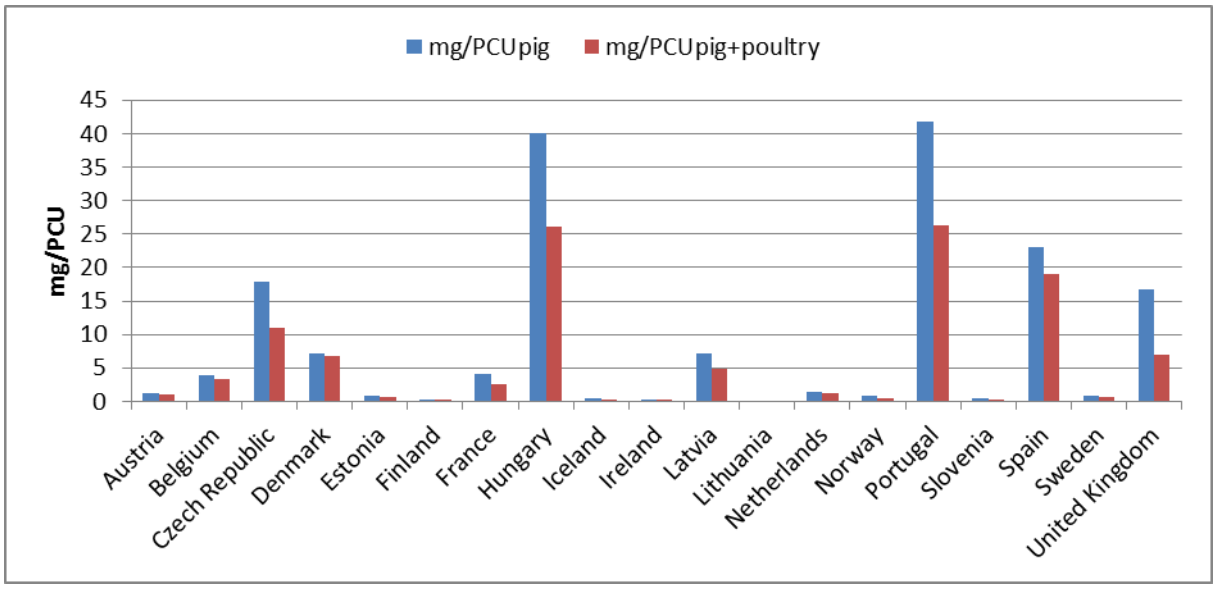

Figure 2. Sales of pleuromutilins expressed as mg/population correction unit (PCU) of pigs and of pigs and poultry.

Almost all of the sales are products formulated for in feed or in water medication (Figure 3), although the relative proportion between the different formulations varies between countries (Figure 4).

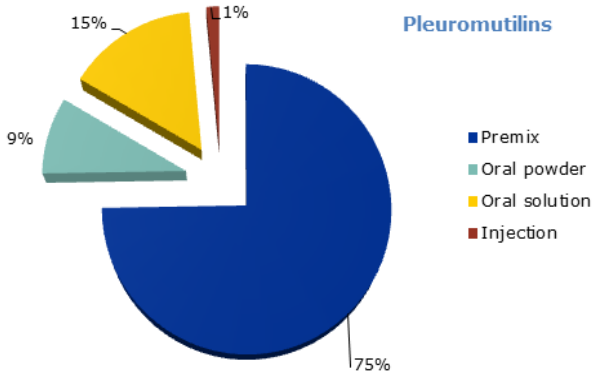

Figure 3. Sales of pleuromutilins by pharmaceutical form in 18 countries (no sales in Lithuania) expressed as percent of total sales in tonnes.

