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Effects of tetracycline and zinc on selection of methicillin-resistant *Staphylococcus aureus* (MRSA) Sequence Type 398 in pigs

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Running title: Selection of porcine MRSA ST398

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Abstract

An *in vivo* experiment was conducted to evaluate the effects of tetracycline and zinc on pig colonization and transmission of methicillin-resistant *Staphylococcus aureus* (MRSA) sequence type (ST) 398. Eight piglets naturally colonized with MRSA ST398 and 8 MRSA-negative piglets of the same age and breed were assigned to three groups treated with tetracycline and zinc (Group 1), zinc (Group 2) or tetracycline alone (Group 3) and one non-treated group (Group 4), each containing two MRSA-positive and two MRSA-negative animals. Two additional non-treated control groups composed of only MRSA- positive (Group 5) and MRSA- negative (Group 6) animals were used to check for stability of MRSA carriage status. Nasal swabs and environmental wipes were collected on Days 0, 7, 14, and 21, and the occurrence of MRSA in each sample was quantified by bacteriological counts on Brilliance™ MRSA agar. Significantly higher nasal MRSA counts were observed in the zinc-treated ($p=0.015$) and tetracycline-treated ($p=0.008$) animals compared to the non-treated animals. Environmental MRSA counts appeared to increase over time in Groups 1 and 2 but such an increase was not statistically significant. MRSA-negative animals housed with MRSA-positive animals became positive in all groups, whereas the carriage status of the animals in Groups 5 and 6 did not change. This study demonstrates that feed supplemented with tetracycline or zinc increases the numbers of MRSA ST398 in the nasal cavity of pigs. Transmission of MRSA from positive to negative animals housed within the same pen was not influenced by exposure to these agents.

Keywords: carriage, epidemiology, heavy metals, transmission

Introduction

Livestock-associated MRSA belonging to sequence type (ST) 398 was first reported in the Netherlands in 2005 (Voss et al., 2005), and has spread in Europe (European Commission. 2009), Canada (Khanna et al., 2008), and in mid-western USA (Smith et al., 2009). It has been hypothesized that the emergence and spread of MRSA ST398 in pigs is driven by the use of tetracycline and zinc oxide (de Neeling et al., 2007, van Duijkeren et al., 2008, Aarestrup et al., 2010). The aim of this *in vivo* experiment was to assess under controlled conditions whether tetracycline and zinc, either alone or in combination, play a role in pig nasal colonization with MRSA ST398. The experiment was also used to assess the effects of the two agents on transmission from MRSA-positive to MRSA-negative animals housed within the same pen.

Materials and Methods

Study design

Eight naturally colonized MRSA-positive and 8 MRSA-negative 2-week old piglets were divided into four groups, each containing two positive and two negative animals. All treatments began after sampling on Day 0. A standard dosage of tetracycline (Terramycin® Vet, 25 mg/kg of body weight) was mixed with pig feed (DLG, Copenhagen, Denmark) and administered orally for seven days in combination with zinc (Group 1) or alone (Group 3). Zinc-oxide was mixed with the same pig feed using a standard dosage used in pig production (2.5g/kg of feed) and administered orally for three weeks either alone (Group 2) or in combination with tetracycline (Group 1). Group 4 was designated as the control group and received no treatment. Two additional non-treated control groups were included in the study. Group 5 contained four 2-week old MRSA-positive piglets and was used to monitor variations in the levels of MRSA in naturally colonized animals. Group 6 contained four 2-week old

MRSA-negative piglets and was used to rule out possible effects of environmental contamination on MRSA nasal carriage status. The experiment was carried out in class II isolation facilities at the Faculty of Life Sciences, University of Copenhagen, and all the procedures used were approved by the Danish National Animal Experiment Inspectorate (License no. 2006/561-1141).

