Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry

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Received 19 July 2002; accepted 26 September 2002

Abstract

Resistance to tetracycline, macrolides and streptomycin was measured for a period of 8 months in soil bacteria obtained from farmland treated with pig manure slurry. This was done by spread plating bacteria on selective media (Luria Bertani (LB) medium supplemented with antibiotics). To account for seasonal variations in numbers of soil bacteria, ratios of resistant bacteria divided by total count on nonselective plates were calculated. Soil samples were collected from four different farms and from a control soil on a fifth farm. The control soil was not amended with animal manure. The occurrence of tetracycline-resistant bacteria was elevated after spread of pig manure slurry but declined throughout the sampling period to a level corresponding to the control soil. Higher load of pig manure slurry yielded higher occurrence of tetracycline resistance after spreading; however, the tetracycline resistance declined to normal occurrence defined by the tetracycline resistance occurrence in the control soil. Concentrations of tetracycline in soil and in pig manure slurry were measured using HPLC. No tetracycline exceeding the detection limit was found in soil samples. Manure slurry concentrations of tetracycline for three of the farms were 42, 81 and 698 µg/l, respectively. For streptomycin and macrolides, only minor variations in resistance levels were detected. Results obtained in this study thus indicate that tetracycline resistance levels in soil are temporarily influenced by the addition of pig manure slurry. The results indicate also that increased amount of pig manure slurry amendment may result in increased levels of tetracycline resistance in the soil.

Keywords: Antibiotic resistance; Soil; Farmland; Pig manure slurry; Tetracycline; Streptomycin; Macrolide

1. Introduction

Bacterial resistance to antibiotics is the result of intensive use of antibiotics in both human medicine and in agriculture (Levy, 1998; Witte, 1998) and can result in treatment failure for both humans and animals. Use of antibiotics has been shown to be associated with the occurrence of antibiotic resistance in fecal samples from pigs (Aarestrup and Carstensen, 1998; Bager et al., 1997), hence creating a reservoir of resistance in animals. When manure slurry from farms is spread on fields, resistant bacteria and antibiotic resistance genes are transferred to this environment creating the possibility of horizontal transfer of resistance genes to the indigenous soil bacteria. Furthermore, antibiotics and residues of antibiotics, which might give resistant bacteria a selective advantage, are transmitted to the farmland with the manure slurry (Halling-Sørensen et al., 2001).

Resistance to antibiotics is often plasmid-mediated, and several microcosm studies have shown plasmid transfer to occur in terrestrial environments (for reviews, see Cresswell and Wellington, 1992; Trevors et al., 1987). Amendment with nutrients enhances activity of the bacterial community and has been shown to have a positive effect on horizontal transfer in both sterile and nonsterile soils (Top et al., 1990). High numbers of parent cells introduced into soil likewise enhance conjugation (Kinkle and Schmidt, 1991; Pukall et al., 1996; Van Elsas et al., 1988), and different plant parts (e.g. the rhizosphere) have furthermore shown to be microhabitats conducive to plasmid transfer (Van Elsas et al., 1988; Kinkle...
and Schmidt, 1991; Normander et al., 1998; Sengeløv et al., 2000). Pig manure has been shown to promote mobilization in farmland, and to contain plasmids conferring antibiotic resistance (Götz and Smalla, 1997; Smalla et al., 2000). Thus, farmland supplied with pig manure slurry is an environment which could favor conjugal gene transfer. This has raised concern about the possible formation of an environmental reservoir of antibiotic resistance genes in farmland that could transfer resistance back to animals or humans via crops. The millions of tons of manure slurry which are spread on farmland worldwide each year might have created reservoirs of resistance, not only to antimicrobials used today, but also to previously used antimicrobials. The aim of this investigation was to evaluate if the use of pig manure slurry as fertilizer has created reservoirs of antibiotic resistance in Danish farmland.

2. Materials and methods

2.1. Data on farms

Soil was sampled from farms on the Danish Island Zealand. Details of the five fields are given in Table 1. Data on spread of pig manure slurry, schedules of emptying of slurry tanks and liters of slurry spread per hectare is based on information provided by the farmers. Values for the use of antibiotics per farm were kindly provided by Per Bundgaard Larsen, Danish Veterinary Institute (DVI), and the data was collected from current assimilation and registration of information on antibiotic use provided by the farmers and their veterinarians to the DVI. In Denmark, veterinarians prescribe all antibiotics for animals.