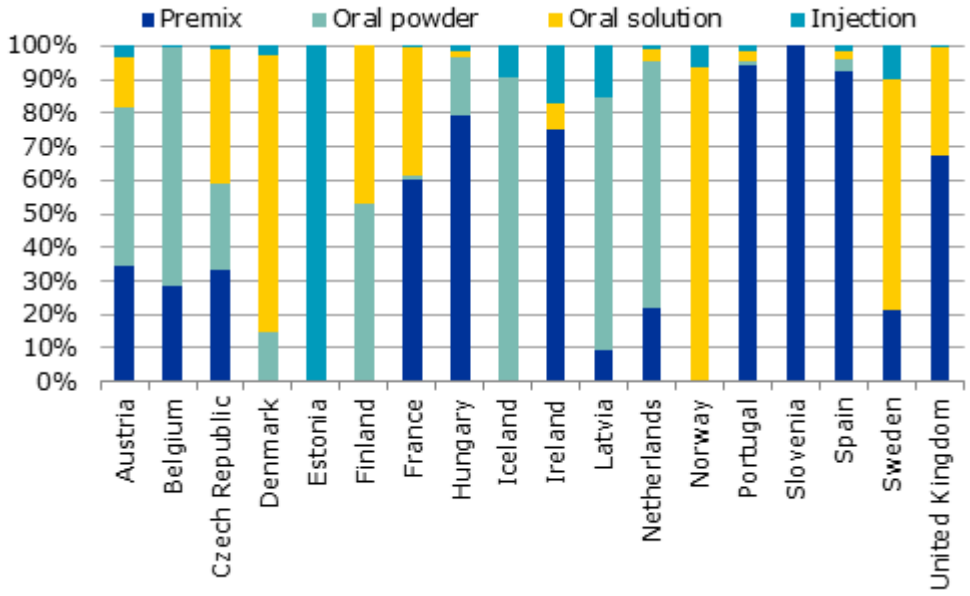

Figure 4. Sales of pleuromutilins in 18 countries (no sales in Lithuania) by pharmaceutical form expressed as percentage of the sales in tonnes in each country.

3. Mechanisms and emergence of resistance in relevant bacteria

Pleuromutilins act by inhibiting protein synthesis by binding to the 50S subunit of the bacterial ribosome. They are strong inhibitors of peptidyl transferase. Resistance derives from chromosomal mutations in the 23 rRNA and *rplC* genes. These chromosomal mutations emerge relatively slowly and in a stepwise fashion and are not transferred horizontally (Giguere, 2006). In addition, resistance genes can be located on plasmids or on transposons like the *vga* genes and the *cfr* gene (Long et al., 2006; Mendes et al., 2011; Novak, 2011; Schwendener and Perreten, 2011; Witte and Cuny, 2011). This type of resistance is transferable between bacteria and bacterial species. The mechanism of antimicrobial resistance varies according to the bacterial species investigated (Gentry et al., 2008; Long et al., 2006; Malbruny et al., 2011; Pringle et al., 2004; Wang et al., 2012a; Wang et al., 2012b; Wang et al., 2012c).

B. hyodysenteriae: The decreased susceptibility to tiamulin in *B. hyodysenteriae* clinical and in laboratory selected isolates has been associated to point mutations in the domain V of the 23S rRNA gene (positions 2032, 2055, 2447, 2499, 2504 and 2572 *Escherichia coli* numbering) and/or the ribosomal protein L3 gene (Hidalgo et al., 2011; Pringle et al., 2004). Mutation in the nucleotide position 2032 seems to cause resistance to pleuromutilins as well as decreased susceptibility to lincosamides (Hidalgo et al., 2011). Tiamulin resistance in *B. hyodysenteriae* develops gradually and in a stepwise manner both in vitro and in vivo suggesting that multiple mutations are needed to achieve high level resistance (Karlsson et al., 2001; Karlsson et al., 2002). The MICs for valnemulin follow those for tiamulin in most cases but are generally a few dilution steps lower (Pringle et al., 2012). No resistance mechanism has yet been detected for *B. pilosicoli* (Pringle et al., 2012).

Staphylococcus species: Resistance in staphylococci can be due to point mutations in the domain V of 23S rRNA or in the *rplC* gene encoding the ribosomal protein L3. Selected mutants of *S. aureus* resistant to linezolid exhibit cross-resistance to tiamulin (Gentry et al., 2007; Miller et al., 2008).

Transferable resistance in *S. aureus* and coagulase-negative staphylococci can be caused by *vga* genes, encoding ABC transporters, which export pleuromutilins, streptogramin A and lincosamides (Gentry et al., 2008; Hauschild et al., 2012). There are 7 known pleuromutilin-streptogramin resistance genes: *vga*(A), *vga*(A)v, *vga*(A)$_{LC}$, *vga*(B), *vga*(C), *vga*(D) and *vga*(E) (Allignet and El Solh, 1997; Allignet et al., 1992; Jung et al., 2010; Kadlec and Schwarz, 2009; Schwendener and Perreten, 2011). Except for *vga*(D) which was found on a plasmid in *Enterococcus faecium* (Jung et al., 2010) all other genes were found on plasmids or transposons of staphylococci. Transferable resistance to pleuromutilins due to *vga* genes has been reported in methicillin-resistant *S. aureus* (MRSA) (Kadlec et al., 2010; Kadlec and Schwarz, 2009). Since 2005, a specific clone of MRSA, ST398 has emerged worldwide in livestock, especially swine (Catry et al., 2010; de Neeling et al., 2007). This clone is referred to as livestock-associated MRSA (LA-MRSA). Mendes, Smith et al. (2011) report the plasmid borne *vga* (A) gene in MRSA ST398 from a pig and a pig farmer in the United States. Kadlec and Schwarz (2009) identified a novel ABC transporter gene *vga*(C) which is located on the multidrug resistance plasmid pKKS825 in MRSA ST398. The *vga* (A)v gene has been detected in MRSA ST49 strains from pigs in Switzerland (Overesch et al., 2011). Porcine MRSA ST398 carrying small plasmids containing *vga*(A) or *vga*(C) genes have been identified in Portugal (Kadlec et al., 2010). Recently a new transposon Tn6133 containing *vga*(E) has been found in porcine MRSA ST398 isolates (Schwendener and Perreten, 2011). The *vga*(E) gene, located on the same transposon, has also been detected in MRSA ST398 in clinical isolates from turkey and cattle as well as from chicken and turkey meat in Germany (Hauschild et al., 2012). This indicates that this resistance gene is disseminating in different countries and different animal species. *S. hominis* clinical isolates with low-level resistance to quinupristin/dalfopristin have also been shown to contain *vga*(A) (Petinaki et al., 2005).

