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SID 5 Research Project Final Report

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Project identification

1. Defra Project code: VM2208
2. Project title:
   Evaluating the new European broth microdilution guidelines for determining the susceptibility of C. jejuni and C. coli recovered from food animals and humans.
3. Contractor organisation(s):
   Veterinary Laboratories Agency
   Woodham Lane
   New haw
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4. Total Defra project costs (agreed fixed price): £ 60,402
5. Project: start date .... 07 January 2008

   end date .... 30 April 2009

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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

In **objective 1** a custom antibiotic microtitre plate for broth microdilution susceptibility testing of *C. jejuni* and *C. coli* was designed to include antibiotics and ranges recommended by the European Food Safety Authority Working Group on developing Harmonised Schemes for Monitoring Antimicrobial Resistance in Zoonotic Agents. The plate also included antibiotics and concentration ranges that covered the breakpoint dilutions traditionally tested by the UK Health Protection Agency and used by the Veterinary Laboratories Agency in previous abattoir surveys. The broth microdilution test, based on the Trek Sensititre™ system, was assessed using *C. jejuni* and *C. coli* isolates from humans, cattle, pigs and broilers. The objectives of the validation and assessment were to (1) evaluate agreement of antimicrobial resistance classification derived by a Sensititre broth microdilution test with the results derived from the traditional agar dilution breakpoint technique (2) assess reproducibility of the broth microdilution test results in two independent laboratories and (3) calculate repeatability of the results derived by the agar dilution breakpoint technique and the broth microdilution test. The study was designed on recommendations from BS EN ISO 16140:2003 and EN ISO 20776-2, but modified to accommodate our objectives. Appropriate sample sizes were calculated and a total of 120 isolates were included in the study. This number ensured a 95% confidence in the results and a power of >70% to significantly detect differences between the test results, if the difference was >5%. Historical antimicrobial resistance patterns were used to try and ensure a minimum of 30:70 split of sensitive and resistant isolates for each antimicrobial at the historical breakpoint as well as a 50:50 division of *C. jejuni* and *C. coli*. The ratio was obtained for the majority of antimicrobials but the low prevalence of resistance to gentamicin and kanamycin in campylobacters from humans and food animals human and animal resulted in a lower proportion of resistant isolates. The final test panel of 120 isolates contained 67 *C. jejuni* isolates and 53 *C. coli* isolates, yielding a species distribution of 56:44. Thirty isolates from each source were included. A blind-coded panel comprising 160 isolates, which included ten isolates each of *C. jejuni* and *C. coli* in triplicate, was dispatched to each of two laboratories.

Resistance to some antimicrobials recorded by the original breakpoint test and used to collate the panel had changed over time, resulting in a less optimally balanced test panel than planned by the study design. This observation was most notable for chloramphenicol, ampicillin and tetracycline. The major contributing factor was that isolates were selected on the basis of their designated resistance or susceptibility in the breakpoint susceptibility test performed on agar. Thus, isolates with true MICs close to the breakpoints of these antimicrobials would have been included in the panel, inevitably introducing some variation into this type of study. Nevertheless, the study found no evidence that the broth microdilution test was inaccurate or in serious disagreement with the agar breakpoint test. The proportions of resistant isolates to kanamycin, gentamicin, streptomycin and erythromycin were very similar between the two tests. However, the broth microdilution test classified slightly more isolates resistant to ciprofloxacin, chloramphenicol and

tetracycline, particularly at the lowest breakpoints applied.

Substantial agreement was also obtained by the broth microdilution test when performed independently in both laboratories for the majority of antimicrobial agents and breakpoints applied. However, the findings suggested a systematic overestimation of MIC values by one of the two laboratories for ampicillin ($p < 0.001$) which warrants further investigation. The combined accordance derived from testing 20 isolates in triplicates was high and fairly stable for both tests. The agar dilution test showed an accordance of 94.5% and the broth microdilution test an average accordance of 96.1%. Lack of accordance was generally associated with results obtained with ampicillin and tetracycline. However, both tests performed very consistently within laboratories. The broth microdilution method was therefore confirmed to be reproducible between laboratories for the majority of antimicrobials and had high levels of accordance even exceeding the traditional agar dilution break-point test. Nevertheless, inconsistencies with some of the test antimicrobials, particularly with ampicillin and tetracycline and to which minor technical and/or interpretative differences between labs may have contributed, warrants further investigation to ensure optimal harmonisation of the method.

Many of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values for antimicrobials included in the Community Reference Laboratory External Quality Assurance Scheme are similar to the traditional breakpoints applied in GB and were included in our validation, but some are species-specific, on the basis of differences in wild type distributions between *C. jejuni* and *C. coli* for the certain antimicrobials. Reproducibility of results at these EUCAST recommended cut-off values laboratories was assessed, by dichotomising the broth microdilution results from each lab at each breakpoint and applying agreement statistics to these values. In general, the new cut-offs changed the agreement of the broth microdilution tests in the two laboratories only slightly; no difference in the level of agreement was observed for gentamicin, while a slight increase was obtained for erythromycin.

Objective 2 aimed to investigate wild type distributions obtained for *C. jejuni* and *C. coli* from humans and food animals and to assess the suitability of the EUCAST designated epidemiological cut-off, for isolates recovered in GB. In order to select isolates of unknown susceptibility from comparable time frames, 200 *C. jejuni* and 100 *C coli* isolates were randomly selected from human and retail meat submissions received by HPA in 2004. Wild type MIC distributions for 99 human and 100 retail meat isolates of *C. jejuni* , and for 49 of each source from *C. coli* for all nine antimicrobials were obtained. MIC distributions were compared with seven of the selected antimicrobials available on the EUCAST reference database (http://www.eucast.org). Distributions of MIC values yielded for the human and retail meat isolates of *C. jejuni* appeared remarkably similar and generally correlated well with the data available for *C. jejuni* isolates in the EUCAST database. However, although some minor differences were observed the wild type population generally fell within MIC values below the recommended epidemiological cut-off.

EUCAST have, however, recommended species-specific cut-off values for erythromycin, gentamicin and streptomycin. When applied to the MIC distributions from *C. jejuni* from our study these fitted well. The data obtained for these antimicrobials from this study demonstrated that MIC distributions for *C. jejuni* and *C. coli* were within the same range, with very similar mode values. Thus no differences in Resistant/Susceptible classifications would have resulted from application of the *C. jejuni* breakpoint to the *C. coli* panel of isolates. It would be appropriate to gather and analyse susceptibility data from subsequent abattoir and food surveys to further investigate these interesting observations.

