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An empirical comparison of isolate-based and sample-based definitions of antimicrobial resistance and their effect on estimates of prevalence

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ABSTRACT

Antimicrobial resistance is primarily a problem in human medicine but there are unquantified links of transmission in both directions between animal and human populations. Quantitative assessment of the costs and benefits of reduced antimicrobial usage in livestock requires robust quantification of transmission of resistance between animals, the environment and the human population. This in turn requires appropriate measurement of resistance. To tackle this we selected two different methods for determining whether a sample is resistant − one based on screening a sample, the other on testing individual isolates. Our overall objective was to explore the differences arising from choice of measurement. A literature search demonstrated the widespread use of testing of individual isolates.

The first aim of this study was to compare, quantitatively, sample level and isolate level screening. Cattle or sheep faecal samples (n = 41) submitted for routine parasitology were tested for antimicrobial resistance in two ways: (1) "streak" direct culture onto plates containing the antimicrobial of interest; (2) determination of minimum inhibitory concentration (MIC) of 8–10 isolates per sample compared to published MIC thresholds. Two antibiotics (ampicillin and nalidixic acid) were tested. With ampicillin, direct culture resulted in more than double the number of resistant samples than the MIC method based on eight individual isolates.

The second aim of this study was to demonstrate the utility of the observed relationship between these two measures of antimicrobial resistance to re-estimate the prevalence of antimicrobial resistance from a previous study, in which we had used "streak" cultures. Boot-strap methods were used to estimate the proportion of samples that would have tested resistant in the historic study, had we used the isolate-based MIC method instead. Our boot-strap results indicate that our estimates of prevalence of antimicrobial resistance would have been considerably lower in the historic study had the MIC method been used.

Finally we conclude that there is no single way of defining a sample as resistant to an antimicrobial agent. The method used greatly affects the estimated prevalence of antimicrobial resistance in a sampled population of animals, thus potentially resulting in misleading results. Comparing methods on the same samples allows us to re-estimate the prevalence from other studies, had other methods for determining resistance been used. The results of this study highlight the importance of establishing what the most appropriate measure of antimicrobial resistance is, for the proposed purpose of the results.

1. Introduction

The primary problem associated with antimicrobial resistance (AMR) is failure of treatment in human medicine. However, resistance does not, generally, originate *de novo* in the patient in which treatment fails; rather it exists in a number of reservoirs within the patient's environment (Woolhouse et al., 2015). Treatment of the patient with an antimicrobial agent then provides a strong selection pressure in which resistant populations can outcompete their non-resistant counterparts. In order to address the interdependence of levels of AMR between a number of potential sources, a "systems map" approach has been suggested (Department of Health, 2014). The "systems maps" proposed are complex, pictorial representations of the inter-connections between reservoirs of potential resistance, possible transmission of resistance, and points of amplification of resistance in the presence of antimicrobials. Ideally we would identify which parts of the map were most amenable to modification and which parts are best targeted to address the main problem, which is resistance in human medicine. To do this

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requires accurate measurement of the component parts of the map. One key measurement is the prevalence of resistance in different bacterial reservoirs that make up the system of AMR and its transfer.

Prevalence estimates depend upon a sampling unit being defined as positive or negative. The number of bacteria in each sampling unit may be very large (e.g. $> 10^9$ per gram of faeces) and, in faeces for example, can vary by several orders of magnitude (Smith and Crabb, 1961). Therefore it is not obvious how many bacteria per sample should be tested nor the threshold for the number of "positive" (i.e. resistant) bacteria that should deem a sample as being resistant. Alternatively there are various methods that seek to test the sample as a whole such as spread plating [e.g. "culturing" on agar (Batura et al., 2010)], streak plating in which samples are serially diluted on the agar through streaking to enable picking of isolates (Amyes et al., 1992; Gunn et al., 2008; Humphry and Gunn, 2014), and detection of genetic markers of resistance via methods such as PCR or sequencing (Waldeisen et al., 2011). It is not clear to us why only one bacterium per sample should be tested to determine a sample as resistant or sensitive.

Comparisons between different methods on single isolates exist (Benedict et al., 2013; Dorado-Garcia et al., 2016; Lo-Ten-Foe et al., 2007; Luangtongkum et al., 2007; Luber et al., 2003) but we are not aware of anything that has been published comparing isolate based methods with whole sample methods. In this paper we quantify the relationship between streak plating and isolate-based methods of measuring resistance by applying both types of method to the same samples. Then, using historic baseline prevalence data for samples based on a sample-level test (streak-plating), we used the quantified relationship to back-calibrate and estimate the consequences had our baseline study used an isolate-based approach. This provides a "proof of concept" of how data such as these can be used to compare prevalence estimates across different studies that use different measures of resistance. Overall we seek to highlight that there is a need for the scientific community to reconsider the validity of taking a single bacterium per sample.