A control soil from farmland, which had only been amended with manure once (75 tons per hectare in 1998) during the last 13 years, was included for comparison.

2.2. Culture conditions

Colony forming unit (CFU) counts of aerobic culturable bacteria recovered from soil were made on Luria Bertani (LB) agar (Sambrook et al., 1989). For specific selection of Gram-positive bacteria, the LB medium was supplied with 3.2 μg ml⁻¹ polymyxin B sulfate and 15 μg ml⁻¹ nalidixic acid. For selection of antibiotic-resistant bacteria, the medium was supplied with 8 μg ml⁻¹ tetracycline hydrochloride or 50 μg ml⁻¹ streptomycin sulfate. For selection of erythromycin-resistant bacteria, the LB was supplemented with 8 μg ml⁻¹ erythromycin, 3.2 μg ml⁻¹ polymyxin B and 15 μg ml⁻¹ nalidixic acid. The two last-mentioned antimicrobials were added to limit the growth of Gram-negative bacteria, which are resistant to erythromycin in the concentrations tested here. All LB agar plates and Columbia agar plates (Oxoid, UK) supplemented with 5% bovine blood. The LB and blood agar were amended with antibiotics as described above. Counts from blood agar were used.

2.3. Sampling of soil and isolation of bacteria

Sampling was performed four times in the year 2000: six weeks before spread of pig manure slurry, 3–5 days after spread, 1 and 1/2 months after spread and 5 months after spread.

Soil was collected 10 m from the border of the field. Five to ten centimeters of the top surface soil was removed, and 10 samples of roughly 1 kg of soil were collected approximately 1 m apart for further studies. In cases where the pig manure slurry was spread by hoses (e.g. on the wheat fields), sampling after spreading was preferably done in the trail tracks, if they could be identified. Immediately after sampling, the soil was brought to the laboratory and manually homogenized in plastic bags before taking subsamples. Bacteria from 10-g soil samples (wet weight) were recovered in 90 ml 0.9% NaCl by shaking in a water bath at 120 rpm and 25 °C for 1 h. The soil particles were then allowed to settle for approximately 15 min. One hundred microliters of serial tenfold

<table>
<thead>
<tr>
<th>Farm</th>
<th>Crop</th>
<th>Plowing of pig manure slurry</th>
<th>Spread of pig manure slurry (l/ha)</th>
<th>Antimicrobial use (g active compound)abc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tetracyclines</td>
</tr>
<tr>
<td>A</td>
<td>winter wheat</td>
<td>no</td>
<td>20.000</td>
<td>285.0</td>
</tr>
<tr>
<td>B</td>
<td>winter wheat</td>
<td>no</td>
<td>25.000</td>
<td>344.6</td>
</tr>
<tr>
<td>C</td>
<td>winter wheat</td>
<td>no</td>
<td>31.000</td>
<td>6229.4</td>
</tr>
<tr>
<td>D</td>
<td>beet</td>
<td>yes</td>
<td>30.000</td>
<td>NA</td>
</tr>
<tr>
<td>Control</td>
<td>spring barley</td>
<td>–</td>
<td>0</td>
<td>–</td>
</tr>
</tbody>
</table>

a Antimicrobials used during the period from the previous emptying of the manure slurry plant up to spread. Farms A and B: October 1999–April 2000, Farm C: September 1999–April 2000. All months are inclusive.

b ATC-group: Tetracyclines, Q01A; lincosamides, Q01F.

c NA: Not applicable.

d No macrolides were used. Cross-resistance between lincosamide and macrolide resistance is frequent (Weisblum, 1995).
dilutions were plated on LB agar and respective selective agar. The remaining soil was stored at \(-80\, ^\circ \text{C}\) for tetracycline concentration analysis (see below). The LB, LB-polymerxyn B-nalidixic acid and LB-streptomycin agar plates were incubated at \(25\, ^\circ \text{C}\) for 2 days before CFU enumeration. The LB-tetracycline and LB-erythromycin-polymerxyn B-nalidixic acid plates were incubated at \(25\, ^\circ \text{C}\) for 3 days before CFU enumeration.