Further issues arose with tetracycline and ampicillin. The traditional GB breakpoint value of 8 mg/l for classification of resistance for tetracycline appeared to give similar results to that of EUCAST (2 mg/l), for both *C. jejuni* and *C. coli*, because of the low number of isolates yielding MIC values between 2 and 8 mg/l. The range and mode of wild type ampicillin MIC distributions for ampicillin for the two *Campylobacter* species differed slightly, with *C. coli* having a mode value 1 MIC step higher than *C. jejuni*. However, compared with the EUCAST reference our the mode MIC value obtained for both species was very close to the *C. jejuni* and *C. coli* epidemiological cut-offs. This unexpected observation may be attributed to skewing of the distribution by isolates with reduced susceptibility to this antimicrobial, although potential variability with this antimicrobial that may be attributed to the systematic differences in results between laboratories should be explored.

In objective 3 following recommendations and outcomes were produced from the study results:
1. An SOP for broth microdilution test for *C. jejuni/coli* as a specific National Reference Method. Standardisation of procedures in all test laboratories submitting susceptibility data on human and food animal isolate susceptibility is necessary to minimise differences in interpretation relating to end point which may cause systematic differences in test results between laboratories.
2. Participation in EU ring trial or similar should be mandatory for national labs involved in this work and funding made available to ensure both human/food and veterinary National Refererence Laboratories for susceptibility testing can participate in such ring trials.

3. As the standard control strain *C. jejuni* (ATCC 33560) has MICs below designated cut-off values for the majority of antimicrobials and the range proposed for ampicillin for *C. coli* strain ATCC 33559 is 4-16 mg/l it would be appropriate to include additional control isolates to include one multi-resistant strain of *C. jejuni* and of *C. coli* , preferably from the ar-CRL EQAS system to ensure authenticity.
4. Generally for *C. jejuni* our wild type distribution data fitted well with EUCAST epidemiological cut-off values, including that for clinically relevant antimicrobials ciprofloxacin and erythromycin. Application of the BSAC breakpoint of 0.5 would have resulted in few isolates being classified as susceptible.
5. Although EUCAST *C. coli* -specific breakpoints have been adopted by EFSA for erythromycin, gentamicin and streptomycin our data showed very similar MIC distributions to those obtained for *C. jejuni*. This observation warrants further investigation by analysis of broiler and other similar randomly selected populations susceptibility tested using this method.
6. The EUCAST cut-off value for chloramphenicol, classifying R>16 mg/l, supported the data obtained from objectives 1 and 2 of this project better than the traditional GB breakpoint of 8 mg/l. However, there appears to be no clear distinction between S and R populations for tetracycline and ampicillin.
7. The results provide data on the MIC distribution for imipenem which may be used clinically in the treatment of invasive campylobacter infections in humans. Although imipenem is not required in EU surveillance monitoring this data may be of clinical relevance for assessment of treatment in a limited number of human infections and will therefore be passed to BSAC.

Project Report to Defra

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   - the scientific objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Transfer).

Objectives as set out in the contract

Objective 01. Validate a broth microdilution sensititre test which uses EU- recommended ranges for Campylobacter, for susceptibility testing of *C. jejuni* and *C. coli* from human and veterinary sources.

Objective 02. Investigate wild type distributions obtained for *C. jejuni* and *C. coli* and evaluate suitability of epidemiological cut-off points recommended by the EU Antimicrobial Resistance CRL for GB isolates from a variety of hosts.

Objective 03. Produce recommendations for joint reporting of human and animal campylobacter antimicrobial resistance surveillance which facilitates comparison with data produced in other EU countries.

Extent to which objectives have been tacked and met
All objectives have been tackled. Objectives 1 and 2 have been fully completed. Recommendations suggested by the project team have been submitted to VLA and HPA representatives on BSAC and DARC Groups for discussion.

The overall aim of the project was to agree, evaluate and apply a harmonised broth microdilution protocol for susceptibility testing of *C. jejuni* and *C. coli* recovered from human, food animal and food products. The project formed three key objectives:

01. Validate a broth microdilution sensititre test which includes EU- recommended antibiotics and ranges for Campylobacter, for susceptibility testing of *C. jejuni* and *C. coli* from human and veterinary sources

Prescribing internationally accepted antibiotic susceptibility test methods for *Campylobacter* spp. has proven difficult, because approved guidance documents and interpretive criteria until recently were limited to the newest National Committee for Clinical Laboratory Standards (NCCLS), later Clinical Laboratory Standards Institute (CLSI), guidelines. Multiple methods are currently used throughout the EU public health and veterinary laboratory network for the antimicrobial susceptibility testing for human, food and veterinary campylobacters. However, the use of harmonised methods for monitoring resistance is a pre-requisite for the provision of high quality comparable data on which to base policy decisions. The test originally developed for surveillance-level antimicrobial susceptibility testing of *C. jejuni* and *C. coli* in England and Wales, by the Campylobacter Reference Unit of the Laboratory of Enteric Pathogens at the Health Protection Agency (HPA) Colindale, comprised agar incorporation at a breakpoint level, for nine different antibiotics (Thwaites and Frost, 1999). Breakpoints were based on those published in 1996 by the British Society for Antimicrobial Chemotherapy (BSAC), except for that of erythromycin which was based around NCCLS guidelines. This test has been used by the HPA Campylobacter Reference Unit to perform susceptibility testing on campylobacters gathered from clinical samples and retail products from numerous studies and projects between 1993 and the present day, with only minor modifications, including alterations to ampicillin breakpoints and an additional breakpoint of 128mg/l for tetracycline. The 1999 and 2003 GB abattoir surveys for prevalence of resistance in *Campylobacter jejuni* and *C. coli* from food animals used the same test to ensure harmonised testing of human clinical, food and food animal isolates and provide results which could be compared directly with results from man and food.