Transferable resistance against five chemically distinct classes of antimicrobials (oxazolidinones, phenicols, streptogramin A, lincosamides and pleuromutilins) is mediated by the gene *cfr* encoding a rRNA methylase (Kehrenberg et al., 2007; Kehrenberg and Schwarz, 2006; Shore et al., 2010; Witte and Cuny, 2011). These antimicrobials bind to overlapping sites at the peptidyl transferase center. Each of these classes of antimicrobials contains important drugs that are used in human and veterinary medicine. This gene has been reported from several countries including Germany, Denmark, Spain, Ireland and China. It has been found in humans and animals, including pigs (Gopegui et al., 2012; Kehrenberg et al., 2009; Morales et al., 2010; Shore et al., 2010; Wang et al., 2012c). The gene was first detected on a plasmid originating from a bovine strain of the coagulase negative *Staphylococcus sciuri* (Schwarz et al., 2000) and has also been found in other coagulase-negative staphylococci (Kehrenberg et al., 2007; Kehrenberg and Schwarz, 2006). The *cfr* gene has also been found on a plasmid in porcine MRSA and MSSA of different clonal lineages (ST398 and ST9) (Kehrenberg et al., 2009). Recently the *cfr* gene has been detected in a Panton-Valentine-Leukocidin positive MRSA of ST8 SCC*mec* type IV (USA300) (Shore et al., 2010). USA300 is a major community acquired MRSA causing skin and soft tissue infections in the United States of America and worldwide. A new multidrug resistance conjugative plasmid (pERGB) containing *cfr*, *tet*(L) (encoding tetracycline resistance), ant('4)-Ia (encoding tobramycin resistance) and *dfr*K (encoding trimethoprim resistance) was detected in a linezolid resistant MRSA strain with sequence type ST125. This MRSA strain was isolated from two patients with chronic obstructive pulmonary disease in Spain and both patients had been treated with linezolid (Gopegui et al., 2012). An outbreak of linezolid resistant *cfr*-positive MRSA has been reported in a Spanish hospital (Sanchez Garcia et al., 2010).

Recently, the enterococcal ABC transporter gene *Isa*(E) conferring resistance to pleuromutilins, lincosamides and streptogramin A has been detected in MSSA and MRSA (Wendlandt et al., 2012), suggesting exchange of this gene between *Enterococcus* spp. and *Staphylococcus aureus*.

E. coli: *Cfr*-mediated resistance has also been detected in *E. coli* (Long et al., 2006; Wang et al., 2012a). Analysis of 1230 *E. coli* isolates from pigs, ducks and chickens in China revealed one *cfr*

positive isolate originating from a nasal swab of a pig. In addition to *cfr* this isolates also harboured the florfenicol resistance gene floR (Wang et al., 2012a).

Proteus vulgaris: The *cfr* gene has been reported in one *Proteus vulgaris*. This isolate was found when screening 557 nasal swabs of Chinese swine for florfenicol resistance (Wang et al., 2011). The isolate was also positive for the *floR* gene.

Enterococcus species: Liu et al. (2012a) and Liu et al. (2012b) reported the occurrence of the *cfr* gene in *Enterococcus faecalis* isolated from bovine and pig faeces in China. Insertion elements have been detected on a plasmid containing *cfr* and are thought to play an important role in the dissemination of resistance genes (Liu et al., 2012b). In *Enterococcus faecalis* resistance to pleuromutilins, streptogramin A and lincosamides is mediated by the *Isa*(A) gene (Wendlandt et al., 2012).

