Using data on resistance prevalence per sample in the surveillance of antimicrobial resistance

A. R. Vieira1,2*, S. Wu1,2, L. B. Jensen1, A. Dalsgaard2, H. Houe2, H. C. Wegener1, D. M. A. Lo Fo Wong3 and H.-D. Emborg1

1Department of Microbiology and Risk Assessment, National Food Institute, Technical University of Denmark, Søborg, Denmark; 2Faculty of Life Science, University of Copenhagen, Frederiksberg, Denmark; 3Department of Food Safety, Zoonoses and Foodborne Diseases, WHO, Geneva, Switzerland

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Objectives: In most existing antimicrobial resistance monitoring programmes, one single bacterial colony from each collected sample is susceptibility tested against a panel of antimicrobials. Detecting the proportion of colonies resistant to different antimicrobials in each sample can provide quantitative data on antimicrobial resistance (resistance prevalence per sample).

Methods: In this study, a total of 98 faecal samples from slaughter pigs were tested for tetracycline and sulphonamide resistance in Escherichia coli using the single colony method, and these results were compared with the results obtained using the resistance prevalence per sample method.

Results: The results obtained by the resistance prevalence per sample method showed a lower occurrence of resistance. Tetracycline resistance in E. coli was found in 36.7% of the samples using the single colony method, while the mean tetracycline resistance prevalence was 22.5% using the resistance prevalence per sample method. Similarly, sulphonamide resistance was 32.7% using the single colony method and 19.6% when using the resistance prevalence per sample method. Although different estimates were obtained by each method, the correlation test and the regression model demonstrated that there is a significant association between the results obtained using both methods (P value <0.01) for both antimicrobials tested.

Conclusions: To support risk assessment and analysis of the association between consumption of antimicrobials and occurrence of resistance, there is a need to move towards a more quantitative approach when dealing with antimicrobial resistance in a population, and the resistance prevalence per sample method can provide some of this additional information.

Keywords: E. coli, quantitative, method

Introduction

In most existing antimicrobial resistance monitoring programmes, one single bacterial colony from each collected food, food animal and human sample is susceptibility tested against a panel of antimicrobials.1,2 This approach provides data on several antimicrobial agents at the same time for randomly selected and well-characterized isolates that can be stored for further study. However, this method does not allow determination of the variability in antimicrobial resistance in terms of proportion of resistant isolates within the sample. Furthermore, this method is not optimal for the detection of antimicrobial-resistant isolates occurring at low prevalences.3 Some studies have investigated the number of colonies selected per sample for antimicrobial susceptibility testing in order to describe the variation in susceptibility within the sample.1,5 Quantitative estimation of antimicrobial resistance using data on resistance prevalence per sample, e.g. by detecting the proportion of Escherichia coli colonies resistant to different antimicrobials, is not commonly used in the existing antimicrobial surveillance programmes. Further, no studies are available that have compared results obtained by testing single colonies and
Materials and methods

Collection of faecal samples
As part of the Danish integrated antimicrobial resistance monitoring and research programme (DANMAP), faecal samples from healthy pigs are collected monthly by meat inspection staff at the time of slaughter and sent for laboratory analyses. A total of 102 faecal samples were randomly selected among the samples collected by DANMAP between March and October 2006. Each sample was tested by the resistance prevalence per sample method and the single colony method.

Bacteriological analysis and resistance prevalence per sample method
The method used to determine the prevalence of resistance per sample was modified from a previous study. One gram of faecal sample was used to make 10-fold dilutions using 0.9% sterile saline up to $10^{-2}$. Subsequently, 100 µL of each dilution from $10^{-2}$ to $10^{-3}$ was plated onto a MacConkey agar (Oxoid, UK) plate. At the same time, 100 µL of each dilution from $10^{0}$ to $10^{-3}$ was plated onto a MacConkey agar plate supplemented with 512 mg/L sulfamethizole and another four plates with 8 mg/L tetracycline, respectively. Such concentrations of sulfamethizole and tetracycline in MacConkey plates were validated in a pilot study, where 512 mg/L sulfamethizole and another four plates with 8 mg/L tetracycline were able to distinguish sulfamethizole- and tetracycline-resistant E. coli from susceptible E. coli, compared with Mueller–Hinton II agar (BD Diagnostics, USA) in MIC testing (data not shown). Plates containing between 20 and 200 colonies were selected and colonies with typical E. coli morphology and appearance were counted after incubation for 18–24 h at 37°C. This method provides quantitative data at the sample level by detecting the proportion of E. coli colonies resistant to each antimicrobial in the test panel and is, in the following, referred to as the resistance prevalence per sample method.

Isolation of E. coli and antimicrobial susceptibility testing
Faecal samples were streaked directly onto Drigalski plates (State Serum Institute, Denmark) and incubated overnight at 37°C. The presumptive E. coli colonies were transferred onto CHROMAgar ECC plates (CHROMagar Microbiology, France) and incubated at 37°C overnight. One E. coli-like colony on CHROMAgar from each sample was subcultured and used for susceptibility testing by the broth microdilution method using a commercially dehydrated panel (Trek Diagnostic System, UK), as performed in DANMAP. However, for this study, only the results of sulfamethizole and tetracycline testing were analysed.