The current test does not provide quantitative results, instead providing information about how a strain behaves at one concentration of the antibiotic. There is also a requirement to harmonise methods at the European level so that comparisons can be made between member states. Commercial semi-mechanised microbroth techniques do provide quantitative trends over a range of antibiotic concentrations and are increasingly favoured by major veterinary resistance surveillance programmes (for example in the Netherlands and Sweden http://www.cidc-lelystad.wur.nl/NL/publicaties/rapporten/maran/; www.sva.se/en/Startpage/Engelsk-malgruppsnavigering/animalhealth/Antibiotic-Resistence/Monitoring-/**SVARM**-reports/) with a set of recommendations for antibiotics and concentration ranges recently set by an EFSA task force (2008). The quantitative broth dilution susceptibility method selected for use as a potential harmonised VLA/HPA test was based on the commonly used Sensititre™ system (Trek diagnostics, East Grinstead). However, it was unknown how this test system would perform against the established break-point test and this study was designed to compare the two test systems to determine the performance and precision of the broth microdilution test for classifying antimicrobial resistance in *C. jejuni* and *C. coli.*

The aims of this first objective were to:
1) evaluate agreement of antimicrobial resistance classification derived by a Sensititre broth microdilution test with the results derived from the traditional agar dilution breakpoint technique
2) assess reproducibility of the broth microdilution test results in two independent laboratories.
3) calculate repeatability of the results derived by the agar dilution breakpoint technique and the broth microdilution test

1.1 Methods
1.1.1 Designing the plate for broth microdilution

A VetMIC™ plate was available commercially from the SVA in Sweden, but the antibiotics and concentration ranges were not optimal for the project and considered unsuitable for our purpose. A standardised European plate for Campylobacter using a 96-microtitre plate format up had also been proposed, but again was unsuitable for UK purposes, as it did not include all the antimicrobials and concentration ranges that were traditionally used in the agar breakpoint test. At the first project meeting between HPA, VMD, Defra and VLA the antimicrobials and concentration ranges and breakpoints required for both human and veterinary considerations was agreed and are shown in Table 1. The proposed custom designed Sensititre™ plate included all antibiotics and ranges recommended by the EFSA Working Group for susceptibility testing of campylobacters from broilers (Anonymous, 2007). An extended range for tetracycline to 256 mg/l to incorporate the current agar breakpoint used by HPA

was incorporated as well as an extension of the erythromycin range to include 0.125 mg/l, to ensure the plate covered the new BSAC breakpoint of 0.5 mg/l (Fig. 1). Results from the VLA abattoir surveys of 2008 revealed a potential increase in chloramphenicol resistance using the traditionally applied 8 mg/l breakpoint which consensus opinion indicated may have been artefactual. To address this issue, a range of chloramphenicol concentrations:1-32 mg/l was included on the plates and in addition two further breakpoints (4 & 16 mg/l) were tested by agar dilution. Ampicillin was included on the plate as it has been traditionally tested and ß-lactam compounds are likely to be continued to be used in veterinary and human medicine. However, as HPA breakpoints had changed from 8 to 32 mg/l over time, both values were included for agar breakpoint testing in this study. Kanamycin was included on the plate because it was tested in the traditional HPA breakpoint test (Table 1). Finally impenem, while not traditionally included for susceptibility testing of this organism, was included due to its use for treatment of invasive disease, such as pyrexia of unknown origins (PUO) or septicaemia in humans. Trimethoprim was included on the plate at a single concentration. Campylobacters are intrinsically resistant to this antimicrobial and thus has been traditionally included in UK campylobacter susceptibility tests as a control. The plates used in the studies were purchased from the manufacturer in a single batch to eliminate batch variation.

Table 1. Antimicrobial agents, breakpoints or antimicrobial concentration ranges for each method included in the study

Antibiotic	Breakpoint (mg/l) tested by agar dilution	MIC range (mg/l) tested by broth microdilution
Ampicillin	8, **32**	0.5-128
Chloramphenicol	4, **8**, 16	1-32
Nalidixic acid	**16**	0.5-64
Ciprofloxacin	0.5, **1**, 2	0.032-32
Erythromycin	2, **4**, 8	0.125-128
Tetracycline	**8, 128**	0.125-256
Kanamycin	**16**	1-128
Streptomycin	2, 4	0.25-64
Gentamicin	**4**	0.125-32
Imipenem	ND	0.04-4

[a] Values in bold indicate agar dilution breakpoints traditionally used by HPA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Tet 0.125	Tet 0.25	Tet 0.5	Tet 1	Tet 2	Tet 4	Tet 8	Tet 16	Tet 32	Tet 64	Tet 128	Tet 256
B	IMI 0.004	IMI 0.008	IMI 0.016	IMI 0.032	IMI 0.064	IMI 0.125	IMI 0.25	IMI 0.5	IMI 1	IMI 2	IMI 4	Chl 1
C	Cip 0.032	Cip 0.064	Cip 0.125	Cip 0.25	Cip 0.5	Cip 1	Cip 2	Cip 4	Cip 8	Cip 16	Cip 32	Chl 2
D	Ery 0.125	Ery 0.25	Ery 0.5	Ery 1	Ery 2	Ery 4	Ery 8	Ery 16	Ery 32	Ery 64	Ery 128	Chl 4
E	Str 0.25	Str 0.5	Str 1	Str 2	Str 4	Str 8	Str 16	Str 32	Str 64	Nal 0.5	Nal 8	Chl 8
F	Gen 0.125	Gen 0.25	Gen 0.5	Gen 1	Gen 2	Gen 4	Gen 8	Gen 16	Gen 32	Nal 1	Nal 16	Chl 16
G	Amp 0.5	Amp 1	Amp 2	Amp 4	Amp 8	Amp 16	Amp 32	Amp 64	Amp 128	Nal 2	Nal 32	Chl 32
H	Kan 1	Kan 2	Kan 4	Kan 8	Kan 16	Kan 32	Kan 64	Kan 128	TM 2	Nal 4	Nal 64	Pos Con

Fig 1. Layout of customised plate UKVLC2 agreed for harmonisation of susceptibility testing for human and veterinary *C. jejuni* and *C. coli.*

Tet – tetracycline
IMI – imipenem
Cip – ciprofloxacin
Str – streptomycin
Ery – erythromycin
Gen - gentamicin
Amp – ampicillin
Kan – kanamycin
Chl – chloramphenicol
Nal – nalidixic acid
TM – trimethoprim

1.1.2 Calculation of required sample sizes

The study design was based on EN ISO 16140:2003, which describes validation of alternative methods in food and animal feed stuffs and EN ISO 2776-2:2007, which describes the minimum requirements for evaluation of performance of antimicrobial susceptibility test devices. To assess agreement at any single cut-off point, the outcomes from both tests were considered binary (resistant/sensitive) for the validation objective. ISO 16140:2003 recommends testing of 60 samples in each sub-group by each test to prove relative accuracy of two qualitative methods. EN: ISO 2776-2:2007 requires testing of at least 100 isolates of similar genus. No justifications or accuracy parameters were provided for the recommended samples sizes and sample sizes were calculated independently of recommendations to ensure that sufficient power and confidence in non-significant results of test comparison was obtained.