Bacillus species: The *cfr* gene located on a plasmid has been found in a *Bacillus* species isolated from a nasal swab of a pig in China (Wang et al., 2012b). The plasmid also carried a novel streptomycin resistance gene. Another *Bacillus* isolate, containing *cfr* and *erm*(B) conferring resistance to macrolides, lincosamides and streptogramin B located on a plasmid and fexA conferring resistance to florfenicol located on the chromosomal DNA was found in an isolated from swine faeces in China (Dai et al., 2010).

Streptococcus agalactiae: Cross-resistance to pleuromutilins, lincosamides and streptogramin A has been found to be caused by a novel gene called *Isa*(C). Expression of this gene in *S. agalactiae* led to increased minimal inhibitory concentrations (MICs) of lincomycin, clindamycin, dalfopristin, and tiamulin (Malbruny et al., 2011). The gene was found in 18 clinical isolates from humans in New Zealand (Malbruny et al., 2011).

Mycoplasma gallisepticum: Li et al. (2010a) studied the in vitro development of resistance to tiamulin and valnemulin in *Mycoplasma gallisepticum*. A single mutation of the 23S rRNA gene could cause elevated tiamulin and valnemulin MICs, but combinations of two or three mutations were necessary to produce high level resistance to these drugs. All mutants were cross-resistant to lincomycin, chloramphenicol and florfenicol and some mutants also to erythromycin, tilmicosin and tylosin (Li et al., 2010b).

Mycobacterium smegmatis: Long et al. (2010) found that single or double mutations at various locations in the 23S rRNA of *Mycobacterium smegmatis* resulted in unpredictable cross resistance between linezolid, chloramphenicol, clindamycin and valnemulin.

Data on resistance mechanisms of *Lawsonia intracellularis* are lacking.

4. Problems of susceptibility testing

Generally, accurate antimicrobial susceptibility testing of anaerobic, fastidious bacteria can be difficult to achieve. Different anaerobes require different supplements to the growth medium and this causes problems with standardisation of the methods. The fastidious nature of *B. hyodysenteriae* and *B. pilosicoli* has hampered standardisation of methods for antimicrobial susceptibility testing. Antimicrobial susceptibility tests of *Brachyspira* spp. are not always performed on a routinely basis and there are no generally approved or recommended standards available. Different methods have been used such as broth dilution, microbroth dilution and agar dilution (Burch, 2005). Published susceptibility testing of *Brachyspira* spp. has been performed predominantly of *B. hyodysenteriae* isolates and by the agar dilution procedure. The most common medium used is trypticase soy agar (TSA) supplemented with 5% bovine or ovine blood. The MIC has been determined as the lowest concentration of the antimicrobial agent that prevents growth or hemolysis. A broth dilution method

has been evaluated for monitoring of antimicrobial susceptibility in *Brachyspira* spp. (Karlsson et al., 2003). MIC quality control ranges for the type strain of *B. hyodysenteriae*, B78T (ATCC® 27164T), has been established in an inter-laboratory study for this method (Pringle et al., 2006a). For pleuromutilins, this method has been compared with agar dilution (Rohde et al., 2004). Both methods gave reproducible results, but the broth method on average gave one dilution lower MICs for both tiamulin and valnemulin.

Antimicrobial susceptibility of *Lawsonia intracellularis* is difficult as this obligate intracellular bacterium needs complicated cell culture systems to grow and published data on their in vitro susceptibility are scarce and include only a very limited number of isolates (McOrist et al., 1995; Wattanaphansak et al., 2009; Yeh et al., 2011).

Internationally accepted interpretative criteria are lacking except for tiamulin for *Actinobacillus* species (Clinical Laboratory Standards Institute (CLSI) 2012). To date, no tiamulin or valnemulin breakpoints have been established for *Brachyspira* species, but breakpoints of ≥ 2µg/ml (Clothier et al., 2011) have been used to classify isolates as resistant to tiamulin. According to Vyt and Hommez (2006) and Karlsson et al. (2003) this breakpoint for tiamulin is too high to indicate decreased susceptibility. On the basis of a field study on clinical efficacy it has been proposed that isolates with MICs ≥ 1µg/ml should be considered as not responding to therapy in vivo (Vyt and Hommez, 2006). Suggestion for interpretative criteria for tiamulin disk diffusion have been made for *Pasteurella multocida*, staphylococci, *Actinobacillus suis*, *Actinobacillus pleuropneumoniae* and *Erysipelothrix rhusiopathiae* (Jones et al., 2002). Burch (2005) suggested a breakpoint of >0.125 µg/ml for valnemulin (75 ppm in feed) using broth dilution and >0.25 µg/ml for the agar dilution method for *Brachyspira* species. For tiamulin (at a dose of 100 ppm) a breakpoint of > 0.5 µg/ml and >1.0 µg/ml was suggested for broth dilution and agar dilution respectively (Burch, 2005). Pringle, Landen et al. (2012) suggest epidemiological cut-off values for monitoring antimicrobial susceptibility in *Brachyspira hyodysenteriae* of >0.25 µg/ml for tiamulin and >0.125 µg/ml for valnemulin.

