Veterinary Microbiology 208 (2017) 1–7

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Effect of different oral oxytetracycline treatment regimes on selection of antimicrobial resistant coliforms in nursery pigs

Ana Herrero-Fresno[a,*], Camilla Zachariasen[a], Nanna Nørholm[a], Anders Holm[b], Lasse Engbo Christiansen[c], John Elmerdahl Olsen[a]

[a] Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark
[b] Danvet K/S, Blåkildevej 17, Hobro, Denmark
[c] Department of Applied Mathematics and Computer Science, Technical University of Denmark, Lyngby, Denmark

ARTICLE INFO

Keywords:
E. coli
Pig
Antimicrobial resistance
Oxytetracycline treatment regime
Co-selection

ABSTRACT

A major concern derived from using antimicrobials in pig production is the development of resistance. This study aimed to assess the impact of selected combinations of oral dose and duration of treatment with oxytetracycline (OTC) on selection of tetracycline resistant (TET-R) coliforms recovered from swine feces. The work encompassed two studies: 1) OTC 5 mg/kg and 20 mg/kg were administered to nursery pigs for 3 and 10 days, respectively, under controlled experimental conditions, and 2) 10 mg/kg, 20 mg/kg and 30 mg/kg OTC were given to a higher number of pigs for 6, 3 and 2 days, respectively, under field conditions. Statistical modeling was applied to analyze trends in the proportion of TET-R coliforms. In the experimental study, no statistical difference in proportion of TET-R coliforms was observed between treatments at the end of the trial (day 18) and compared to day 0. In the field study, treatment had a significant effect on the proportion of TET-R bacteria two days after the end of treatment (2dAT) with the regimes "low dose-six days" and "medium dose-three days" yielding the highest and lowest proportions of TET-R strains, respectively. No indication of co-selection for ampicillin- and sulphonamide -R bacteria was observed for any treatment at 2dAT. By the end of the nursery period, the proportion of TET-R bacteria was not significantly different between treatments and compared to day 0. Our results suggest that similar resistance levels might be obtained by using different treatment regimes regardless of the combinations of oral dose-duration of treatment.

1. Introduction

Antimicrobial resistance is recognized as a global health problem, and the World Health Organization considers it as one of the top health challenges facing the 21st century (FDA, 2000; CDC, 2014). The persistent increase in resistance is alarming, and the occurrence of high resistance levels continues to threaten the ability of both doctors and veterinarians to treat infections.

For many years, the association between antimicrobial resistant bacteria in humans and antimicrobial use in food animals has been debated (Jones and Ricke, 2003; Phillips et al., 2004; Chiller et al., 2004; Alpharma, 2007; Cox and Ricci, 2008; Falgenhauer et al., 2016). Based on a large amount of data, however, it is now evident that use of antimicrobials in food animals impacts human health through direct transfer of resistant bacteria, and more distantly through the food chain and the environment (Levy et al., 1976; Holmberg et al., 1984; Hummel et al., 1986; Fey et al., 2000). Since it is unrealistic and unethical for animal welfare reasons to completely avoid the use of antimicrobials in intensive livestock production, it is important to identify the antimicrobial applications in livestock that might have the highest impact on human health, and to minimize the development of resistance without compromising treatment efficacy.

Escherichia coli is a common facultative anaerobic bacterium in the intestinal microbiota of humans and animals (Karami et al., 2006), and it is therefore one of the commensal bacteria commonly used as an indicator in different types of studies in animals, humans, and food products (Karami et al., 2006; EFSA, 2012). Its ubiquitous presence in mammals and indications of resistance occurrence in the bacterium make it a good candidate for studies on antimicrobial selection pressure in the population (Vieira et al., 2011).