Three colonies per sample, corresponding to a total of 30 isolates per farmland per sampling, from the LB-tetracycline agar plates were chosen and re-streaked twice on the same selective medium. This selection was aimed at obtaining as many resistant morphologically different bacteria as possible from the 10 samples in all. The bacterial isolates were stored at \(-80\, ^\circ \text{C}\) in LB amended with 15% (vol/vol) glycerol.

Two samples of approximately 1 l were collected from the manure slurry tank prior to spread on the same day from each farm and stored at room temperature at the farms until sampling 3–5 days after spread. One hundred microliters of serial tenfold dilutions were plated on LB agar, blood agar and selective LB and blood agar. Ten colonies per manure sample, five from LB agar and five from blood agar, corresponding to a total of 20 manure isolates per farm, were chosen and re-streaked twice on the same selective medium.

The isolates were Gram-stained. A total of 37 Gram-negative soil isolates sampled from farm A, B and C before spreading of manure slurry and 45 Gram-negative soil isolates sampled from farm A, B and C after spreading were screened for the presence of \(tet(A), tet(B)\) and \(tet(C)\). A total of 45 Gram-negative soil isolates sampled from manure slurry from farm A, B and C were screened for the presence of \(tet(A), tet(B)\) and \(tet(C)\).

### 2.4. PCR conditions

Presence of selected antibiotic resistance genes for tetracycline was detected using specific primers for amplification of internal segments of the genes. The primers were: \(tet(A)\) (5’-GTAATTCGACCTGTCGC-3’ and 5’-CTGCTTGGACAAACATTTGCTT-3’), \(tet(B)\) (5’-CTCAGTATTCGCAAGGAGCTT-3’ and 5’-ACTCCCCCTGAGCTTGAGG GC-3’), and \(tet(C)\) (5’-CTCTTTCGCGGATATCCTCC-3’ and 5’-GGTGAAGGCTCTCAAGGCG-3’). The amplicon sizes of \(tet(A), tet(B)\) and \(tet(C)\) were 956, 414 and 505 bp, respectively. DNA was isolated as previously described (Jensen et al., 1998), and \(T_m\) for the individual primers were calculated using the Tm DETERMINATION (Breslauer et al., 1986) available on Internet (http://alces.med.umn.edu/ rawtm.html). The following \(T_m\)’s were used: \(tet(A) 57\, ^\circ \text{C}, tet(B) 52\, ^\circ \text{C}\) and \(tet(C) 65\, ^\circ \text{C}\). All PCRs were conducted with the buffers supplied by the manufacturers using 5 pmol of each primer and 0.25 U Super Taq (HT Biotechnology, UK). All reaction mixtures (25 μl) were overlaid with an equal volume of mineral oil, and the samples were amplified using the following programs: 3 min at \(94\, ^\circ \text{C}\) followed by 25 cycles of 1 min at \(94\, ^\circ \text{C}, 1 \text{ min at } 57\, ^\circ \text{C}\) and 1 min at \(72\, ^\circ \text{C}\) followed by 10 min at \(72\, ^\circ \text{C}\) (tet(A)), 3 min at \(94\, ^\circ \text{C}\) followed by 30 cycles of 1 min at \(94\, ^\circ \text{C}, 1 \text{ min at } 52\, ^\circ \text{C}\) and 1 min at \(72\, ^\circ \text{C}\) followed by 10 min at \(72\, ^\circ \text{C}\) (tet(B)) or 3 min at \(94\, ^\circ \text{C}\) followed by 30 cycles of 1 min at \(94\, ^\circ \text{C}, 1 \text{ min at } 65\, ^\circ \text{C}\) and 1 min at \(72\, ^\circ \text{C}\) followed by 10 min at \(72\, ^\circ \text{C}\) (tet(C)). The PCR products were analyzed by electrophoresis through 1.5% agarose gels and staining with ethidium bromide (Sambrook et al., 1989).

### 2.5. HPLC analysis of tetracyclines

Soil samples and extracts were treated and analyzed with HPLC–MS–MS as previously described by Loke et al. (in press). Previously to all analyses, samples were filtered through a 0.45-μm syringe filter (Minisart® 17598, Sartorius, Göttingen, Germany). Chemical analysis was made on an HPLC system (Waters 2690, Milford, MA, USA) as previously described (Loke et al., in press). The same HPLC column, system and mobile system were used for the pig manure slurry. The samples were analyzed by combining the HPLC system with an MS–MS system as described in Loke et al. (in press).