Table 2 shows the confidence in non-significant results (the tests are equally good) according to sample size and expected difference between the tests. If we test 60 samples and expect that the true difference in S/R result between the tests is 10% and achieve a non-significant result (p>0.05), we can be 67% certain that the tests are similar. If we think the true difference is smaller (5%) the confidence in a non-significant result drops to 15%. A sample size of 120 strains was chosen to compare the two tests.

Table 2. Confidence in non-significant results according to sample size

# of samples	Expected difference	Power
60	10%	67%
60	5%	15%
120	10%	99%
120	5%	70%

1.1.3 Selection of isolates

The test population was selected to represent the variety of antimicrobial resistance phenotypes of *Campylobacter* on which it would be applied, rather than present any 'real-life' population to ensure that the new test was appropriate for different species of *Campylobacter* from different sources. The optimal test population would contain 50% *C. jejuni*, 50% *C. coli*, 25% of human origin, 25% of chicken/meat origin, 25% of pig origin and 25% from cattle/sheep. Ideally all antimicrobial agents would also be presented with a 50/50 division between resistant and susceptible strains. It was not possible to fulfil all criteria and best fit was approximated.

The strains originated from a four strain pools from humans, poultry, pigs and cattle, respectively. The human isolates originated from England and Wales between 2004-2006 and were drawn from the culture collection stored by the HPA Centre for Infections Gastrointestinal Infections Reference Unit (GEZI, formerly the Campylobacter and Helicobacter Reference Unit). Cattle and pig isolates were recovered from intestinal contents during a 12-month abattoir survey undertaken in GB in 2003 (Milnes *et al*, 2008). Isolates from broilers were recovered from caecal contents and broiler meat products from research surveys between 2003 and 2006. The animal strain pools were all stored at VLA Weybridge.

Historical antimicrobial resistance patterns were available for the majority of isolates at the breakpoints, as determined by the agar breakpoint test (Thwaites and Frost, 1999), in force at the time at which the isolates had been initially tested. Streptomycin was not studied in previous surveys, but classification of resistance at newly designated EU breakpoints (2, 4) was determined by agar dilution for a selection of cattle, pig and broiler flock isolates at those breakpoints. These patterns were used to choose the strain panel, which attempted a 50:50 split of sensitive and resistant isolates for each antimicrobial at the historical breakpoint as well as a 50:50 division of *C. jejuni* and *C. coli*. Since an exact 50:50 distribution was not possible for all antimicrobials at multiple breakpoints, a borderline ratio of 30:70 was considered acceptable without adjusting the calculated sample size. Isolates were selected and combined to approximate the ideal study population and the most relevant antimicrobials and their breakpoints were given first priority and the remainder adjusted accordingly. The final test panel contained 67 *C. jejuni* isolates and 53 of *C. coli*, yielding a percentage distribution by species of 56:44. The distributions of resistance to each antimicrobial agent in the testing panel is shown in Table 3.

Table 3. Distribution of previously determined resistance proportions of the isolates selected to validate the broth microdilution test

	Breakpoint	# of isolates resistant	# of isolates susceptible	Distribution R:S
Ampicillin	32	50	70	42:58
Chloramphenicol	8	39	81	33:67
Nalidixic acid	16	66	54	55:45
Ciprofloxacin	1	52	68	43:57
Erythromycin	4	52	68	43:57
Tetracycline	8	75	45	62:38
Tetracycline	128	40	80	33:67
Kanamycin	16	15	105	13:87
Streptomycin[1]	2(j), 4(c)	18	61	23:77
Gentamicin[1]	4	0	90	0:100

[1] Includes missing values due to unknown resistance status

Once the panel was selected, all isolates were given a random study number under which the isolates were prepared and distributed during the study. The laboratory staff conducting the testing were blinded to any other information than the study number and the species of the isolate. Fresh stocks of control isolates *C. jejuni* NCTC 11351 (ATCC 33560) & *C. coli* NCTC 11366 (ATCC 33559) were obtained from The National Collection of Type cultures (London, UK) for use as batch controls. All isolates were stored at -80°C in glycerol broth.

1.1.4 Susceptibility testing

1.1.4.1 Broth Microdilution test

The broth microdilution test is described in detail in Appendix 1. For the preparation of the test inoculum growth from the plate, typically 5-6 colonies, was transferred into 5 ml distilled water (T3339); Trek Diagnostic Systems, East Grinstead, UK) and adjusted to a 0.5 McFarland standard using a calibrated nephelometer (Laboratory 1) or as recommended by BSAC guidelines (Laboratory 2) (http://www.bsac.org.uk/_db/_documents/Chapter_2_Determination_of_MICs_2006.pdf), according to EN ISO 20776-1. The suspension was mixed and 100µl was added to 11ml cation adjusted Mueller-Hinton broth (T3462; Trek Diagnostic Systems) containing 2.5% defibrinated horse blood (TCS Biosciences, Buckingham, UK), to give an inoculum of $5X10^5$ CFU/ml. Each well of the customized Sensititre™ microtitre plates was filled with 100µl of strain suspension. Plates were sealed using an anerobic and microaerophilic film supplied by the manufacturer and incubated in a modular atmosphere controlled system incubator (MACS, Don Whitley Scientific, Shipley, UK) at 37°C under microaerobic conditions (87% nitrogen, 5% oxygen, 3% hydrogen and 5% carbon-dioxide). The test results were evaluated 40-48 h later. The *C. jejuni* and *C. coli* control strains were included in each batch of broth microdilution tests. The MIC Interpretive guidelines of Trek derived from CLSI (2006) were used for determining end-point. Positive growth control wells were always read first and if any showed no growth, results were considered invalid and the test repeated for the isolate in question. Inoculum density and purity checks were carried out as described by EN ISO 20776-1 Quality control ranges for antimicrobials for which CLSI have provided recommendations for *C. jejuni* strain ATCC 33560 (CLSI, 2008).

1.1.4.2 Agar breakpoint test

The agar dilution breakpoint technique was conducted on all isolates and used as reference test for the broth microdilution test. The antimicrobials tested were incorporated into Iso-sensitest agar, which contained 5% laked horse blood as previously described (Frost and Thwaites, 1999). The breakpoint methodology was essentially the same as that originally used, except for the changes and additions to the breakpoints tested for certain antimicrobials as shown in Table 1. Originally the breakpoints were based on BSAC guidelines with the exception of erythromycin, for which no appropriate guidelines existed at that time. Consequently, the NCCLS guideline of 4 mg/l was used. Recently, BSAC have recommended an erythromycin breakpoint of 0.5 mg/l for Campylobacter which is much lower than new EUCAST recommendations in Europe (4 mg/l by the French Society for Microbiology (CA-SFM)) and the USA (4/16 mg/l by CLSI). The HPA has stuck to the original breakpoint to ensure that data between old and current studies is comparable.