5. Occurrence of resistance in bacteria from food producing animals

Brachyspira species: An increase of the MICs of tiamulin and valnemulin for porcine *B. hyodysenteriae* isolates. An increase in MIC's of tiamulin and valnemulin against *B. hyodysenteriae* has been reported in several countries. Reduced in vitro susceptibility of *B. hyodysenteriae* has been reported from Japan (Ohya and Sueyoshi, 2010), Spain (Hidalgo et al., 2011), The Netherlands (Duinhof et al., 2008), Germany (Rohde et al., 2004), Hungary (Molnar, 1996), the United Kingdom (Gresham et al., 1998) and Czech Republic (Lobova et al., 2004; Sperling et al., 2011). A study investigating 20 *Brachyspira intermedia* isolates from layers in Belgium and the Netherlands found that the MIC distribution was monomodal, but with tailing towards higher MIC values, possibly indicating low level acquired resistance in six isolates (Verlinden et al., 2011). Decreased susceptibility to tiamulin has also been found in *B. pilosicoli* isolates from Sweden (Pringle et al., 2006a) and in various *Brachyspira* spp. from the United States of America (Clothier et al., 2011). Seven out of 79 (4.7%) and 4/79 (3.2 %) *Brachyspira* isolates were resistant to tiamulin and valnemulin respectively applying a MIC ≥2 as breakpoint (Clothier et al., 2011). In Spain the susceptibility to tiamulin and valnemulin of *B. hyodysenteriae* decreased in 2008/2009 compared to previous years (Hidalgo et al., 2011). Resistance to pleuromutilins seems to be common in *B. hyodysenteriae* in Spain (Hidalgo et al., 2011). An increase in MICs was also seen in Japan were MICs for tiamulin and valnemulin were low and MIC distribution unimodal from 1985-2000, but higher MICs were recorded from 2001 onward and the distribution had a trend towards a bimodal distribution (Ohya and Sueyoshi, 2010). The MIC90 of Czech *B. hyodysenteriae* isolates increased from 0.25µg/ml in 1997 to 4 µg/ml in 2001 for tiamulin

and from ≤0.031 in 1997 to 8 in 2001 for valnemulin (Lobova et al., 2004). In Germany MIC90 for tiamulin increased from 0.125 µg/ml (1989-1993) to 2-8 µg/ml (2000-2002). For valnemulin the MIC90 increased from 0.063 µg/ml (1989-1993) to 2-4 µg/ml (2000-2002) (Rohde et al., 2004).

Resistance to tiamulin has also been reported in *Haemophilus parasuis* and *Actinobacillus pleuropneumoniae* (Aarestrup et al., 2008). To date, *cfr*-mediated resistance seems to be uncommon in *Enterobacteriaceae* such as *E. coli* and *Proteus vulgaris* (Wang et al., 2012a; Wang et al., 2011).

S. aureus and other staphylococci: A Canadian study found tiamulin MIC's to be significantly higher among MRSA ST398 than among human methicillin-susceptible *Staphylococcus aureus* and non-ST398 MRSA and porcine MSSA isolates (Rubin et al., 2011). Several studies have found *S. aureus* and MRSA isolates resistant to pleuromutilins (Gentry et al., 2008; Hauschild et al., 2012; Kadlec et al., 2009; Mendes et al., 2011). A high percentage of *S. aureus* isolates (40%) from slaughter pigs in Uruguay have been reported as resistant to tiamulin (Meyer et al., 2012). As described above, *cfr* and *vga* related transferable resistance in *S. aureus* including MRSA has been reported in different countries and different clonal lineages, including the livestock-associated MRSA ST398. In China, 149 staphylococcal isolates resistant to florfenicol were found when screening 557 pigs originating from 3 farms by taking nasal swabs. Of these isolates, 33 (22%) were found positive for *cfr* including *S. arlettae, S. saprophyticus, S. cohnii, S. sciuri* and *S. aureus*. Several isolates contained the florfenicol resistance gene fexA in addition to *cfr*. Four different *cfr* carrying plasmids were identified and these plasmids sometimes also harboured other resistance genes such as erm(C) and accA-aphD (Wang et al., 2012c). Co-selection of *cfr* carrying isolates could therefore occur under selective pressure imposed by the use of florfenicol, aminoglycosides or macrolides.

Lawsonia intracellularis: In *Lawsonia intracellularis* no resistance to pleuromutilins has been reported, but only very few isolates have been investigated and accepted interpretative criteria for such susceptibility testing are lacking (McOrist et al., 1995; Wattanaphansak et al., 2009).