Statistical analysis
Each sample presented a sampling date (month), total number of E. coli colonies and the calculated resistance prevalence per sample for tetracycline and sulphonamide. The tetracycline and sulphonamide resistance prevalence for each sample were given as a percentage. From each faecal sample, the number of antimicrobial-resistant E. coli colonies was determined from MacConkey plates supplemented with either tetracycline or sulphonamide and divided by the total number of E. coli enumerated on MacConkey agar plates not supplemented with antimicrobials. MIC values of tetracycline and sulphonamide from the single colony method were dichotomized into resistant or susceptible, according to the breakpoints defined in the DANMAP surveillance programme and added to each observation.

Statistical analyses were performed using the SAS Enterprise Guide. Descriptive analysis was performed for the results obtained using both methods. Resistance prevalence per sample was further stratified into four classes (<1%, 1–10%, 11–50% and 51–100%) and correlation coefficients between the results of the two methods were calculated using the Spearman rank correlation test. Multiple logistic regression analysis was performed both for tetracycline and sulphonamide resistance. The outcome was the result of the single colony method, while the independent variables were the sampling date, total number of E. coli colonies and the resistance prevalence per sample. The criterion for keeping effects in the final model was $P$ value <0.05.

Results
A total of four faecal samples were excluded due to an uncountable number of E. coli colonies on the plates. The total number of E. coli isolated from each sample ranged from 4 to $9.3 \log_{10}$ cfu/g. MIC frequency distributions for tetracycline and sulphonamide presented a clear bimodal distribution with few samples showing MIC values around the recommended breakpoint.

According to the results obtained by the single colony method, 36 (36.7%) of the E. coli samples were resistant to tetracycline, while 32 (32.7%) were resistant to sulphonamide. Ten (10.2%) of the samples were resistant only to tetracycline and six (6.1%) only to sulphonamide. A total of 26 (26.5%) samples were resistant to both antimicrobial agents tested. Using the resistance prevalence per sample method, the unweighted tetracycline resistance mean was 22.5% (95% CI 17.9–27.1) and the median was 12.5%, while the unweighted sulphonamide resistance mean was 19.6% (95% CI 15.3–23.9) and the median was 10.4%. The percentage of samples where the resistance prevalence per sample ≥1% was 89.8% for tetracycline and 87.8% for sulphonamide, while none of the samples had 100%...
of its colonies resistant to any of the two antimicrobials. Resistance prevalence per sample distributions is presented in Figure 1. The distributions for tetracycline and sulphonamide were skewed and most of the samples had few resistant colonies.

The correlation between the results obtained by the two methods was significant for both antimicrobials tested ($P$ value $<0.01$), and the coefficients calculated by the Spearman rank test are given in Table 1. The two final models obtained through logistic regression confirmed the statistically significant direct association ($P$ value $<0.01$) between the results obtained using both methods. Total number of *E. coli* and sampling date were not significant, and were excluded from the models by backward selection.

**Discussion**

Although there is a difference in the prevalence estimates resulting from each method, both the correlation test and the regression model demonstrated that, at the sample level, there is a significant association between the results obtained using these methods. The results obtained by the resistance prevalence per sample method showed lower tetracycline and sulphonamide resistance prevalence among *E. coli* from pigs. This could be explained by a potentially stronger selection pressure on the selection media for the antimicrobial used in the quantitative method, underestimating the resistance prevalence. This pressure could reduce the number of colonies growing on the plates containing the antimicrobials, resulting in the underestimation of the resistance prevalence in each sample. In 2006, the DANMAP programme reported resistance to tetracycline in 28% of the *E. coli* samples and sulphonamide resistance in 26%. These percentages are for the whole year ($n = 148$) and are lower than the figures obtained by the single colony method in this study. The higher number of samples analysed by DANMAP over the whole year might explain the difference.

To our knowledge, this is the first time that antimicrobial susceptibility testing results from the single colony method and the resistance prevalence per sample method have been tested and compared based on analyses of identical samples. Resistance prevalence per sample has been calculated before to detect the antimicrobial resistance of *E. coli* from poultry and poultry farmers. In another study, where the replica plating method was compared with a five colony pick, similar results were obtained in both methods, in addition to further information on the resistance distribution when using the replica plating method.

In conclusion, to support risk assessment there is a need to move towards a more quantitative approach when dealing with resistance levels in a population, and the method employed in this study can provide some of this additional information. It presented results that provided more descriptive measurements and quantitative data compared with those obtained by the single colony method. Further studies evaluating this method, with the inclusion of different antimicrobials and bacterial populations should be carried out, particularly studies screening for emerging or rare occurrence of resistant profiles.

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**References**


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**Quantitative method for resistance prevalence**

Table 1. Correlation between the resistance prevalence per sample method and the single colony method, Spearman’s rank correlation coefficient ($r$) and $P$ value

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Resistance prevalence per sample method (percentage range)</th>
<th>Single colony method (%)</th>
<th>$r$</th>
<th>$P$ value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>susceptible</td>
<td>resistant</td>
<td></td>
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<tr>
<td>Tetracycline</td>
<td>&lt;1</td>
<td>9 (100)</td>
<td>0 (0)</td>
<td>0.399</td>
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<td></td>
<td>1–10</td>
<td>30 (81.1)</td>
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<td></td>
<td>11–50</td>
<td>17 (42.5)</td>
<td>23 (57.5)</td>
<td></td>
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<tr>
<td></td>
<td>51–100</td>
<td>6 (50)</td>
<td>6 (50)</td>
<td></td>
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<tr>
<td>Sulphonamide</td>
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<td>0 (0)</td>
<td>0.495</td>
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<tr>
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<td>17 (47.2)</td>
<td>19 (52.8)</td>
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<td></td>
<td>51–100</td>
<td>3 (30)</td>
<td>7 (70)</td>
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