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

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Running title[<=55 characters]: "Quantifying the effect of AMR measure on prevalence estimates"

#### Highlights

- A literature search demonstrated the widespread use of isolate-level measurement of resistance
- Faecal samples were tested for antimicrobial resistant *E. coli* at both the sample-level and the isolate-level

- Sample-level measurement was more likely to demonstrate resistance
- Using simulation we back predict isolate-level results from an historic study that only used sample-level measurement
- We question the widespread use of single isolate tests as demonstrated by our literature search

## 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 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; Lubert 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.

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.

Keywords: antimicrobial resistance; AMR; antibiotic; measurement; prevalence

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.

## Materials and Methods

### 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.

### 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”.

#### Comparative study laboratory methods

Each sample was ‘streak’ cultured on three plates: a standard MacConkey plate and two containing antibiotic (ampicillin 16mg/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.

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.

#### 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.

#### 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.

#### 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 ampicillin. The number of samples testing resistant to nalidixic acid by streak plating was too few to allow the procedure to be run on just these samples.

#### Historic sample-level prevalence study used for an illustration of re-calibration

Historic sample-level data, based on plate streaking as described previously (Gunn et al., 2008; Humphry and Gunn, 2014), were used to provide an illustration of the consequence of different definitions of a resistant sample. From a randomised survey of healthy animals, faeces samples were taken (1086 lambs from 104 randomly selected sheep farms, 312 calves aged less than 6 weeks, and 804 adult cattle were collected from 100 randomly selected farms throughout the Highlands and Islands of Scotland). Faecal samples were tested using streak plating (see above) on three plates in total, one without antibiotic (i.e. control) and two with either of the respective two antibiotics (ampicillin & nalidixic acid). A sample was defined as being resistant if any isolates which grew on an antibiotic-containing plate were demonstrated to be *E. coli*. Hereafter these data will be referred to as the baseline study data.

#### Simulated re-estimation

A bootstrap approach was used to obtain 95% percentiles for the re-calibrated estimated animal level prevalence from the baseline study data of healthy animals. This was done for both antibiotics: ampicillin and nalidixic acid. By bootstrapping from the comparative study data we allow for clustering for which there was evidence in the comparative study data (see Results).

For each resistant sample (one sample per animal) in our baseline study (streak plate), a randomly selected resistant streak-plate sample from our comparative study was selected. The predicted results from testing a single isolate, and eight isolates were simulated by randomly sampling with replacement from the corresponding data from the comparative study. If one or more of the isolates in each of these random selections were resistant isolates, then the baseline sample was deemed to be simulated as “EUCAST1sim” or “EUCAST8sim” resistant for the one and eight isolate sampling respectively. Then, using these predicted results for resistance, we calculated the population prevalence simulated as if a single isolate, or eight isolates had been tested using the EUCAST/BSAC criteria. This process was repeated 1000 times to provide a 95% percentile interval for the original baseline study prevalence based on bootstrapping from the comparative study.

The prevalence that we report for the baseline study and the simulated re-estimation is the proportion of samples positive with one sample per animal in the baseline study. This boot-strapping method is illustrated in Figure 1 in the form of a flow diagram.

## Results

### Literature sample

From the fifty most “relevant” (according to the search engine in Web of Science) papers returned, we were able, through the SRUC online library system to access 18 papers for examination (see appendix for full list of these papers). Of the 18 papers we accessed, eight did not state the number of isolates tested per sample, four papers were not applicable (e.g. review papers), five specifically stated that they tested one isolate per sample, and one paper declared that they tested “up to two colonies per plate”.

### Comparative study

In total, 41 samples were tested. An insufficient number of confirmed *E. coli* isolates were obtained from one sample to be included in the data analysis. Of the remaining 40 samples: three showed resistance by streak plating to nalidixic acid; fourteen showed resistance by streak plating to ampicillin. The 40 samples all yielded eight or more isolates from the control plate for further testing.

Table 2 gives cross-classification of samples measured as resistant or not according to the streak plate method and according to the testing of eight isolates for their MIC (EUCAST/BSAC method). One or more isolates from one of these samples is interpreted here as defining the sample as resistant using EUCAST/BSAC thresholds. Using the exact McNemar test we found that the difference in “marginal proportions” (i.e. prevalence using each method) was statistically significant at the 5% level in the case of ampicillin ( $p=0.008$ ) but not for nalidixic acid ( $p=0.5$ ).

Note that no isolates were classified as resistant using the EUCAST method from samples that were classified as sensitive using the streak plate method.

Table 3 provides estimates and confidence intervals for the conditional probability of a result dependent on a result using the alternative method. The confidence intervals are relatively wide due to the relatively small sample size. It is noticeable that the estimates for the conditional probabilities for ampicillin and nalidixic acid are close to one another. Whilst taking into account the large confidence intervals, it is noteworthy that there is a large overlap of confidence ranges -except in the case of  $\Pr(\text{sample tests sensitive with$

streak plating given that the sample contributed 0 resistant isolates from eight) in which the overlap is smaller.