### 2.6. Predicted soil concentrations

Worst-case predicted environmental concentrations (PECs) in the soil were also estimated for the tetracyclines and lincomycin as a consequence of soil amendment with pig manure slurry containing residues of excreted drug using the equations proposed in Halling-Sørensen et al. (2001).

Estimations were based on the assumption that 80% and 60% of the dosed tetracyclines (Aiello, 1998) and lincomycin (Aiello, 1998) were excreted unchanged or as metabolites (lincomycin). Furthermore, the estimations were also based on the assumption that the soil density was estimated to be 1500 kg/m³. The compounds were as a worst-case scenario assumed to be persistent during storage of manure slurry in the tank.

### 2.7. Data analysis

Samples with resistant bacteria below the detection limit (1 CFU per 0.01 g of soil) were not included. Pig manure slurry CFUs were taken as average of two samples.

To investigate the importance of the number of resistant bacteria (CFU) spread per hectare (ha) a new variable load (CFU/ha) was calculated using the following load on different fields, the relative load (rload) was calculated as \(\text{rload} = \text{load}/\Sigma\text{load}\), where load, is the load on farm \(i\) \((i = 1, 2, 3, 4)\) and \(\Sigma\text{load}\) is the sum of the load on

\[\text{rload}_i = \frac{\text{load}_i}{\sum \text{load}_j}\]

where \(i\) represents the farm and \(j\) the sample on each farm.
the four farms. In the model described in the following, it was assumed that the load last year was the same as the load observed in the study period.

One objective of the current research is to quantify the proportion of resistant soil bacteria on farmland treated with pig manure slurry, and the extent to which the proportion is affected by the amount of amended manure and time since spread. The relations were analyzed by means of a mixed-effect model allowing incorporating both fixed and random effects. The components in the fixed part of the model were regressions on log_{10}(time) and load both modeled by a linear term. Since the purpose is a general expression of the decay of the proportion of resistant soil bacteria, random slopes were included in the regression of log_{10}(time) at the upper hierarchical levels of the model (farm-level). This results in the following model equation:

$$\log_{10}(prop) = X\beta + b_{farm} \times \log_{10}(time) + \varepsilon$$

In this model, prop is the proportion of resistant soil bacteria, $X$ is the observation matrix, $\beta$ is the fixed effects (intercept, slope and load), $b_{farm}$ is a random (Gaussian) variable with mean zero and variance interpretable as the variability of the slope of log_{10}(time) at farm levels and time is the time in days since last spreading of manure. So far, nothing has been said about the 10 repeated measurements within each farm. In the model, an additional compound symmetry correlation structure in $\varepsilon$, which assumes equal correlation among all within-group errors pertaining to the same group, is assumed for measurements within each farm. The correlation parameter $\rho$ is generally referred to as the intraclass correlation coefficient (Table 2).

The parameters in the model were estimated by restricted maximum likelihood (REML) using the lme-function in S-PLUS 6.0.

3. Results

3.1. Counter-selection of Gram-negative bacteria

To obtain erythromycin-resistant isolates, it was necessary to counter-select the Gram-negative bacteria since these are intrinsically resistant to macrolides in the concentration used in this study. Different concentrations of polymyxin and nalidixic acid were added to LB medium, and the percentages of Gram-negative and Gram-positive bacteria from soil samples were determined by Gram staining. The percentages of Gram-negative isolates in farmland not amended with manure were (total number of isolates stained) 78% (77), 98% (44), 95% (57) and 98% (40) for LB, LB + 25 µg ml^{-1} nalidixic acid (Nal), LB + 3.2 µg ml^{-1} polymyxin (Pol) + 15 µg ml^{-1} Nal and LB + 3.2 µg ml^{-1} Pol + 25 µg ml^{-1} Nal, respectively. The corresponding percentages of Gram-negative isolates in farmland amended with manure were (total number of isolates stained) 45% (60), 84% (31), 100% (54) and 100% (42), respectively. A concentration of 3.2 µg ml^{-1} polymyxin B and 15 µg ml^{-1} nalidixic acid was chosen.