In Denmark, the swine sector accounted for ca. 80% of the veterinary use of antimicrobials in 2012 and tetracycline was the most frequently used drug in pig production (Apley et al., 1998; McDermott et al., 2002; DANMAP, 2013), commonly administered to treat

* Corresponding author at: Department of Veterinary Disease Biology, University of Copenhagen, Stigbøjlen 4, 1870, Frederiksberg, Denamrk.
E-mail address: ahefr@sund.ku.dk (A. Herrero-Fresno).

http://dx.doi.org/10.1016/j.vetmic.2017.07.005
Received 21 September 2016; Received in revised form 8 June 2017; Accepted 6 July 2017

intestinal diseases in nursery pigs (Roberts, 1996; DANMAP, 2013). Approximately 36% of *E. coli* isolates were tetracycline resistant (TET-R) in the Danish antimicrobial resistance surveillance system of pigs in 2012 (DANMAP, 2013). Furthermore, this surveillance has shown high levels of TET-R *E. coli* (over 30%) over the past five years, suggesting that TET-R *E. coli* are endemic in the pig production. Tetracycline is also used in humans, accounting for 11% of the total consumption of antimicrobials in the Danish health care sector in 2012 (DANMAP, 2013), and resistance to this antimicrobial is very common in *E. coli* from humans (Calva et al., 1996; Karami et al., 2006; Marshall and Levy, 2011).

In previous studies, where a mathematical model was developed and used to simulate selection of tetracycline resistance following treatment (Græsbøll et al., 2014; Ahmad et al., 2015), it was predicted that all the doses tested led to a temporary advantage for TET-R strains compared to the sensitive ones in the intestine of nursery pigs. It was also predicted that the total amount of antibiotic used and the duration of treatment affected selection of resistance, as well as the time it took for the intestinal flora to get back to equilibrium. Based on these observations, the aim of the present work, encompassing two in vivo studies, was to analyze the effect of different tetracycline treatment regimes on emergence and selection of TET-R coliforms in the gut of nursery pigs. Also, co-selection for ampicillin (AMP) and sulphonamide (SUL) resistant bacteria was investigated.

2. Material and methods

2.1. Animals, experimental setup and ethical issues

Two studies, an experimental trial and a study under field conditions were performed.

For the experimental study, 24 seven-to-eight weeks old nursery cross-bred sex-mixed pigs (11–18 kg) were purchased from a specific-pathogen-free farm in Denmark. The animals were housed in a level 1 isolation unit at University of Copenhagen and weighed at least once a week. Animal experiments were carried out according to the Animals Scientific Act and after having obtained the license and approval of the Danish National Animal Experiment Inspectorate (license no. 2009/561-1675). At the end of the study, all pigs were euthanized by captive bolt pistol penetration followed by bleeding.

Pigs in the experimental trial were divided into five groups housed in five well-separated pens avoiding contact between them. After one week of acclimatization, four groups including five pigs each received a specific oxytetracyline (OTC) treatment as follows: groups 1 and 2; low dose of antibiotic (5 mg/kg) for three and 10 days (Do5.Dur3 and Do5.Dur10), groups 3 and 4; high dose of antibiotic (20 mg/kg) for three and 10 days (Do20.Dur3 and Do20.Dur10). Group 5, was not treated (Do0.Dur0) (Table 1). Terramycin®Vet. 20% solution (Orion Pharm, Copenhagen, Denmark) was orally and individually administered to all pigs at the specific dose in nutri-drink (Nutricia, Allerød, Denmark).

For the field study, 120 pigs were randomly selected at one specific farm in Denmark where pigs were housed under regular pig production conditions. Permission to perform these experiments was granted by the Danish Medicines Agency (license no. 2011090862/2012053751) and a written "Owner informed consent" was signed by the owner of the herd involved in the study.