1.1.5 Statistical evaluation

1.1.5.1 Evaluation of agreement between tests

The MIC values from the broth microdilution test were collapsed into a binary variable of S/R at the breakpoint value of the agar-based breakpoint test, which was used as an anchor point for comparative purposes, and the R/S results were described. Thus, for an agar breakpoint of 1, an MIC value of >1 was classified as resistant and the remaining recorded as susceptible. Sensitivity and specificity relative to the agar dilution breakpoints were calculated to assess the ability of the broth microdilution test to correctly identify resistant and sensitive isolates, respectively. The agreement between the results of the two tests to classify isolates as resistant or sensitive at each breakpoint was evaluated by kappa values and statistics. The kappa index measured agreement between pairs of binary outcomes at each breakpoint for each strain, which occurred beyond chance and provided an index value between 0 and 1. The magnitude of kappa, the agreement that occurred beyond chance and agreement was assessed on the scale: <0.2 = slight, 0.2-0.4 = fair, 04-0.6 = moderate, 0.6-0.8= substantial, >0.8 almost perfect. The kappa statistics also provided a p-value, which indicated whether the results agreed beyond chance (H_0 = the agreement occurred by chance). The similarity of resistant and sensitive proportions was assessed using McNemar statistics (H_0=the proportions identified by the two tests are similar).

1.1.5.2 Reproducibility

The panel was tested in parallel using the broth microdilution test in two laboratories.
Reproducibility of binary (R/S) results derived by the broth microdilution test in the two laboratories was assessed using kappa and McNemar statistics as described above. Any antimicrobials exhibiting differences beyond chance were explored further by assessment of essential agreement. Essential agreement was defined as an MIC result that is within +/- 1 MIC dilution step of the MIC value of the comparative result. Essential agreement is considered acceptable as described by BS EN ISO 20776-2:2007.

1.1.5.3 Repeatability

A random selection of 10 *C. jejuni* and 10 *C. coli* isolates from the original panel were tested in triplicate by the broth microdilution test as recommended by EN ISO 16140:2003. The triplicates were mixed randomly within the original panel and assigned a study number at random as for the other isolates. This ensured that laboratory staff were blinded to which isolates were triplicates. The consistency of MIC values within each strain triplicate cluster obtained by the broth microdilution test was assessed and the cluster classified into full agreement (the same MIC value), acceptable variance (2 consecutive MIC values only) and unacceptable variance (non-consecutive or >2 MIC values). The probability that two replicates gave the same result each time they were tested within the same laboratory, was calculated for each of the twenty sets of antimicrobial results. This probability was collated to

accordance scores (0-100) for the agar dilution breakpoint technique and the broth diffusion test in each laboratory as recommended by BS EN ISO 16140:2003.

1.2 Results

In general the overall prevalence of resistance in the panel had fallen since the original agar breakpoint based testing of the isolates, yielding slightly less optimal distributions of R:S in the test panel than expected (Tables 3, 4).

Table 4. Prevalence of resistance classified by two different tests and the level of non concurrence between them

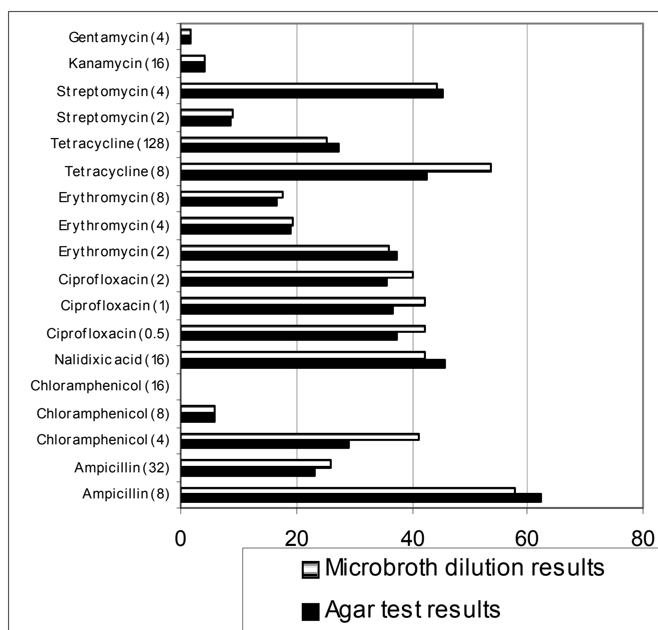
AB	Breakpoint	% resistant by agar break-point test	% resistant by broth microdilution test	# of isolates which do not concur	# of isolates where MIC >1 step away from breakpoint
Ampicillin	8	62.5	58.0	29	13
Ampicillin	32	23.3	26.1	9	2
Chloramphenicol	4	29.2	41.2	26	1
Chloramphenicol	8	5.8	5.9	6	1
Chloramphenicol	16	0.0	0.0	0	0
Nalidixic acid	16	45.8	42.2	19	12
Ciprofloxacin	0.5	37.5	42.2	11	9
Ciprofloxacin	1	36.7	42.2	11	11
Ciprofloxacin	2	35.8	40.3	11	11
Erythromycin	2	37.5	36.1	26	6
Erythromycin	4	19.2	19.3	8	5
Erythromycin	8	16.7	17.7	5	5
Tetracycline	8	42.5	53.8	19	16
Tetracycline	128	27.5	25.2	21	5
Streptomycin*	2	8.5	9.0	3	2
Streptomycin^	4	45.2	44.2	6	5
Kanamycin	16	4.2	4.2	0	0
Gentamicin	4	1.7	1.7	0	0

***C. jejuni* only (n=67); ^ *C. coli* only (n=52)

1.2.1 Agreement between tests

The proportions of resistant isolates to kanamycin, gentamicin, streptomycin and erythromycin were very similar for both tests, indicating good agreement between the two tests for these antimicrobials. However, for the remaining antimicrobial agents and concentrations, the broth microdilution test classified slightly more isolates as resistant than the agar dilution breakpoint test (Fig. 2). The overestimation was largest for chloramphenicol (at 4 mg/l) and tetracycline (8 mg/l) and for some of the isolates the imprecision was larger than one MIC concentration step from the designated break-point (Table 4). For both these antimicrobials, the differences between the test results disappeared at the higher breakpoints applied, suggesting that, at least in part, this effect may be attributable to the position of the breakpoint in relation to the normal distribution of susceptible and resistant populations, as well as the degree of separation (if any) which occurs between the MIC values of resistant and susceptible isolates for a given antimicrobial.