2. Materials and methods

2.1. Literature survey

To provide evidence regarding the use of individual isolates in relevant published studies we carried out a literature search. The search terms "prevalence antimicrobial resistance livestock" were entered into the online literature database "Web of Science". The search hits were ordered in decreasing "relevance" to the search terms and the 50 most relevant hits were then sought through the SRUC online access system. Any papers that were accessible were then read to determine whether the microbiological test was a sample based method (such as streak plating or spread plating) or a method based on isolates and, if so, how many isolates per sample.

2.2. Comparative study sampling

Sub-samples were taken from 41 faecal samples submitted for routine parasitological (i.e. non-bacterial) screening from cattle (25 samples) and sheep (16 samples) to the SAC (Scottish Agricultural College) Veterinary Investigation Centre, Inverness between August 2013 and July 2014. This study we call the "comparative study".

2.3. Comparative study laboratory methods

Each sample was 'streak' cultured on three plates: a standard MacConkey plate and two containing antibiotic (ampicillin 16 mg/L or nalidixic acid 15 mg/L). The streaking process on the plates involved sequentially streaking sub-samples from one streak to the next with the result that the concentration of sample decreased with each consecutive streak on a plate.

Table 1
The two antibiotics, the concentrations (μg/mL) used in the agar plates for testing the sample using the plate streak method and the MIC breakpoints chosen to determine an isolate's categorisation as sensitive/resistant.

Units in μg/mL	Ampicillin	Nalidixic acid
Concentration used in streak plate	16	15
Sensitive threshold for isolate MIC	MIC[a] ≤8	MIC[b] ≤16

[a] EUCAST, 2015.
[b] BSAC, 2012.

Where present, one putative *E. coli* colony from each of the two antibiotic-containing plates was randomly selected resulting in 0–2 "resistant" isolates. From the standard (non-antibiotic) plate 8–10 colonies were selected in addition to the "resistant" isolates to make up a total of ten isolates selected per sample in order to make full use of the ten wells per row on the test plates. These ten morphologically typical lactose fermenting colonies were selected and identified as *E. coli* based on their reactions in oxidase, indole, urease and Simmon's citrate tests (Cowan et al., 1993). They were then tested for the Minimum Inhibitory Concentration (MIC) for ampicillin, and nalidixic acid using concentrations from an appropriate standard with priority given to EUCAST ("European Committee on Antimicrobial Susceptibility Testing") (see Table 1) breakpoints, or, if these were unavailable (in the case of nalidixic acid) then we used BSAC (British Society for Antimicrobial Chemotherapy) breakpoints.

We have not assumed any level of sensitivity or specificity for either of the tests used. This is because it is not clear that there is a gold standard. Instead we calculate the conditional probabilities of each test dependent on the result of the other.

2.4. Comparative data analysis

Only samples from which eight or more validated *E. coli* isolates were identified from the control plate, and tested for MIC were included in the analysis. Where a sample resulted in more than eight isolates being tested (maximum of ten) a sub-sample of eight was randomly selected from these data in order to achieve statistical balance. Hereafter these data will be referred to as the comparative study data.

2.5. Statistical tests within the comparative study

A McNemar exact test (package *exact2x2* in R, (Fay, 2010)) was used to test whether the apparent difference in "marginal proportions" (i.e. prevalence using each method) was statistically significant.

The conditional probabilities and confidence intervals were calculated assuming a binomial process using exact binomial confidence limits (using *binom.test* in R), relating the probability of a sample testing resistant or sensitive using one test conditional on the result from the other test.

2.6. Assessing clustering (over-dispersion)

The assessment of statistical clustering (aka "over-dispersion") of resistant isolates was carried out with a quasi-binomial model in comparison to a null model of a binomial distribution based on a single overall proportion of isolates resistant.

We defined each isolate as resistant or sensitive according to the relevant EUCAST or BSAC definition. Results were aggregated at the sample level and a binomial logistic model was run. This was re-run as a quasi-binomial model (which allows for clustering) and a chi-squared test used to test the significance of the dispersion accounted for in the quasi-binomial model (Dobson, 2002). This procedure was used on all samples for both the ampicillin and nalidixic results and on the subset of samples for which streak plating tested resistance in the case of