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\*From the six samples that contributed at least one ampicillin resistant isolate there were, in total, 18 resistant isolates and thirty sensitive isolates. From the one sample that contributed at least one nalidixic resistant isolate there were six resistant and two sensitive isolates.

#### Evidence of clustering

Evidence of clustering is presented visually (Figure 2) as well as analysed statistically. The histogram of the actual data has “fatter tails” than the expected number (the points) suggesting clustering in the data.

In the case of nalidixic acid only three samples tested resistant using the streak plating, of these three samples, two had no isolates testing resistant and the third sample had six out of the eight isolates testing resistant. The proportion of isolates resistant from samples testing resistant (to nalidixic acid using streak plating) is therefore  $6/24 = 0.25$  (0.10, 0.47). The probability of a single sample (testing resistant using streak plating) giving six or more isolates resistant if we assume a background isolate level prevalence of 0.25 amongst those samples testing resistant using streak plating, may be calculated from the cumulative distribution function of the binomial and is  $p=0.004$  for a single, specified sample and  $p=0.012$  for any one of three samples. Therefore despite the low numbers of resistant samples, this low p-value suggests clustering may also be present for nalidixic acid.

More formal statistical testing for clustering was also employed. In the case of ampicillin the procedure could be run on all samples and also on only those samples that tested resistant using streak plating. In the case of nalidixic acid the number of samples that tested resistant using streak plating was low and therefore the statistical procedure was only appropriate when applied to all samples. In the case of ampicillin the p-values ( $1.3 \times 10^{-20}$  including all samples and  $2 \times 10^{-7}$  for just those samples which tested resistant using streak plating) strongly suggest clustering. Similarly for nalidixic acid, a p-value of  $1.8 \times 10^{-30}$  strongly suggests clustering when all samples were included. We therefore chose to use the bootstrap approach for back calculation rather than rely on a binomial assumption for the distribution of positive isolates.

#### Phenotypic diversity

Figure 3 illustrates the distribution of MIC values from samples broken down by the status of each sample according to the streak-plating sample-level method. Note that samples that tested resistant by streak-plating contained isolates from both sides of the breakpoint.

### Simulated re-estimation

The simulated estimates for the prevalences based on one or eight isolates from a control plate (EUCAST1sim and EUCAST8sim) are substantially lower than the original streak plating estimates collected in the baseline prevalence survey (Tables 4, 5).

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## Discussion

Our literature search demonstrated that studies reporting prevalence of antimicrobial resistance commonly estimate the resistance using just one isolate per sample. The justification for this choice of measurement over alternatives, for example the whole sample streak plating method, does not appear to be considered. Results presented in this paper demonstrate that the method used affects the prevalence estimate greatly. The data presented allowed us to relate, quantitatively, one method (isolate-based) with another (streak plating). Other studies have looked at the relationship between different methods based on testing the same isolates (Benedict et al., 2013; Dorado-Garcia et al., 2016; Lo-Ten-Foe et al., 2007; Luangtongkum et al., 2007; Luber et al., 2003) but work comparing sample based testing with isolate based testing has not been reported to the authors' knowledge. We report here such a comparison. This comparison is based on a small sample size but never the less gives interesting results for quantifying the relationship between the two different methods of testing for antimicrobial resistance. We see from the 2 by 2 classification tables (Results) that when compared to a sample of eight isolates tested for MIC and classified under EUCAST or BSAC guidelines, the streak plating method appears more likely to categorise a sample as resistant. This is either because the method is more sensitive or less specific (or a combination of both) than the measured MIC-based isolate method for both ampicillin and nalidixic acid. Our thesis is that there is no single gold standard for defining a sample as resistant or sensitive. It may be tempting however to assume 100% specificity, that is, when resistance is identified by *any* method, then it is assumed that the sample contains phenotypically resistant bacteria of the species of interest. For the purposes of this study we do not need to make this assumption and therefore we do not do so. In short

we have not chosen either method, or a combination of the two, as being a gold standard test.

The distribution of MIC values (Figure 3) for isolates taken from samples deemed sensitive and resistant using the streak plate method suggest a bimodal distribution (Martinez, 2014) for both ampicillin and nalidixic acid. Since these appear to cluster on either side of the MIC threshold for defining resistance, this suggests that the cut-off used has relevance to the bacterial population. It is clear that streak plate sensitive samples only provided sensitive isolates whilst the streak plate resistant samples included isolates from both resistant and sensitive sub-populations. For both ampicillin and nalidixic acid, the majority of isolates from samples found to be resistant using streak plating were themselves found to be sensitive using the measured MIC and the relevant MIC threshold. This is further evidence of phenotypic diversity amongst isolates within a single sample deemed resistant by streak plating (Humphry et al., 2002).