2a.



2b.

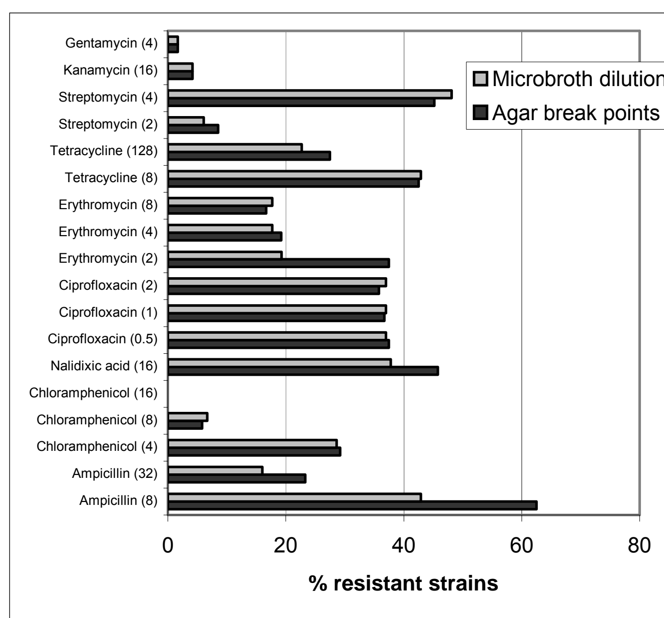


Fig. 2. Percentage of isolates classified as resistant by agar dilution and broth microdilution tests respectively (n=119) in Laboratory 1 (a) and 2 (b).

In Laboratory 1 the broth microdilution test consistently overestimated the proportion of resistant isolates compared to the agar breakpoint test for ciprofloxacin and the slight imprecision appeared to be independent of breakpoint concentration applied (Fig 2a). Surprisingly, the majority of the misclassified isolates were more than one MIC away from the breakpoint. The broth microdilution test overestimated the proportion of ampicillin resistant isolates slightly at the breakpoint of 32 mg/l, but slightly underestimated it the breakpoint of 8 mg/l.

All kappa values in Table 5a show that agreement between the agar dilution and broth microdilution methods was higher than expected by chance ($p<0.05$), however the strength of agreement varied by antimicrobial agent and concentration. Full agreement between the classification of resistant isolates by both tests were observed for kanamycin and gentamicin. There was substantial agreement between the two tests for classification of resistance to nalidixic acid and streptomycin; although the relative sensitivity and specificity of the broth microdilution test could have been higher, the results for the two tests were not significantly different. The agreement for determination of ciprofloxacin resistance for the two tests was very good and appeared to be independent of the concentration tested. The broth microdilution test had a consistent high relative sensitivity and specificity for classifying isolates as resistant or sensitive to ciprofloxacin at different breakpoints and any misclassification was equally distributed. Detection of resistance to erythromycin at higher breakpoints (4 and 8 mg/l) also showed good agreement between the tests. However, at 2 mg/l, the broth microdilution test was less likely to classify isolates as resistant compared with the agar test, with a relative sensitivity of 68.9% (Table 4), which may result in an under-estimation of resistant isolates at that breakpoint. Nevertheless, the difference between the relative sensitivity and relative specificity was not significantly different ($p=0.845$).

Classifications for chloramphenicol were only in moderate agreement between the two tests. At 4 mg/l, which is lower than the traditionally applied HPA breakpoint, the relative sensitivity and specificity were significantly different (Table 4). The observed low specificity of the broth microdilution test (the ability to identify sensitive isolates as sensitive) is illustrated in the overestimation observed in Fig. 2a. The level of agreement was also high when comparing the resistance for the two tests at the traditionally applied breakpoint concentration of 8 mg/l. However, the relative sensitivity was low at this break-point. The discrepancy between the two measures of test validation was due to the low proportion of isolates resistant at this breakpoint included in the study panel (7%). The overestimation of tetracycline resistance at the breakpoint concentration of 8 mg/l shown by Fig. 2a is a result of high relative sensitivity combined with a significantly lower relative specificity of the broth microdilution test.

Table 5a. Agreement between agar dilution and broth microdilution test results derived in Laboratory 1.

Antimicrobial	Breakpoint	Agreement %	Kappa	McNemar p-value	Relative sensitivity %	Relative specificity %
Ampicillin	8	75.6	0.493	0.458	77.0	73.3
Ampicillin	32	92.4	0.797	0.508	89.3	93.4
Chloramphenicol	4	78.2	0.529	0.009	82.7	76.2
Chloramphenicol	8	95.9	0.545	1.00	57.2	97.3
Chloramphenicol	16	100	NA	NA	NA	NA
Nalidixic acid	16	84.0	0.677	0.359	78.2	89.1
Ciprofloxacin	0.5	90.1	0.808	0.227	93.3	89.2
Ciprofloxacin	1	90.0	0.790	0.146	93.1	88.0
Ciprofloxacin	2	90.8	0.805	0.227	93.1	89.5
Erythromycin	2	78.2	0.531	0.845	68.9	83.8
Erythromycin	4	93.3	0.784	1.000	82.6	95.8
Erythromycin	8	95.8	0.853	1.000	97.0	90.0
Tetracycline	8	84.0	0.684	0.004	94.1	76.5
Tetracycline	128	82.4	0.547	0.664	63.6	89.5
Streptomycin*	2	93.6	0.632	1.00	60.0	97.6
Streptomycin^	4	80.7	0.614	0.688	75.0	86.7
Gentamicin	4	100	1.000	1.00	100	100
Kanamycin	16	100	1.000	1.00	100	100

*_C. jejuni_ only (n=67); ^ _C. coli_ only (n=52)

In Laboratory 2 the broth microdilution test also classified slightly fewer isolates more resistant than the agar breakpoint test, but not as many as Laboratory 1 (Fig. 2b). For erythromycin and streptomycin the microdilution test underestimated the proportion of resistant strains compared to agar dilution breakpoint test at lower breakpoints. The classifications were significantly different for the two tests for erythromycin at 2 mg/l (Table 5b).