6. Possible links between the use of pleuromutilins and other antimicrobials in animals and resistance in bacteria of animal origin

Brachyspira species: The lack of authorised and effective drugs for the treatment of swine dysentery has increased the use of pleuromutilins, and this probably explains the emergence of resistant strains. In a Belgian study the MIC's of pleuromutilins for *B. hyodysenteriae* isolates from 17 farms were correlated with clinical efficacy of the drugs in the treatment of swine dysentery; 88% of the swine farms (n=15) that performed well were associated with susceptible isolates, whereas unfavourable clinical outcomes were associated with decreased susceptibility on two farrow-to-finish farms (Vyt and Hommez, 2006). In the Netherlands tiamulin resistant *B. hyodysenteriae* isolates were cultured from the faeces of pigs. The isolates were also resistant against lincomycin, tylosin, doxycycline, and tylvalosin. The repeated use of tiamulin on the affected farm was assumed to be the main cause of the development of resistance to the drug (Duinhof et al., 2008). Generally the use of pleuromutilins is high in Spain, Portugal and Czech Republic and relatively high percentages of *Brachyspira* isolates resistant to pleuromutilins have also been reported from Spain (Hidalgo et al., 2011) and Czech Republic (Lobova et al., 2004; Sperling et al., 2011). Multidrug-resistant and pleuromutilin-resistant *B. hyodysenteriae* isolates were associated with farms with endemic incidence of swine dysentery (Sperling et al., 2011). Increased consumption of pleuromutilins has been incriminated as cause for the increase in MICs of tiamulin and valnemulin (Lobova et al., 2004).

Staphylococci: It has been suggested that the use of pleuromutilins very likely selects for the emergence of *cfr* in animal isolates of staphylococci (Witte and Cuny, 2011). It must be noted that many isolates resistant to pleuromutilins are multidrug resistant. Mobile elements containing genes mediating resistance to pleuromutilins often contain resistance genes that confer resistance to other classes of antimicrobials. Therefore not only the use of pleuromutilins, but also the use of other antimicrobials can select for pleuromutilin resistance through co-selection. Plasmids carrying *vga*(C) genes have been found to contain the tetracycline resistance gene *tet*(L), the kanamycin/neomycin resistance gene *aadD* and the trimethoprim resistance gene *dfrK* and therefore co-selection of *vga*(C) under selective pressure by the use of the other antimicrobials can potentially occur (Kadlec and Schwarz, 2009). Isolates harbouring the *vga*(E) gene were also resistant to beta-lactams, tetracyclines, trimethoprim, macrolides and lincosamides, spectinomycin and tiamulin and resistant or less susceptible to quinopristin/dalfopristin, which is used in human medicine to treat (severe) infections caused by MRSA and vancomycin resistant enterococci (Hauschild et al., 2012). Staphylococci carrying *cfr* were multidrug-resistant, resistance to erythromycin, tetracycline, spectinomycin, clindamycin and streptomycin being most common and three of six *cfr* positive isolates also carried the florfenicol resistance gene *fexA* (Kehrenberg and Schwarz, 2006).

Antibiotic usage records for Chinese pig farms indicate that multiple antimicrobial drugs, including florfenicol, lincomycin and tiamulin have been used on farms were *cfr* positive isolates have been found suggesting that selective pressure might have played a role (Wang et al., 2012b; Wang et al., 2011).

7. Impact on animal health and production

For most indications for which pleuromutilins are authorised there are alternative substances except for swine dysentery where high prevalence of resistance against alternative antimicrobials exists in many Member States. Therefore, pleuromutilins are the only remaining treatment option for this indication. Thus impact of resistance to pleuromutilins on animal health and production is likely to be highest in the case of swine dysentery. In herds affected by this infection, the disease usually has a considerable impact on animal health as well as on production economy (Hampson et al., 2006; Wood and Lysons, 1988). Due to the lack of commercial vaccines, control and treatment of swine dysentery depends on the use of effective antimicrobial drugs. In most EU Member States there are no national programmes for control of swine dysentery.

Occurrence of resistance among *B. hyodysenteriae* to antimicrobial agents commonly used for treatment of swine dysentery such as macrolides (tylosin) and lincosamides is common (Hidalgo et al., 2011; Ohya and Sueyoshi, 2010; Sperling et al., 2011). Therefore the number of antimicrobials available for the treatment of swine dysentery is limited. Alternatives such as carbadox or olaquindox, which are used in the United States of America, are not authorised in the EU. Thus, in many cases pleuromutilins are the only potentially effective choice among antimicrobials with swine dysentery as authorised indication. However, isolates with reduced susceptibility to pleuromutilins have emerged among *B. hyodysenteriae* in many countries (Duinhof et al., 2008; Hidalgo et al., 2011; Karlsson et al., 2004; Lobova et al., 2004; Ohya and Sueyoshi, 2010; Rohde et al., 2004; Sperling et al., 2011). Several of these reports document an increase in proportion of isolates with decreased susceptibility over time (Hidalgo et al., 2011; Ohya and Sueyoshi, 2010; Sperling et al., 2011), and in some cases therapy failure is described (Rohde et al., 2004; Sperling et al., 2011; Vyt and Hommez, 2006).