We found strong evidence of clustering. This evidence means that resistant *E. coli* are not homogeneously or randomly dispersed amongst samples. If clustering were 100% complete then a sample would be either all resistant or all sensitive. Such a situation would mean that taking a single isolate per sample would be sufficient to describe the sample. In practice, the data show that there is good evidence of a mixture of resistant and sensitive isolates within a resistant sample even with the clustering observed.

Antimicrobial resistance is a complex system. There are different species of bacteria within a sample, and different antimicrobials to consider. Even when we restrict our view to a single bacterial species and a single antimicrobial, different clones (colony forming units) within a sample will demonstrate the ability to grow in different concentrations of antimicrobial (MIC). Alternatively, given what we know of the ability of resistant genes to cross species boundaries within intestinal systems (horizontal

transfer) (Huddleston, 2014), we might believe that we need to consider the presence of resistance genes throughout the whole bacterial population. The description of a sample as “susceptible” or “resistant” therefore represents a single binary summary of the whole bacterial population, its sampling and testing processes. We could think of this population summary as being analogous to providing a summary statistic from a sample. We do not believe that the summary statistic fully describes the population, but it may be useful as an indication of one aspect of the population's distribution.

We know that the density of bacteria in faeces can be very high and can vary from animal to animal by orders of magnitude (Smith and Crabb, 1961) and we know that the isolate-level prevalence within a sample of *E. coli* that are resistant can vary greatly (Humphry et al., 2002). Therefore even when we test as many as eight isolates we identify fewer samples as being resistant compared to when we test using the streak plating method. It is valuable to demonstrate this empirically and to give quantitative estimates to the relationship between methods. The streak plating method is inherently capable of screening a very large, but not quantified, number of isolates at one time. We also note that it is common for studies of prevalence of resistance to test one isolate per sample (see appendix) and this is likely to affect resistance prevalence estimates to a greater extent than in the case of eight isolates per sample (Table 2). This suggests that streak plating would be more sensitive or less specific than testing one or eight isolates per sample - with the caveat that there is no gold standard.

To show one potential use of our results, we back-calculated our prevalence estimate from a randomly sampled baseline historic study had we used one or eight isolates determined resistant or susceptible using a breakpoint defined method (Table 2). This back-calibration is only applicable if we assume that the relationship between the two tests was the same in the population considered by the historic baseline study as it was

in our more recent comparative study. The most important assumption is that the frequency distribution of number of resistant isolates within a sample that tests resistant using streak plating has stayed approximately the same. The similarity in the conditional probabilities (Table 3) between ampicillin and nalidixic acid indicate that for faecal samples, the statistical relationship between streak plating results and isolate based results may be fairly consistent across populations. The wide confidence intervals around these conditional probabilities is a consequence of the low numbers of samples in our study. In the absence of any knowledge to the contrary, assuming that relationship to be the same is the most parsimonious position to start - until evidence is found to the contrary. Again, our back-calibrated estimates for prevalence of resistance, using one or eight isolates, are substantially lower than those originally reported, based upon the streak plating method. Our primary conclusion is that the prevalence estimates that researchers produce are a function of the laboratory method of summarising the multitude of latent “data” that are the population of bacteria of interest in a sample.

Given how much the results we present depend on the method used, it is our belief that the scientific community, even at this stage, ought to be open-minded to using the best available method to answer a particular question. For example, highly sensitive methods such as streak plating, spread plating or genetic screening for resistance genes through PCR or whole gene sequencing might become more relevant as we attempt to understand the antibiotic resistance “systems map” better (Department of Health, 2014). There are environments (such as livestock holdings or hospitals) in which antibiotic use, through natural selection pressure, might quickly and substantially amplify even very low levels of resistance. Therefore using a measure that is effective at identifying resistance even when present in low levels, may be a better predictor of future clinical problems or transfer of resistance than a method which focusses on a small number of isolates.

There is another important potential advantage of systems of measurement that screen samples of bacteria rather than only testing individual picked isolates. Screening samples inherently takes into account, without necessarily measuring directly, the bacterial density of the sample (Humphry and Gunn, 2014). When modelling risk of transfer of bacteria from one environment to another, the bacterial density is an important contributor to the risk (Gerhardts et al., 2012). Streak plating, plate screening and sample screening for genetic markers of resistance fulfil this function of *inherently* accounting for bacterial density within their system of measurement. Methods such as streak plating do not however, by themselves, offer estimates of bacterial density, but they are positively correlated with bacterial density (Humphry and Gunn, 2014).

In conclusion, we hope we have presented sufficient evidence to support our thesis that the prevalence estimate of antibiotic resistance is greatly dependent on the method used. The extent of this we believe may surprise many. Sample level methods, not based on individual isolates, but screening the sample as a whole can be much more sensitive at identifying low within-sample prevalences of resistance. We present the argument that sample level screening techniques in the future should be an important part of the research community's armoury when trying to populate systems level models such as the "systems map" (Department of Health, 2014). Understanding which screening measure is best for particular clinical or research questions should therefore be one component of the widespread and escalating research effort demanded by society.