Table 5b. Agreement between agar dilution and broth microdilution test results derived in Laboratory 2

Antimicrobial	Breakpoint	Agreement %	Kappa	McNemar p-value	Relative sensitivity %	Relative specificity %
Ampicillin	8	73.1	0.4815	<0.001	62.7	90.9
Ampicillin	32	90.7	0.711	0.012	64.3	98.9
Chloramphenicol	4	84.0	0.612	1.000	71.4	89.3
Chloramphenicol	8	92.4	0.360	1.000	42.9	95.5
Chloramphenicol	16	na	na	na	na	na
Nalidixic acid	16	88.2	0.760	0.013	78.2	96.9
Ciprofloxacin	0.5	96.6	0.889	1.000	95.6	98.7
Ciprofloxacin	1	98.3	0.964	1.000	97.7	98.7
Ciprofloxacin	2	97.5	0.946	1.000	97.4	97.7
Erythromycin	2	78.2	0.486	<0.001	46.7	97.3
Erythromycin	4	96.6	0.889	0.625	87.0	99.0
Erythromycin	8	99.2	0.971	1.000	100	99.0
Tetracycline	8	86.6	0.703	0.077	92.2	82.4
Tetracycline	128	88.2	0.689	0.180	69.7	95.4
Streptomycin*	2	97.8	0.846	1.000	75.0	100
Streptomycin^	4	87.1	0.746	0.125	76.5	100
Gentamicin	4	100	1.000	na	100	100
Kanamycin	16	100	1.000	na	100	100

*_C. jejuni_ only (n=67); ^ _C. coli_ only (n=52)

For the majority of the isolates in the panel, the imprecision could be related to a difference of one doubling dilution MIC step from the original breakpoint. For ampicillin the broth microdilution method was significantly more likely to underestimate resistance, at both 8 and 32 mg/l, compared with the agar breakpoint test (p=0.02). Nalidixic acid resistance was also significantly underestimated by the microdilution test and 43% of the non-concurring strains were more than one doubling dilution step from the breakpoint classification.

1.2.2 Reproducibility between independent laboratories

There was very good agreement with no evidence of statistical difference between the results obtained by the broth microdilution test in both laboratories for the majority of antimicrobial agents and breakpoints (Table 6). There was total agreement for the classifications obtained by each laboratory for gentamicin, kanamycin. However, the proportion of resistance to ampicillin at both breakpoints was significantly different between the laboratories. The difference was smaller, when essential agreement was considered (acceptable variation +/- 1 dilution i.e. step in MIC value), but for ampicillin quite a few of the differences in the MIC values obtained were still in the unacceptable range. The majority of these were also above the zero difference line (Fig. 3), which suggests a systematic overestimation of MIC values by Laboratory 1, compared to Laboratory 2. The significant p-value (<0.001) shown for this antimicrobial (Table 6) was caused by the one-sided difference.

Table 6. Agreement and difference between results derived using a broth microdilution test, when conducted in two different laboratories.

Antimicrobial agent	breakpoint (mg/l)	Agreement	Kappa	McNemars p-value
Ampicillin	8	80.5	0.618	<0.001
Ampicillin	32	89.8	0.700	<0.001
Chloramphenicol	4	78.8	0.544	0.004
Chloramphenicol	8	94.1	0.502	1.000
Chloramphenicol	16	na	na	na
Nalidixic acid	16	89.8	0.788	0.388
Ciprofloxacin	0.5	90.7	0.805	0.227
Ciprofloxacin	1	90.7	0.805	0.227
Ciprofloxacin	2	90.7	0.803	0.549
Erythromycin	2	78.8	0.486	<0.001
Erythromycin	4	95.8	0.858	1.000
Erythromycin	8	97.5	0.912	1.000
Tetracycline	8	93.2	0.864	0.289
Tetracycline	128	89.0	0.670	0.581
Streptomycin	2	98.5	0.881	na
Streptomycin	4	92.3	0.846	0.625
Gentamicin	4	100	1.000	na
Kanamycin	16	100	1.000	na

**C. jejuni* only (n=67); ^ *C. coli* only (n=52)

Significant inconsistencies in MIC values were also observed for chloramphenicol and erythromycin, but these were most marked at the lowest of breakpoints tested, which were 1 dilution step below the traditionally applied breakpoint for those antimicrobials (Table 6). For chloramphenicol the differences were almost all within the acceptable range, whereas erythromycin still had results outside this range (Fig. 3). However, the majority of these differences were within two doubling dilutions of each other.

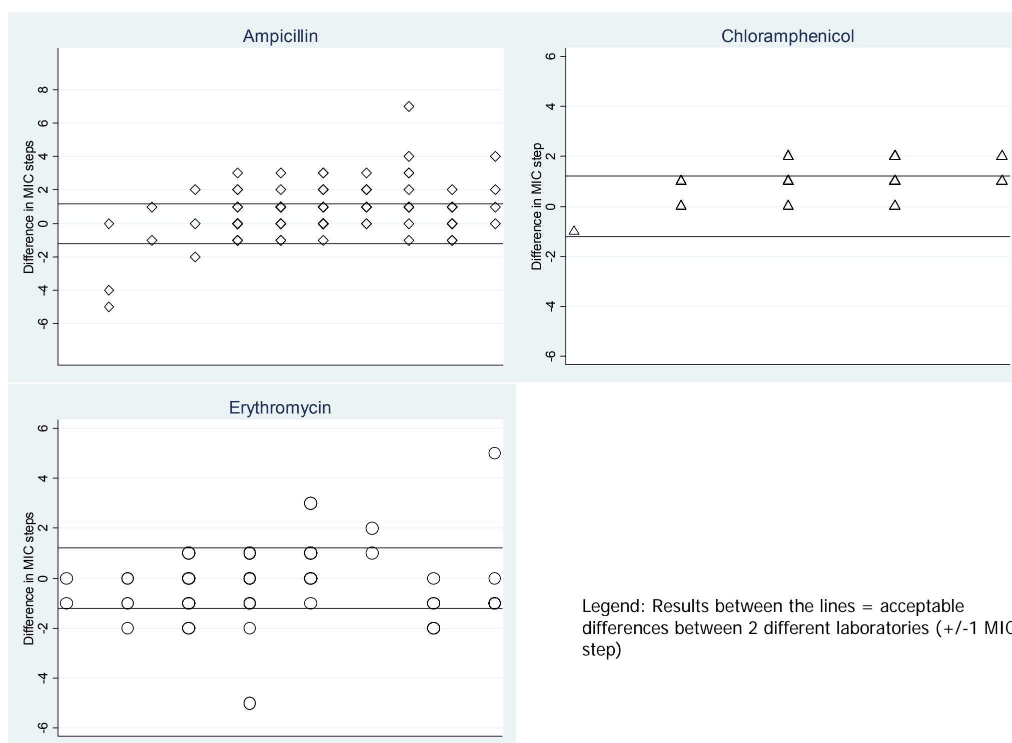


Fig 3. Difference in MIC and essential agreement between broth microdilution results measured in two independent laboratories

1.2.3 Repeatability

The results for the triplicate testing in Laboratory 1 indicated that chloramphenicol, erythromycin and gentamicin were very precise with a maximum of two isolates with unacceptable variance between results (Table 7). Results obtained for kanamycin, tetracycline and ampicillin were less precise, with unacceptable variance observed for 20% or more of the isolates.