Lack of effective treatment options of swine dysentery would have considerable consequences for production economy due to mortality, impaired growth and secondary costs (Hampson et al., 2006). Depopulation of the farm and replacement with non-infected animals may in such cases be a last resort measure (Hampson et al., 2006). To summarise, the loss of pleuromutilins as effective tools to

treat swine dysentery because of a further increase in resistance or as a consequence of restrictions would present a considerable threat to the pig health, welfare and productivity.

8. Potential impact on human health

To date only one product containing pleuromutilins (retapamulin) is authorised for humans for topical use only. Concerns about lack of sufficient bioavailability, gastrointestinal side effects, hepatotoxicity, and the challenging side-chain chemistry of pleuromutilins labelled these drugs as difficult and hazardous to develop and several companies stopped their efforts in developing these drugs for human medicine (Novak, 2011). A new product BC-3781 is now being developed for oral and intravenous administration to humans with serious multidrug-resistant skin infections and respiratory infections, including MRSA (Sader et al., 2012a; Sader et al., 2012b). Investigations exploring pleuromutilins for the treatment of *Mycobacterium tuberculosis* infections in humans are ongoing (Lotesta et al., 2011). Therefore, potential implications of the emergence of resistance to pleuromutilins in *S. aureus* and MRSA, including the livestock associated MRSA ST398 need to be considered. The emergence of successful epidemic clones of MRSA, like ST8-Iva/USA300 and ST125 carrying plasmids containing *cfr* is cause for concern and warrants close surveillance, those PVL positive clones have not been reported in the EU pig population. Transfer of such plasmids between different bacteria and different hosts, including humans could potentially occur. The gene confers resistance to several important antimicrobials used in human medicine, such as oxazolidones and streptogramin A. Therefore, the emergence of this resistance gene in animals might pose a threat to human medicine as they might compromise empirical treatment of human MRSA infections. To date linezolid resistance in *S. aureus* of human origin is still uncommon. Resistance to linezolid can be mediated by chromosomal mutations, but also through the acquisition of *cfr* by horizontal transfer (Stefani et al., 2010). An outbreak involving 12 patients with linezolid resistant MRSA has been reported in an intensive care unit in a Spanish hospital. Eleven of these patients had been treated with linezolid. In addition 3 patients in other wards were also infected with the linezolid resistant MRSA. All 15 isolates from the outbreak carried *cfr.* Six patients died and one death was directly attributed to the resistant MRSA (Morales et al., 2010; Sanchez Garcia et al., 2010). Contact with animals was not investigated. Retapamulin demonstrated excellent in vitro activity against MSSA and MRSA strains, but not against MRSA isolates harbouring the *cfr* gene (Candel et al., 2011).

Retapamulin MICs of ≥2 µg/ml were found in only 6 out of 5676 clinical *S. aureus* isolates. The ABC proteins Vga(Av) and Vga(A) were responsible for the reduced susceptibility to pleuromutilins in these six isolates (Gentry et al., 2008). Livestock-associated MRSA containing the plasmid-borne *vga*(A) gene has been reported from pigs and a pig farmer in the United States indicating that zoonotic transmission may occur (Mendes et al., 2011).

A special concern is the recent emergence of *cfr*-encoded plasmid-mediated linezolid resistance in *Enterococcus faecalis* and *Enterococcus faecium* in human clinical isolates in several countries, including Thailand (Diaz et al., 2012) and the United Kingdom (http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1317135991530).

9. Summary assessment

Pleuromutilins are antimicrobial agents that are mainly used in veterinary medicine, especially in swine and to a lesser extent in poultry and rabbits. In pigs, tiamulin and valnemulin are used to treat swine dysentery, spirochaetal diarrhoea, porcine proliferative enteropathy, enzootic pneumonia and other infections where Mycoplasma is involved.

The vast majority of the sales of pleuromutilins comprise oral medication. Data on sales of pleuromutilins in different countries presented in figure 2 indicate that the amounts of pleuromutilins used vary markedly between countries. One possible explanation for this might be that in some countries, these substances are used more widely for treatment and prevention of not only swine dysentery but also porcine respiratory disease complex associated with *Mycoplasma spp.* and of infections with *Lawsonia intracellularis*. Other possible explanations might be differences in the prevalence of swine dysentery between the MSs and a high prevalence of resistance to alternative antimicrobials used to treat swine dysentery, e.g. the macrolides in countries with the highest use. A better understanding of the various factors explaining the observed differences would be valuable to support responsible use initiatives.