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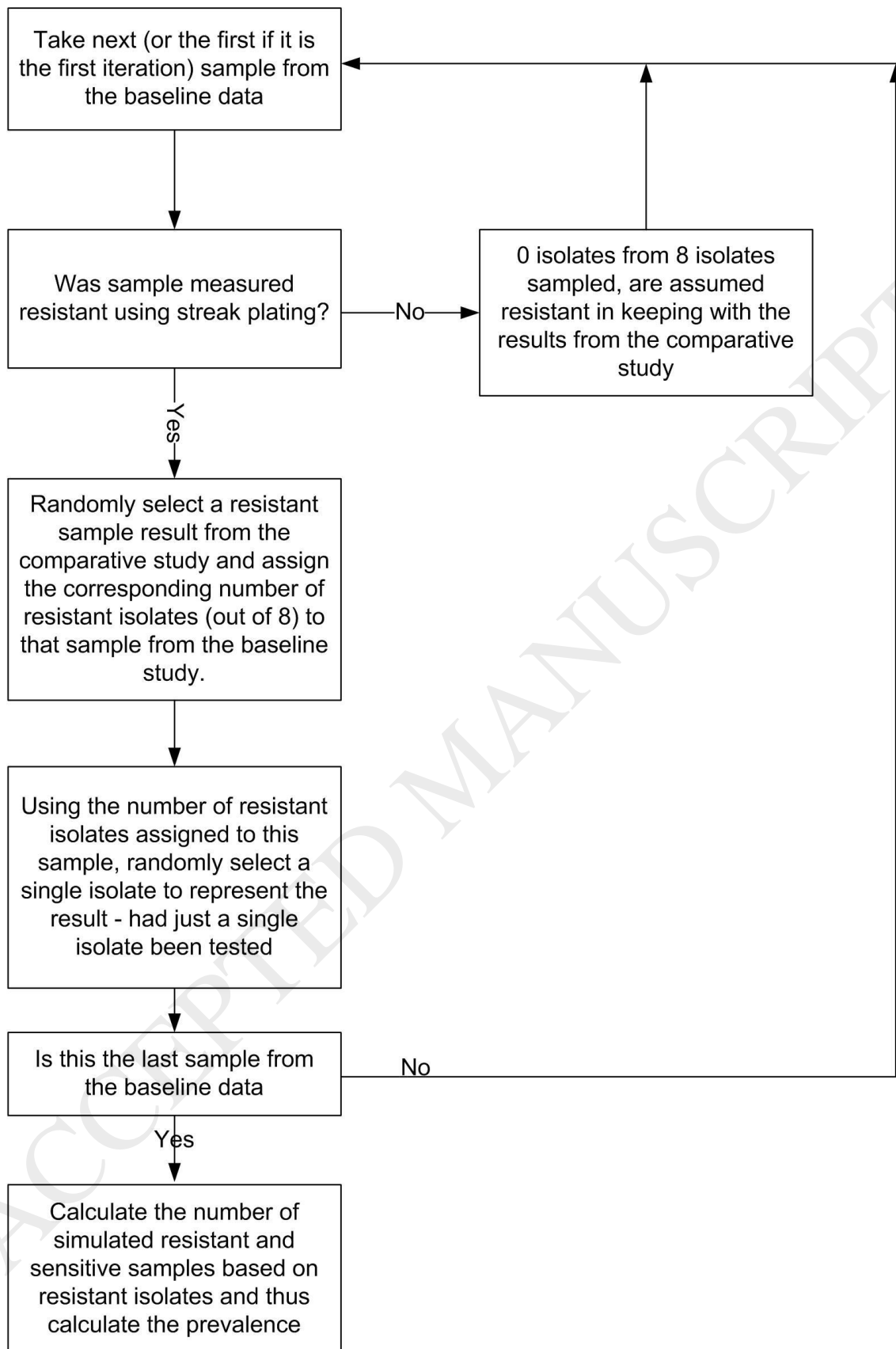


Figure 1. A flow diagram illustrating the algorithm for simulating results from the baseline data as if the samples had been tested using the isolate based method.