The nursery pigs used in the field study were divided into six pens containing 20 pigs each. Treatment with OTC was started at week four after weaning. Pigs in different pens received the following OTC treatments (in duplicate): groups 1 and 2; low dose of antibiotic (10 mg/kg) for six days (Do10.Dur6), groups 3 and 4; medium dose (20 mg/kg) for three days (Do20.Dur3) and groups 5 and 6; high dose (30 mg/kg) for two days (Do30.Dur2). It was not possible to include a control group under field conditions. All treatments were implemented at the pen level, and were randomly allocated to pen by draw. Terramycin®Vet. 20% was administered orally through drinking water, through a dosing pump, and it was controlled that all the medicine was consumed within 24 h (Larsen et al., 2016).

2.2. Collection and microbiological analysis of fecal samples

Fecal samples (ca. 5 g) were collected from the rectum of all the pigs prior to antimicrobial treatment (day 0) and every second day over a period of 18 days (experimental study) and before starting the treatment (day 0), at two and 10 days after having finished the treatment (2dAT and 10dAT), as well as by the end of the nursery period (EN; 20 days after day 0) (field study). At every collection time CFU counts were performed. For this, serial 10-fold dilutions in PBS were prepared and inoculated on MacConkey agar (Oxoid, Thermo Scientific, Roskilde, Denmark) without antibiotic and on MacConkey agar supplemented with 16 μg/ml TET (both studies) and 16 μg/ml AMP or 250 μg/ml SUL (field study). Antibiotics were purchased from Sigma (Sigma-Aldrich, Copenhagen, Denmark). All counts were determined by the spot method (Cavaco et al., 2008). Briefly, 20 μl of each dilution was inoculated as a spot on two plates, followed by 24 h of incubation at 37 °C. Deep red colonies with a diameter of > 0.5 mm were counted. The species of one hundred such colonies had previously been tested by MaldiToff and all of them were shown to be *E. coli* (Katakweba et al., 2015). Such a control was not performed in the current study, and we will use the term coliforms, even though they are likely to be *E. coli*.

2.3. Statistical analysis

Average $\log_{10}$ transformed CFU of TET-R coliforms was compared between treatment groups (experimental study) using ANOVA with Turkey's multiple comparison test in GraphPad Prism, version 6.0 (GraphPad software, La Jolla, USA). Statistical modelling of CFU data was performed using a generalized linear model in the statistical software R (Version 3.2.5). The count data was assumed to be Poisson distributed, and in case of over dispersion this was relaxed to so-called quasi-poisson. The dilution was used as offset for each count. For the two distributional assumptions, ChiSq- and F-test with Pearson residuals (Venables and Ripley, 2002) were performed, respectively. In all

Table 1
Tetracycline treatment regimes used in experimental and field studies.

Study	Dose/duration (days)	Number of pigs (group)	Collection time (Day)[a]	Antibiotics used for selection
Experimental	Low (5 mg/kg)/3	5 (1)	0,2,4,6,8,10,12,14,16,18	TET, no antibiotic
	High (20 kg/kg)/3	5 (2)	0,2,4,6,8,10,12,14,16,18	TET, no antibiotic
	Low (5 mg/kg)/10	5 (3)	0,2,4,6,8,10,12,14,16,18	TET, no antibiotic
	High (20 kg/kg)/10	5 (4)	0,2,4,6,8,10,12,14,16,18	TET, no antibiotic
	0/0	4 (5)	0,2,4,6,8,10,12,14,16,18	TET, no antibiotic
Field	Low (10 mg/kg)/6	40 (1 and 2)	SN,0,2dAT,10dAT,EN	AMP, TET, SUL, no antibiotic
	Medium (20 kg/kg)/3	40 (3 and 4)	SN,0,2dAT,10dAT,EN	AMP, TET, SUL, no antibiotic
	High (30 mg/kg)/2	40 (5 and 6)	SN,0,2dAT,10dAT,EN	AMP, TET, SUL, no antibiotic

[a] Day 0; day starting the treatment, day 2dAT and 10dAT: two and 10 days after having finished the treatment. SN; start of nursery period, EN; end of nursery period.