MRSA quantification and characterization

Nasal swabs and environmental surfaces were collected on Days 0, 7, 14 and 21. Environmental surface samples were taken from all four sides of the pen using Sodibox wipes (Sodibox, France). MRSA were semi-quantitatively assessed by bacteriological counts on Brilliance™ MRSA agar (Oxoid, United Kingdom). Nasal swabs and environmental wipes were resuspended in 1 mL and 100 mL saline, respectively, and vortexed for 15-30 sec. Thereafter, 1mL of each bacterial suspension was centrifuged at 6000 x g for 5 min and the pellet resuspended in 100µL of saline and spread plated on Brilliance™ MRSA agar. Characteristic MRSA colonies (denim blue colonies with blue edges) were counted following overnight incubation at 37°C. In parallel, the swabs were enriched in 5mL Mueller Hinton broth (MHB) containing 6.5% NaCl and incubated overnight at 37°C. For samples that yielded no MRSA in the bacteriological counts, 10µL of the enrichment broth was streaked onto Brilliance™ MRSA agar and incubated overnight at 37°C to confirm the absence of MRSA.

One colony per Brilliance™ MRSA plate was subcultured onto 5% blood agar and confirmed to be MRSA by *mecA* PCR (Zhang et al., 2004). All isolates were plated on MH agar containing 16µg/mL tetracycline or 2mM zinc-chloride (Sigma-Aldrich, Denmark) to evaluate phenotypic resistance, and genes conferring tetracycline and zinc resistance was assessed by PCR (Aarestrup et al., 2000, Cavaco et al., 2010). *Cfr9I*-pulse-field gel electrophoresis (PFGE) (Bosch et al., 2010) was performed on all animal and environmental isolates (n=19) from Group 1 to confirm transmission of MRSA between

piglets and the environment within a pen. One representative of each pulsotype was further characterized by *spa* typing (Harmsen et al., 2003).

Statistical analysis

The designed groups (Groups 1-4) were reorganized into treatment groups: tetracycline-treated (Groups 1 and 2) vs. non-tetracycline treated (Groups 3 and 4), and zinc-treated (Groups 1 and 3) vs. non-zinc treated (Groups 2 and 4). This was done to increase the statistical power. The counts of MRSA were compared between treatment groups using Poisson regression and taking into account the repeated structure of the data, using the Genmod procedure in SAS version 9.2 (SAS Institute, Cary, NC, USA). Nasal and environmental counts from zinc-treated, tetracycline-treated and non-treated were compared to determine if a specific treatment regimen selected for MRSA in the nasal cavity of the piglets and in the pen environment, respectively. Control groups 5 (untreated MRSA-positive piglets) and 6 (untreated MRSA-negative piglets) were not included in the statistical analysis as they were not relevant to assess the effects of treatment. This analysis was done including and excluding the counts on Day 0 to determine if the initial load of MRSA affected the MRSA counts on Days 7 to 21. Since there was no effect, and no animals received treatment prior to sampling on Day 0, counts on Day 0 were excluded from the analysis.

Results

Mean sample-day MRSA counts in the different treatment groups are shown in Figure 1a. Both zinc-treated ($p=0.015$) and tetracycline-treated ($p=0.008$) animals had significantly higher MRSA counts in the nasal cavity of treated animals compared to non-treated animals. The average increase was in the order of 4-6 folds. Tetracycline-treated pigs had an average count of 167 colony forming units

(CFU)/sampling (range: 2-1024 CFU/swab) compared to 55 CFU/sampling (range: 0-664 CFU/swab) for non-tetracycline treated pigs, and zinc-treated pigs had an average count of 221 CFU/sampling (range: 0-1024 CFU/swab) compared to 46 CFU/sampling (range: 5-540 CFU/swab) in non-treated pigs. No significant interaction between tetracycline and zinc was detected ($p=0.62$). The counts on Days 7, 14 and 21 were not affected by the animal being positive on Day 0 ($p=0.67$). During the experiment, MRSA counts (CFU/sampling) from environmental surfaces increased in both treatment groups (Figure 1b). However, this finding was based on a limited number of observations and there was no statistical association between environmental MRSA counts and treatment with zinc ($p=0.09$) or tetracycline ($p=0.11$). Nasal swabs and environmental wipes from Groups 5 and 6 were MRSA-positive and -negative, respectively, throughout the experiment. All samples not yielding MRSA by direct selective plating on Brilliance™ MRSA agar were also negative following enrichment.