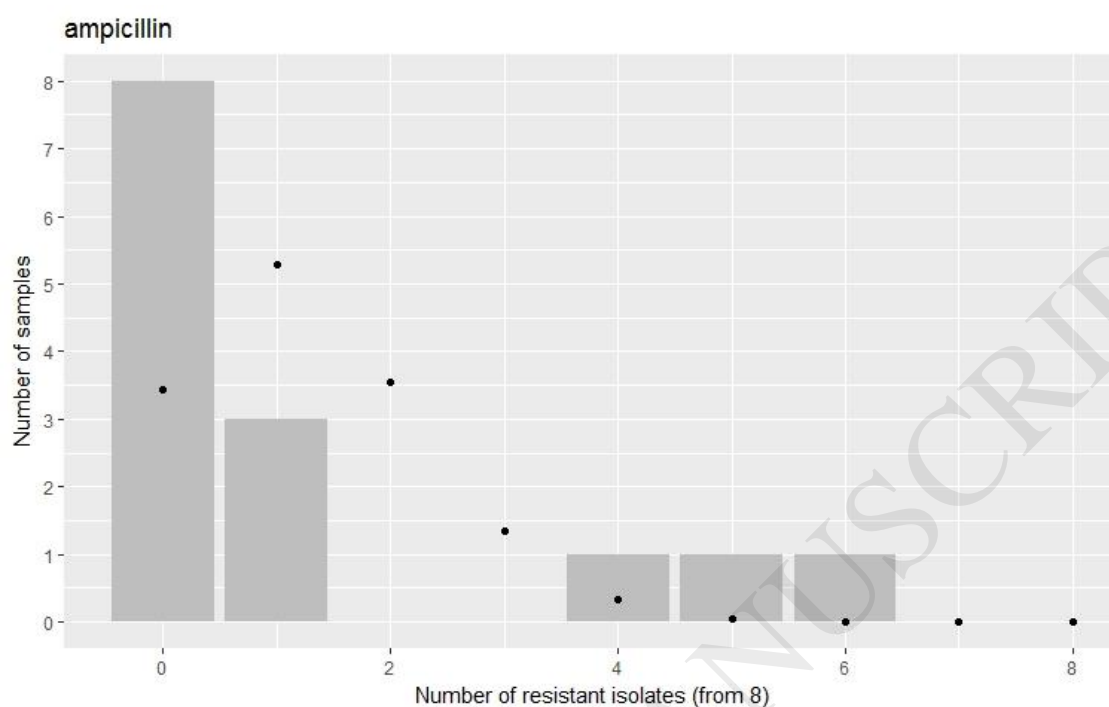


Figure 2. Histogram for the frequency of samples based on the number of resistant isolates (out of eight tested from each sample) from those samples that tested resistant to ampicillin using streak plating. The black dots give the expected number of samples if the process were a purely binomial process with a single isolate-level prevalence for all samples that tested resistant using streak plating.