3.2. Occurrence of antimicrobial resistant bacteria in farmland and in pig manure slurry

Observed and predicted levels of tetracycline resistance in farmland are presented in Fig. 1. Data is presented as the fraction of resistant bacteria, which is the ratio of tetracycline-resistant aerobic counts of culturable bacteria to the total aerobic counts of bacteria culturable on LB medium (Table 3 and Fig. 1). Fig. 1 shows the development through 1 year in the fractions of tetracycline-resistant bacteria after spread of pig manure slurry for farms A, B, C and D. The soil collected prior to spreading of manure slurry represents also measurements 1 year after spreading, since manure slurry is spread on the soil each year in the spring. The fractions of tetracycline-resistant bacteria declined after spread of pig manure slurry for all four farms, whereas no decline was observed in the control soil. All samples sampled during the year from the control soil are therefore presented as a box–whisker plot in the right side of Fig. 1. For all farms, the fractions of tetracycline-resistant bacteria declined to normal occurrence of tetracycline resistance in soil here defined by the distribution of the pooled control samples from untreated soil (Fig. 1, right). The numbers of tetracycline-resistant bacteria from two pig manure slurry samples were 2.87 \times 10^7 \pm 4.45 \times 10^6, 8.75 \times 10^7 \pm 3.75 \times 10^7, 7.55 \times 10^7 \pm 5.02 \times 10^7 and 7.15 \times 10^6 \pm 2.12 \times 10^5 CFU/ml, for farms A, B, C and D, respectively. Load values (CFU/ha) were calculated for each farm as

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Estimate</th>
<th>Approximate 95% confidence intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-1.41</td>
<td>(-1.76, -1.058)</td>
</tr>
<tr>
<td>Load</td>
<td>0.360</td>
<td>(-0.110, 0.830)</td>
</tr>
<tr>
<td>Logtime</td>
<td>-0.131</td>
<td>(-0.203, -0.0598)</td>
</tr>
<tr>
<td>Slope variance</td>
<td>0.0290</td>
<td>(0.00567, 0.148)</td>
</tr>
<tr>
<td>Within-group standard error</td>
<td>0.398</td>
<td>(0.351, 0.451)</td>
</tr>
<tr>
<td>Intraclass correlation coefficient</td>
<td>0.255</td>
<td>(0.160, 0.364)</td>
</tr>
</tbody>
</table>

$\beta$ is the fixed effects (intercept, slope and load), $b_{farm}$ is a random (Gaussian) variable with mean zero and variance interpretable as the variability of the slope of log_{10}(time) at farm levels. The correlation parameter $\rho$ is generally referred to as the intraclass correlation coefficient assuming equal correlation among all within-group errors pertaining to the same group within each farm.
concentration of resistant bacteria in the manure (CFU/ml) times the volume manure spread per hectare (l/ha). To be able to compare the load on different fields, the relative load (rload) was calculated as \( r\)\(_{load}^i = \frac{\text{load}_i}{\sum \text{load}_i} \), where \( \text{load}_i \) is the load on farm \( i (i = 1, 2, 3, 4) \) and \( \sum \text{load}_i \) is the sum of the load on the four farms (Tables 1 and 4). The occurrence of proportional tetracycline resistance corresponded to the load values of farms A, B, C and D (0.43, 1.63, 1.79 and 0.16, respectively). At all sampling times, the predicted fractions of tetracycline-resistant bacteria were C>B>A>D (Fig. 1). Furthermore, the predicted level of tetracycline resistance in the soil corresponding to 1 year after spread was distributed according to the load. The predicted proportions as shown among the distribution of the control isolates in Fig. 2 strongly indicate the relation between load and resistance.

No significant decline in the fraction of streptomycin and erythromycin-resistant bacteria during the year was observed (data not shown). The fraction of erythromycin-resistant bacteria was calculated as the ratio of aerobic culturable Gram-positive erythromycin-resistant bacteria to the total counts of aerobic Gram-positive bacteria able to grow on LB amended with polymyxin and nalidixic acid.

The numbers of streptomycin-resistant CFU in the pig manure slurry were \( 3.35 \times 10^5 \pm 4.95 \times 10^4 \), \( 4.60 \times 10^7 \pm 1.13 \times 10^2 \), \( 2.31 \times 10^7 \pm 1.12 \times 10^7 \) and \( 2.55 \times 10^7 \pm 5.02 \times 10^6 \) per milliliter for farm A, B, C and D, respectively.