MRSA-negative animals housed together with MRSA-positive were positive at Day 7 and remained positive for the duration of the experiment irrespective of the group to which they were assigned. All isolates exhibited resistance to tetracycline and zinc as a result of the presence of two tetracycline resistance genes *tet(K)* and *tet(M)*, and *czrC*, which confers resistance to zinc. The 19 isolates typed by PFGE had the same pulsotype and belonged to *spa* type t034, which is associated with ST398.

Discussion

We demonstrated that treatment with tetracycline and zinc under controlled experimental conditions results in significantly higher counts of MRSA ST398 in the nasal cavity of pigs. These results indicate that either compound exerts a co-selective pressure in favour of MRSA ST398 colonization. Although it may be reasonable to assume that higher levels of MRSA in the nasal cavity may facilitate animal-to-

animal spread at the herd level, however the epidemiological significance of our results is uncertain since the efficiency of MRSA spread within farms may not be related to the numbers of nasal MRSA in individual pigs. MRSA transmission from positive to negative animals was also observed in the absence of antimicrobial selection pressure (Group 4), suggesting that short-term exposure to tetracycline and zinc is unlikely to enhance transmission. However, it cannot be excluded that prolonged use of these agents over time may result in a cumulative effect and have an impact on MRSA transmission by increasing the levels of environmental contamination.

Tetracycline is the most prescribed antibiotic for pigs in Denmark (Anonymous, 2008). Zinc is a commonly used pig feed additive in weaning piglets as it has growth promoting effects (Hill et al., 2000; Katouli et al., 1999). Heavy metals such as copper have been shown to play a role in the spread of antibiotic resistant bacteria by co-resistance i.e. presence of different resistance determinants on the same genetic element (Hasman et al., 2006). In MRSA, methicillin resistance is mediated by *mecA*, which is carried on Staphylococcal Cassette Chromosome *mec* (SCC*mec*). Zinc oxide has the potential to co-select specifically for MRSA ST398 since *czrC*, which encodes for cadmium and zinc resistance, is present within SCC*mec* elements in ST398 (Cavaco et al., 2010). Tetracycline resistance may not be a determinative factor of methicillin resistance in ST398, since *tet(K)* and *tet(M)* are located on other mobile genetic elements (Schmitz et al., 2001, de Vries et al., 2009), and can be found in both MSSA and MRSA ST398 isolates.

A limitation of this study is the inherent difficulty in quantifying the amount of bacteria in the nasal cavity. Similar semi-quantitative approaches have been used to quantify nasal *S. aureus* in humans (Nouwens et al., 2004; Mermel et al., 2010). In general, high variability of the MRSA counts in individual animals was observed and such variability could be related to the effects of repeated nasal

swabbing within a short period, which could interfere with the natural colonization patterns, or merely reflect the natural variation in the levels of MRSA in individual animals over time.

In the present study we show that tetracycline and zinc, which are commonly used in pig production, select for significantly higher counts of MRSA ST398 in the nasal cavity of treated pigs. Although the study has some intrinsic methodological limitations concerning the semi-quantitative method used for MRSA quantification and the relative short duration of the experiment, it provides useful quantitative data for modelling the spread of MRSA at the herd-level, and adds novel information on the relationship between antibiotic use and occurrence of resistant bacteria in animal farming.

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231

232 **Figures legend**

233 Figure 1: a) Mean MRSA counts (CFU/sampling) from nasal swabs in the two treatment groups versus
234 the non-treated group. b) Mean MRSA counts (CFU/sampling) from environmental surface samples in
235 the two treatment groups versus the non-treated group.

Figure 1