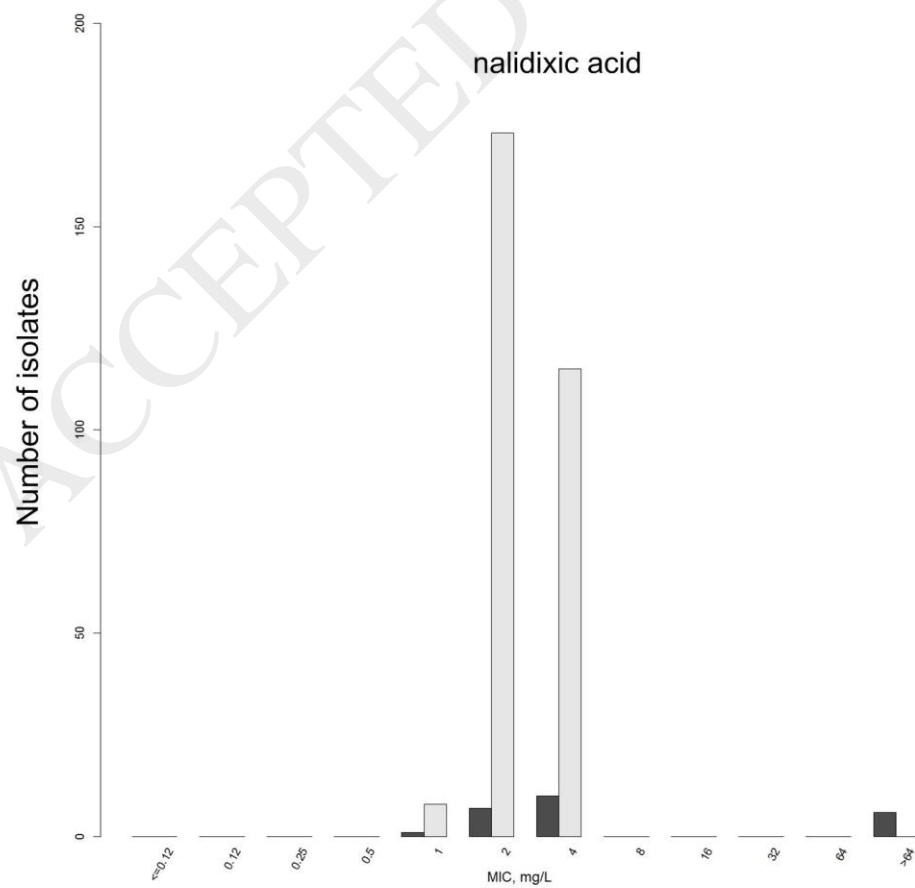
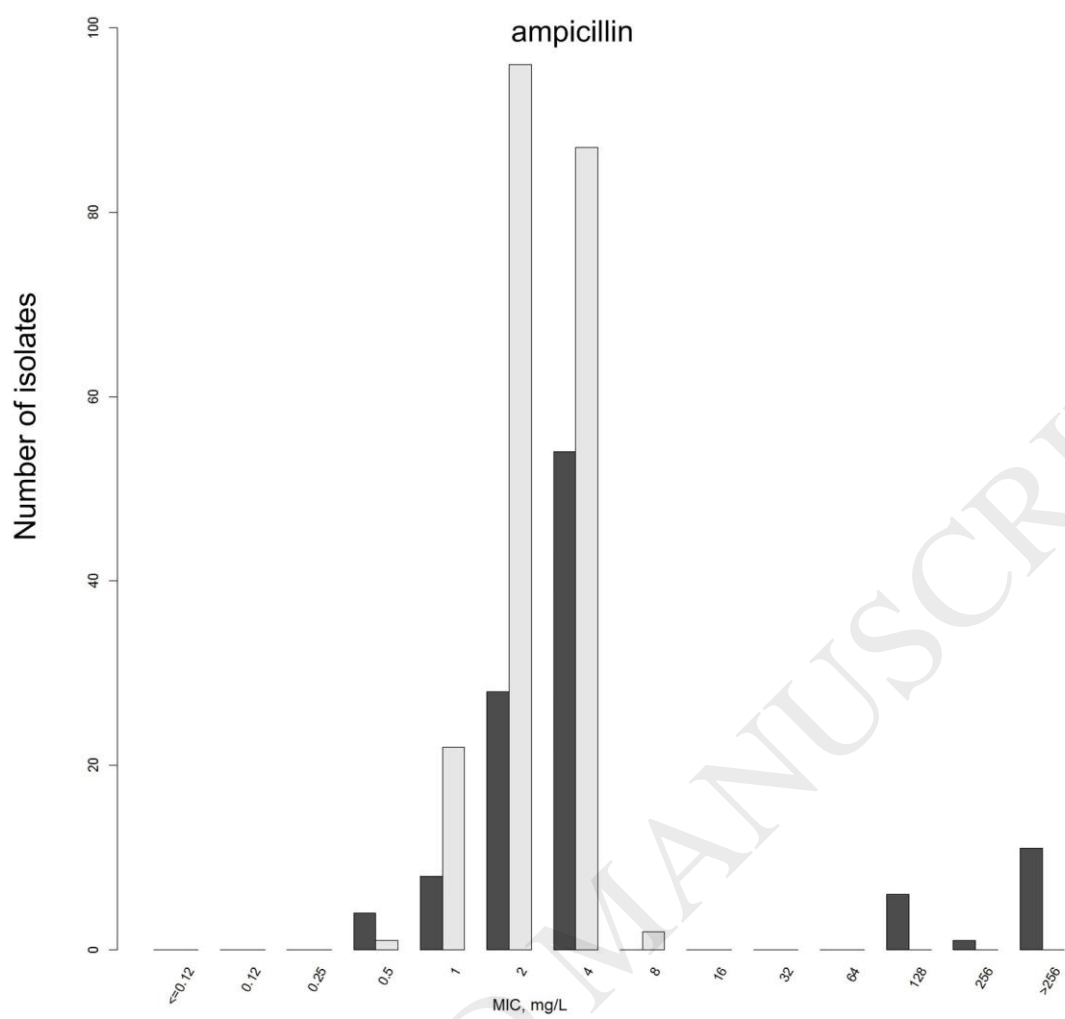


Figure 3. Frequency of MICs to ampicillin or nalidixic acid for each of the eight *E. coli* isolates from each of the forty samples in the comparative study. Light bars indicate isolates taken from samples that tested sensitive to antibiotic when tested using the streak plating method. Dark grey indicates isolates taken from samples that tested resistant to antibiotic when tested using the streak plating method. The EUCAST guidelines state that a threshold MIC of over eight mg/L should be deemed resistant. For nalidixic acid the BSAC guidelines state that a threshold MIC of over 16 mg/L should be deemed resistant. The results for ampicillin are in the top graph, results for nalidixic acid are in the bottom graph.



Table 1: The two antibiotics, the concentrations ( $\mu\text{g}/\text{mL}$ ) 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 $\mu\text{g}/\text{mL}$	Ampicillin	Nalidixic acid
Concentration used in streak plate	16	15
Sensitive threshold for isolate MIC	MIC <sup>a</sup> $\leq$ 8	MIC <sup>b</sup> $\leq$ 16

a. EUCAST, 2015;

b. BSAC, 2012;

## Appendix I

Eighteen references accessed online under the search terms: "*prevalence antimicrobial resistance livestock*" within the Web of Knowledge database:

Reference	Description of sampling method	Clinical sample or not clinical sample
(Argudín and Butayea, 2016)	implied one isolate per sample but not explicit	Not clinical
(Alonso et al., 2016)	"Up to 2 colonies per plate were selected for posterior identification	Not clinical
(Katakweba et al., 2016)	Number of isolates per sample not stated	Not clinical
(Horigan et al., 2016)	Review	
(Hanon et al., 2015)	"one isolate/faecal sample"	Not clinical
(Van Boeckel et al., 2015)	Review of usage	
(Guerra et al., 2014)	Number of isolates per sample not stated	Not clinical
(Schwaiger et al., 2014)	Number of isolates per sample not stated	Not clinical
(Burow et al., 2014)	Review - unstated number of isolates per sample	Treatment and control groups
(Roug et al., 2013)	Number of isolates per sample not stated	Not clinical
(Thai et al., 2012)	Number of isolates per sample not stated	Not clinical