The numbers of tetracycline-resistant CFU in the pig manure slurry were \( 2.15 \times 10^7 \pm 1.90 \times 10^6 \), \( 1.07 \times 10^6 \pm 3.82 \times 10^5 \) and \( 1.60 \times 10^6 \pm 4.24 \times 10^5 \) per milliliter for farm A, B, C and D, respectively.

3.3. Concentrations of tetracycline and lincosamide in soil and pig manure slurry

On the three farms (A, B and C), 285, 345 and 6229 g of tetracyclines and 42.4, 7.2 and 345.3 g of lincomycin, Table 3

<table>
<thead>
<tr>
<th>Farm</th>
<th>Before spreading</th>
<th>3–5 days after spreading</th>
<th>Six weeks after spreading</th>
<th>Five months after spreading</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.88E–02 ± 7.73E–03</td>
<td>7.92E–02 ± 5.47E–02</td>
<td>4.96E–02 ± 6.37E–02</td>
<td>2.84E–02 ± 1.50E–02</td>
</tr>
<tr>
<td>B</td>
<td>3.05E–02 ± 1.41E–02</td>
<td>1.66E–01 ± 1.90E–01</td>
<td>6.43E–02 ± 3.19E–02</td>
<td>1.87E–02 ± 8.67E–03</td>
</tr>
<tr>
<td>C</td>
<td>7.11E–02 ± 2.15E–02</td>
<td>1.59E–01 ± 7.20E–02</td>
<td>6.07E–02 ± 6.04E–02</td>
<td>1.68E–02 ± 1.11E–02</td>
</tr>
<tr>
<td>D</td>
<td>5.89E–03 ± 3.36E–03</td>
<td>3.29E–02 ± 3.15E–02</td>
<td>1.14E–02 ± 1.61E–02</td>
<td>1.46E–02 ± 1.04E–02</td>
</tr>
<tr>
<td>Control</td>
<td>8.64E–03 ± 4.35E–03</td>
<td>3.47E–02 ± 2.15E–02</td>
<td>1.81E–02 ± 1.39E–02</td>
<td>1.25E–02 ± 4.53E–03</td>
</tr>
</tbody>
</table>

Soil samples were taken from five different fields on five different farms.
respectively, were therapeutically administered to the pigs during the period of manure slurry accumulation (Table 1). No macrolides were used. Concentrations of lincosamides were measured due to frequent cross-resistance between lincosamides and macrolides (Weisblum, 1995). The PECs in soil on farms A, B and C were estimated to 2.3, 6.6, and 85.8 μg/kg soil of tetracyclines, and 0.26, 0.10, and 3.6 μg/kg soil of lincomycin, respectively.

Soils sampled before spreading were also analyzed by HPLC, but no tetracycline concentrations exceeding the limit of detection (LOD) were found. The LOD for both chlorotetracycline (CTC) and oxytetracycline (OTC) was found to be 82 μg/kg soil. Similarly, we found for all the tetracycline degradation products LODs of more than 150 μg/kg soil. The LODs were calculated as described in Loke et al. (in press).

The pig manure slurry concentration (average of two samples) of tetracyclines on farms A, B and C were 42, 81 and 698 μg/l, respectively. The corresponding estimated soils concentrations were 0.6, 1.3 and 14.5 μg/kg soil.

3.4. Tetracycline resistance determinants in soil isolates

Gram-negative isolates from farms A, B and C soil and pig manure slurry samples were screened by PCR for the presence of the tetracycline resistance determinants tet(A), tet(B) and tet(C) on the basis of previous determinant identification in intestinal bacteria from pigs (Lee et al., 1993; Marshall et al., 1983). A total of 37 tetracycline-resistant Gram-negative isolates (15, 10 and 12 isolates from farm A, B and C soils, respectively) from the soil samples before spread of pig manure slurry were screened. In only three isolates (two from farm A and one from farm B) positive amplicons for tet(A) were detected. No amplicons for tet(B) or tet(C) were detected. From soil sampled 3–5 days after spread, one of 45 tetracycline-resistant Gram-negative isolates (15 from each farm) was positive for tet(B) (isolate from farm A), none for tet(A) or tet(C). In pig manure slurry samples, only one of 45 Gram-negative tetracycline-resistant isolates (15 from each farm) was positive for tet(B) (isolate from farm B), and none was found positive for tet(A) or tet(C).