Decreased susceptibility of *B. hyodysenteriae* to pleuromutilins develops slowly and is caused by chromosomal mutations. The reported increase of the MICs of tiamulin and valnemulin against porcine *B. hyodysenteriae* isolates from different European countries is nevertheless alarming, as there is only a limited number of antimicrobials available for the treatment of swine dysentery and resistance to these antimicrobials is already very common. Considering that swine dysentery is a common and economic important disease a possible future loss of effective treatment could have considerable consequences for swine production.

Given the potential impact of resistance to pleuromutilins in *B. hyodysenteriae* on pig health, welfare and production, there is a need to include *B. hyodysenteriae* in national resistance monitoring programmes. Establishing approved standards for the methods used for susceptibility testing and accepted criteria for the interpretation of the results could help to monitor the development of resistance.

Strategies to control or eradicate the infection from a herd or region could be implemented in order to reduce the continuous need for pleuromutilins on farms were swine dysentery is endemic. Such strategies rely on the supply of breeding animals that are certified free from *B. hyodysenteriae* and in most cases utilise strategic treatment with pleuromutilins for a limited period as part of the eradication protocol (Vyt et al., 2007; Vyt and Hommez, 2006). Successful programmes are in place in e.g. Sweden, Norway and Finland (Pringle et al., 2012). Another option to reduce the use of pleuromutilins would be to reserve this class of antimicrobials for the treatment of swine dysentery as alternative treatments for the other indications are available. Alternative strategies for the control of swine dysentery e.g. development of new antimicrobials, development of vaccines, increased hygiene and better management could be explored. Initiatives targeting responsible use of pleuromutilins could potentially reduce the risks associated with further emergence of resistance in *B. hyodysenteriae*.

To date the importance of pleuromutilins in human medicine is limited as only one product for topical treatment is authorised, but products for systemic use in humans with infections caused by multidrug-resistant bacteria are being developed. Therefore the importance of pleuromutilins for humans might increase in the future.

A special concern relating to human and veterinary medicine is the emergence of resistance to pleuromutilins in staphylococci (including MRSA) and enterococci, which can be located on mobile elements like plasmids and transposons and thus be horizontally transmitted (Kadlec et al., 2010; Kadlec and Schwarz, 2009; Witte and Cuny, 2011). A special concern are the *vga* genes conferring cross-resistance to pleuromutilins, streptogramin A and lincosamides and the *cfr* genes with an even broader spectrum conferring resistance to pleuromutilins, lincosamides, streptogramin A, phenicols and oxazolidinones.

Colonisation of animals with livestock-associated MRSA ST398 can lead to clinical infections in animals and zoonotic infections in humans and severe cases have been documented (Catry et al., 2010). The

prevalence of MRSA in pigs is very high in many Member States (Catry et al., 2010; de Neeling et al., 2007) and in such situations there is a high potential that the use of pleuromutilins for the treatment or prevention of other disease like swine dysentery further selects for pleuromutilin resistant staphylococci, including MRSA. The *vga* and *cfr* genes have been detected in isolates from humans and animals in many different countries (Gentry et al., 2008; Gopegui et al., 2012; Kadlec and Schwarz, 2009; Mendes et al., 2011; Shore et al., 2010; Witte and Cuny, 2011), and *cfr*-mediated resistance has been detected in several bacterial species, indicating inter-species and inter-genus transfer (Liu et al., 2012b; Long et al., 2006; Wang et al., 2012a; Wang et al., 2012b; Wang et al., 2011). Resistance selection and spread between animals and humans might jeopardise the efficacy of antimicrobial agents. The emergence of these resistance genes in animals pose a potential threat to human medicine as they might compromise empirical treatment of human MRSA infections. The use of linezolid or dalfopristin in humans may also select for resistant staphylococci and enterococci which might also be transmitted between humans, but also from humans to animals. As the pleuromutilin resistant isolates are often multidrug-resistant co-selection under selective pressure by numerous other antimicrobial agents in human and veterinary medicine may potentially occur. Nevertheless, resistance seems to be emerging and to further access the situation, there is a need for the surveillance of bacteria especially staphylococci and enterococci from both animals and humans, for the presence of *vga* and/or *cfr* genes.

Co-selection for pleuromutilins with many different antimicrobials can potentially occur due to multidrug resistance genes and therefore prudent use of all antimicrobials in animals and humans is warranted.

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