(Kreausukon et al., 2012)	Number of isolates per sample not stated	Not clinical
(Egger et al., 2012)	Number of isolates per sample not stated	Not clinical
(Hur et al., 2012)	Review	
(Williams et al., 2011)	Faecal broth sample	Not clinical
(Guenther et al., 2010a)	One colony per sample	Not clinical
(Guenther et al., 2010b)	One colony per sample	Not clinical
(Padungtod and Kaneene, 2006)	One isolate per sample went through to AST testing	Some clinical

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Figure Caption

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Fig-1

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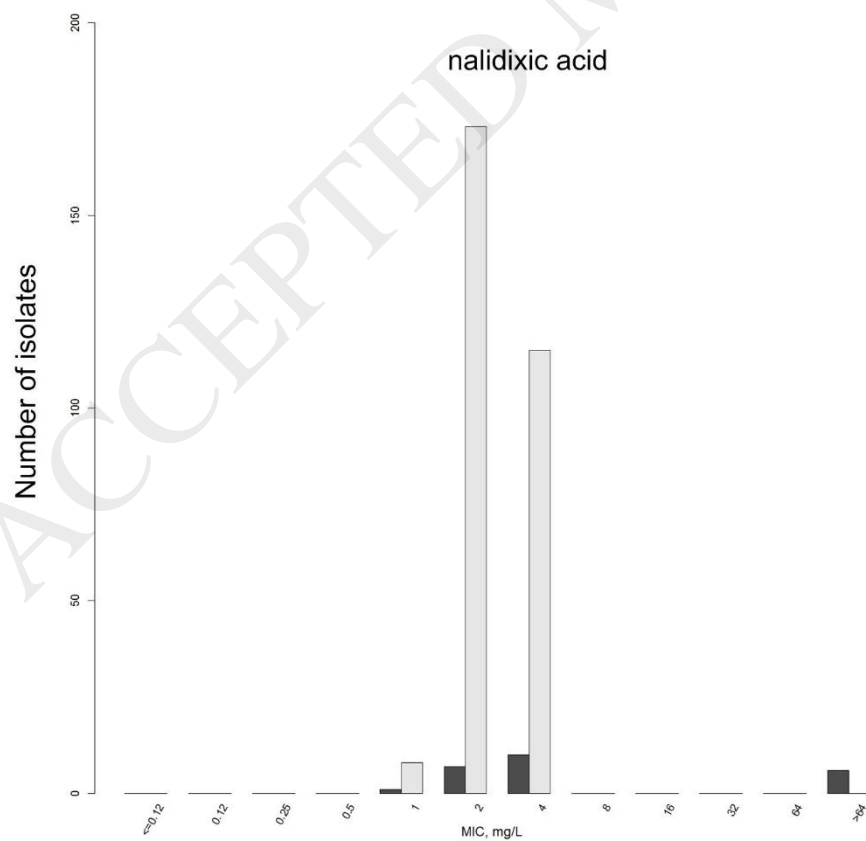
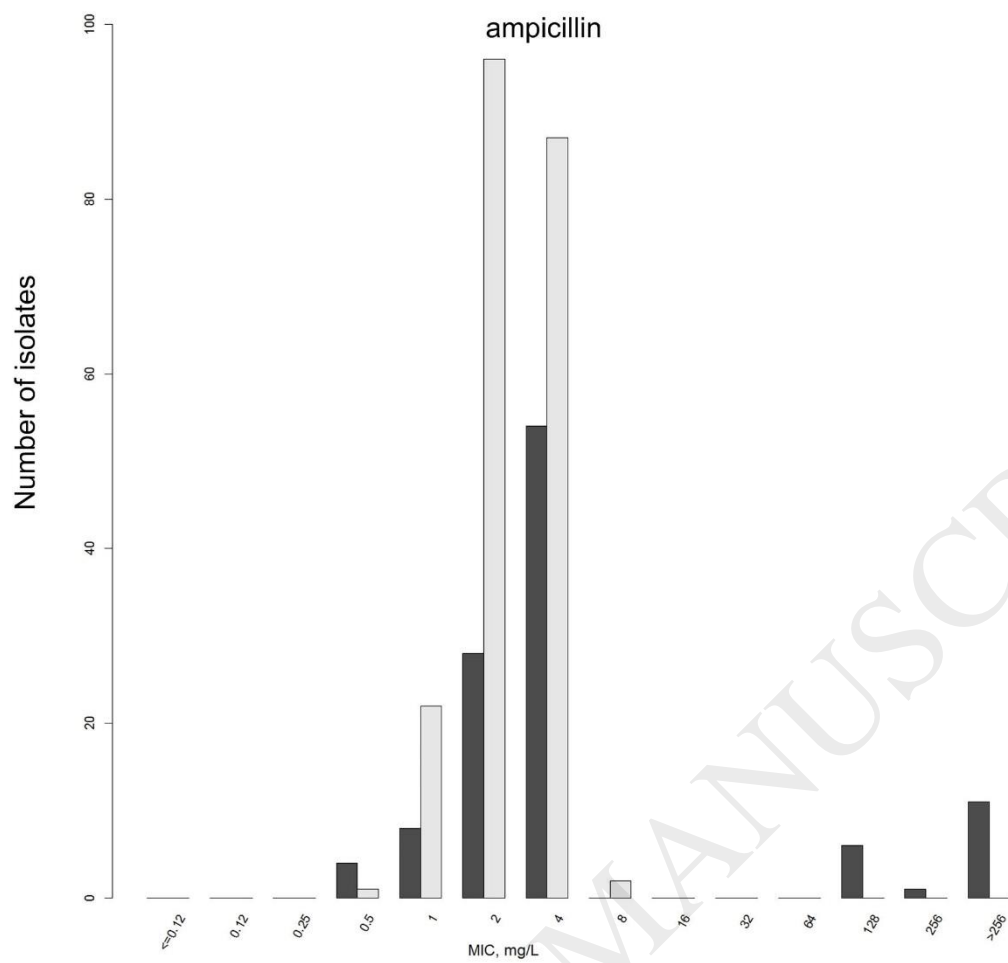