4. Discussion

Substantial numbers of intestinal bacteria of animal origin are spread on farmland by disposal of manure, and spread of antibiotic resistance genes from bacteria in the manure to bacteria indigenous to soil could create an undesirable reservoir of antibiotic resistance in the soil. Variations in resistance levels before and after spread of animal manure have previously been observed for Pseudomonas spp. and for the Bacillus cereus group (Jensen et al., 2001). In the present study, we investigated the short-term and long-term effects of spread of pig manure slurry on occurrence of antibiotic resistance to tetracycline, macrolide and streptomycin in farmland. The long-term effects were evaluated by comparison to a nonmanured control soil. The antibiotics were chosen on the basis of their present and previous usage in animal husbandry in Denmark (DANMAP, 2000).

A decline in occurrence of tetracycline-resistant bacteria was observed after spread of pig manure slurry for all farms (Fig. 1). The reduced fraction of tetracycline-resistant bacteria in farm D soil as compared to farms A, B and C (Fig. 1) may be due to the lowest load of resistant bacteria combined with plowing of the manure slurry or because another crop was grown (Table 1). The temporary increase in tetracycline-resistant bacteria is probably caused by the addition of resistant intestinal bacteria present in the pig manure slurry with a limited survival potential in the environment (Sandvang et al., 2000). However, gene transfer between the intestinal bacteria and the bacteria indie-
nous to soil could have taken place in the manured soil. Addition of nutrients has been shown to enhance mobilization of plasmids (Top et al., 1990) and conjugation (Richaume et al., 1992), and high cell numbers have also been shown to increase gene transfer (Kinkl and Schmidt, 1991; Van Elsas et al., 1988). Pig manure slurry is rich in nutrients, and in this study we found up to $8.75 \times 10^7$ tetracycline-resistant bacteria per milliliter. CFU counts from blood plates were used as they were closer to the actual numbers of aerobic, culturable bacteria spread on the fields than counts on LB agar (data not shown). Studies on horizontal transfer as a result of spread of manure slurry are scarce, but enhancement of mobilization by addition of manure in a field study has been reported (Götz and Smalla, 1997). Only temporary changes in prevalence of tetracycline resistance were observed in this study since the fractions of the tetracycline-resistant bacteria all declined to the normal level of tetracycline resistance as compared to the non-manured control soil. Thus, either the extent of horizontal transfer to indigenous soil bacteria was absent or so low, that the pool of resistant soil bacteria already present in the soil hid it or the transformed cells did not survive due to lack of selective pressure in the soil. Approximately 5 months after spread, the levels of tetracycline resistance on all farms were within the range of the samples from the control soil. However, two issues indicate a promoting effect of pig manure slurry on the long-term tetracycline resistance levels in soil. (A) The predicted values of tetracycline resistance were correlated to load as illustrated in Fig. 2. (B) Comparing the measured levels of tetracycline resistance before spread from farms A, B, C and D showed a correlation between the load of pig manure slurry and levels of tetracycline resistance (Fig. 1). Use of antibiotics increases presence of antibiotic-resistant bacteria in the intestine (Bager et al., 1997; Smith et al., 1991; Van den Bogaard, 1997). To detect the selective pressure present in the soil, the concentrations of tetracycline in the soil sampled before spreading were measured using HPLC for farms A, B and C. In addition, concentrations of tetracycline in soils from farms A, B and C after spreading were estimated on the basis of the respective antimicrobial use at the farms. Using HPLC, no concentration of tetracycline in soil above the detection limit of 82 µg/kg soil was observed. The concentrations of tetracycline in soil were estimated to be between 2 and 85 µg/kg soil. These results supported data obtained using HPLC, as the PECs were lower than the sensitivity of the method. No correlation between estimated antibiotic soil concentrations of tetracycline and numbers of tetracycline-resistant bacteria in samples from different farms was observed. The fractions of tetracycline-resistant bacteria were in the same order of magnitude in all three farms (A, B and C, Fig. 1). This may point to the fact that the tetracycline residues in the manure slurry itself are not contributing substantially to the overall number of tetracycline-resistant bacteria in the soil. The actual measured concentrations of tetracyclines in the pig manure slurry were only between 15% and 20% of the theoretical concentration (calculation based on doses and produced manure slurry), indicating that the compound either degraded in the manure tank during storage of the slurry until spread, or was adsorbed to the slurry particles (Loke et al., 2002). Based on the actual pig manure slurry concentrations, soil concentrations were predicted to be between 0.6 and 14 µg/kg of tetracyclines. This supports that tetracyclines were present in the soil.

Levels of streptomycin (an aminoglycoside) and erythromycin (a macrolide) resistance were included in this study due to high usage of these antimicrobials in previous years both for growth promotion (macrolides) and for therapy (DANMAP, 2000). Only minor variations in streptomycin resistance levels were detected during the sampling year (data not shown). Although streptomycin was not used on any of the farms, between $2.3 \times 10^7$ and $4.6 \times 10^7$ streptomycin-resistant bacteria per milliliter were found in the pig manure slurry. However, the high numbers of streptomycin-resistant bacteria in the pig manure slurry did not seem to have any permanent effect on the streptomycin resistance levels in the soils, since similar levels of streptomycin resistance were found in the non-manured control soil (data not shown).

Selection of macrolide resistant bacteria is problematic due to the intrinsic resistance of Gram-negative bacteria in lower concentrations of macrolides. A combination of polymyxin and nalidixic acid (Granato and Ellner, 1976) showed effective counter-selection of Gram-negative bacteria from soil samples. No macrolides were used for therapy in the study period, and only limited registered use of lincosamides, giving cross-resistance to macrolides, on the farms was found. In the pig manure slurry, up to $1.6 \times 10^6$ erythromycin (macrolide)-resistant bacteria was found per milliliter, but no accumulation of resistance in the farm soils from previous usage was detected when compared to the control soil (data not shown). This observation suggests that previous high quantities of macrolides used for growth promotion (e.g. tylosin; Aarestrup and Carstensen, 1998) may not have caused any accumulation of resistance in the manured farmland. Correspondingly, a short degradation half-life in soil—manure slurries was found for tylosin (Ingerslev and Halling-Sørensen, 2001). The levels of lincosamides were calculated. The soil concentrations for the lincosamides were estimated in the range of 0.26—3.6 µg/kg. Thus, the lincomycin residues in the manure slurry did, like the tetracyclines, not seem to contribute considerably to the overall numbers of resistant bacteria, as PEC in farm C was higher by a factor of 14 than farm A.

Tetracycline resistance determinants tet(A), tet(B) and tet(C) have previously been identified in intestinal bacteria from pigs. Numbers of isolates that hybridized with DNA probes for tet(A), tet(B) or tet(C) of fecal isolates from pigs constituted 48% (Lee et al., 1993), and tet(B) was the most dominant tetracycline resistance determinant among Enterobacteriaceae (Marshall et al., 1983). In this study, indige-
nous soil bacteria were screened using PCR for the presence of these determinants to determine if the environment constituted a reservoir of these resistance genes. Very few positive isolates were, however, found, both in the pig manure slurry and the soil samples. Thus, previous most positive isolates were, however, found, both in the pig manure slurry or in the soil, and the screening could not be used as an indication for gene transfer.

In conclusion, large numbers of antibiotic-resistant bacteria are added to the farmland by spread of pig manure slurry. The greater part of these bacteria apparently only survive for a limited time, and the occurrence of antimicrobial resistance declines to a level corresponding to a non-manured control soil. The antibiotic residues present in pig manure slurry do not seem to contribute substantially to selection of antimicrobial resistance in the soil. However, the amount of pig manure slurry spread on the soil may have an effect on occurrence of tetracycline resistance.

Acknowledgements

The excellent technical assistance by Susanne Thorsen, Lise Christensen and Jeanette Knudsen from the Danish Veterinary Institute is highly acknowledged. We thank Per Bundgaard Larsen, DVI, for providing the data on antibiotic use and for help in interpretation of these data. Cooperation extended by the respective farmers is gratefully acknowledged. This investigation was funded by grants from Veterinary Environmental Research (1997–2000), the Danish Directorate for Development, Ministry of Food, Agriculture and Fisheries and from the Danish Research Agency, ref. 53-00-0279.

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