Table 2. A cross classification of resistance to ampicillin and nalidixic acid for samples tested using the measured EUCAST or BSAC isolate MIC based method and the sample level streak plating method from samples taken in the calibration study.

			Streak plating method	
			Sensitive	Resistant
MIC method.	Ampicillin	Sensitive	26	8
Sample resistant		Resistant	0	6*
if at least one out	<hr/>			
of eight isolates	Nalidixic acid	Sensitive	37	2
resistant.		Resistant	0	1*

Table 3. The conditional probabilities and confidence intervals (calculated assuming a binomial process using `binom.test()` in R) relating the probability of a sample testing resistant or sensitive using one test depending on the result from the other test. The symbol “|” is the statistical symbol for the conditional “given that”.

	Ampicillin	Nalidixic acid
Pr(resistant isolates >0   sample tested resistant with streak plating)	0.43 (0.18, 0.71)	0.33 (0.008, 0.91)
Pr(resistant isolates >0   sample tested sensitive with streak plating)	0 (0.00, 0.13)	0 (0.00, 0.09)
Pr(a single isolate is resistant   sample tested resistant with streak plating)	0.16 (0.10, 0.24)	0.25 (0.10, 0.47)
Pr(a single isolate is resistant   sample tested sensitive with streak plating)	0 (0.00, 0.13)	0 (0.00, 0.09)

Pr(sample tests sensitive with streak plating   sample contributed 0 resistant isolates from eight)	0.76 (0.59, 0.89)	0.95 (0.83, 0.99)
Pr(sample tests resistant with streak plating   sample contributed >0 resistant isolates from eight)	1.00 (0.54, 1.00)	1.00 (0.025, 1.00)

Table 4. The predicted base-line animal-level prevalences for resistance to ampicillin, simulated and based on either single or eight isolates. Prevalence predictions presented with their percentile intervals in comparison to the prevalence estimated originally using streak plating.

	Animal-level (i.e. sample-level) prevalence estimate of ampicillin resistance		
	Streak plate animal-level prevalence from original baseline survey	Simulated median and 95% percentile interval for animal-level prevalence had one isolate been tested (EUCAST1sim)	Simulated median and 95% percentile interval for animal-level prevalence had eight isolates been tested (EUCAST8sim)
Calves	88%	14% (11%, 18%)	31% (25%, 36%)
Adult Cattle	47%	7.6% (6.0%, 9.5%)	17% (14%, 19%)
Sheep	19%	3.1% (2.2%, 4.1%)	6.7% (5.5%, 7.9%)

Table 5. The predicted base-line animal-level prevalences for resistance to nalidixic acid, simulated and based on either single or eight isolates. Prevalence predictions presented with their percentile intervals in comparison to the prevalence estimated originally using streak plating.

Animal-level (i.e. sample-level) prevalence estimate of nalidixic acid resistance			
	Streak plate animal-level prevalence from original baseline survey	Simulated median and 95% percentile interval for animal-level prevalence had one isolate been tested (EUCAST1sim)	Simulated median and 95% percentile interval for animal-level prevalence had eight isolates been tested (EUCAST8sim)
Calves	6.69%	1.59% (0.64%, 2.87%)	2.23% (0.96%, 3.50%)
Adult Cattle	1.84%	0.49% (0.12%, 0.86%)	0.61% (0.12%, 1.10%)
Sheep	0.64%	0.16% (0%, 0.40%)	0.16% (0%, 0.40%)