Table 7. Number of isolates showing consistency in MIC values in the broth dilution test in both laboratories, when testing 20 isolates in triplicate.

Antimicrobial	Laboratory 1			Laboratory 2		
	No variance MIC	Acceptable variance in MIC[1]	'unacceptable' variance in MIC[2]	No variance MIC	Acceptable variance in MIC[1]	'unacceptable' variance in MIC[2]
Ampicillin	8	8	4	11	8	1
Chloramphenicol	14	6	0	13	6	1
Nalidixic acid	9	8	3	12	4	4
Ciprofloxacin	9	8	3	11	6	3
Erythromycin	9	9	2	6	13	1
Tetracycline	8	7	5	11	5	4
Kanamycin	7	7	6	9	9	2
Streptomycin	8	10	2	7	12	1
Gentamicin	13	6	1	16	4	0

[1]One doubling dilution difference only; [2] more than doubling dilution difference

The results for the triplicate testing in Laboratory 2 showed that results for ampicillin, chloramphenicol, gentamicin, erythromycin, streptomycin and kanamycin were very precise with a maximum of two isolates with unacceptable variance between results (Table 7). In this laboratory nalidixic acid, tetracycline and, to a lesser extent, ciprofloxacin had a higher level of unacceptable variance, for a maximum of 20% of the isolates. Nevertheless, the combined accordance derived from testing 20 isolates in triplicates was high, and fairly stable, for both tests in both laboratories. The agar dilution test showed an accordance of 94.5% and the broth microdilution test an average accordance of 96.1%. In Laboratory 1, the accordance was 95.0%, whereas it was slightly higher in Laboratory 2: 97.1%. Both tests appeared to perform very consistently within laboratories.

1.3 Comparing susceptibility classifications obtained from traditional breakpoint and EU epidemiological cut-offs

EFSA has published epidemiological cut-off values recommended by EUCAST for 5 antimicrobials, some of which are species-specific (Anonymous, 2007) (Table 8). EUCAST have also recently published additional epidemiological cut-off values for chloramphenicol and nalidixic acid (Table 8).

Table 8. Epidemiological cut-off values recommended by EUCAST and applied to EFSA monitoring programme

Antimicrobial	MIC (mg/l) **R is >**	
	C. jejuni	C. coli
Chloramphenicol*	16	16
Ciprofloxacin	1	1
Erythromycin	4	16
Gentamicin	1	2
Nalidixic Acid*	16	32
Streptomycin	2	4
Tetracycline	2	2

* Antimicrobials not part of the EFSA monitoring programme, but for which EUCAST has designated cut-off values and are included in the Community Reference Laboratory External Quality Assurance Scheme

At the time of study design, no traditional breakpoints for streptomycin were available and the EUCAST cut-off values were applied and have been described in Objective 1.2. Many of the EUCAST cut-off values are similar to the traditional breakpoints applied in GB and thus the suitability of the broth microdilution tests at these cut-off values were also included in Objective 1.2 and will not be described further.

For the values which had not already been evaluated previously, no agar breakpoint test results existed. Instead, reproducibility of similar results at these EUCAST recommended cut-off values laboratories was assessed, by dichotomising the broth microdilution results from each lab at each breakpoint and applying agreement statistics on these values. Agreement was interpreted as a proxy for confidence in and stability of the classification i.e. if both laboratories obtained same result; it is more likely to be precise. However, many of the EUCAST recommendations are species-specific and since the original sample-size did not allow for stratification on

species, the confidence in a non-significant p-value is limited. This should be kept in mind, when interpreting species-specific validation results as we would only be likely to detect large differences between laboratories.

In general, the new cut-offs only changed the agreement of the broth microdilution tests in the two laboratories slightly (Table 9).

<u>Chloramphenicol</u>
Comparisons for the EUCAST cut-off value for chloramphenicol have been previously described as this value was included in our comparison of the two tests (Tables 5a and 5b). The findings clearly show that while there was good agreement when resistance to this antimicrobial was classified as >8 or >16 mg/l, total agreement was only obtained at the higher cut-off value. Panel isolates classified as resistant using the traditional breakpoint of 8 mg/l would be considered susceptible by applying the recommended epidemiological cut-off value.

<u>Ciprofloxacin</u>
Although ciprofloxacin cut-off values were the same as traditionally applied in GB, species-specific analysis were added in this section. Minor changes in kappa and McNemar values were seen, but these were most likely attributable to the equivalent smaller sample size. The stratification into species showed that the majority of disagreement occurred in *C. jejuni* rather than *C. coli*. A new EUCAST cut-off of 32 mg/l for nalidixic acid was applied to the *C. coli* isolates in the panel and the level of agreement dropped slightly (Table 9). The reduction in agreement was almost exclusively due to isolates yielding MIC values of 32 mg/l in Laboratory 1, and thus classified as susceptible by Laboratory 1, but had MIC values of 64 in Laboratory 2 and was thus classified as resistant to ciprofloxacin. Application of the new cut-off is likely decrease the proportion of resistant *C. coli* isolates in any population. This should be kept in mind, when interpreting temporal trends.

Table 9. Comparison of classifications obtained from broth microdilution tests performed in VLA and HPA laboratories at traditional and EUCAST cut-off values for antibiotics included in the EU ar-CRL Campylobacter EQAS scheme

Antimicrobial	Cut-off[a] (mg/l)	Campylobacter species*	Agreement	Kappa	McNemars p-value
Chloramphenicol	8[b]	Both	94.1	0.502	1.000
Chloramphenicol	16[c]	Both	na	na	na
Nalidixic acid	16[b]	Both	89.8	0.788	0.388
Nalidixic acid	16[c]	*jejuni*	87.9	0.748	0.289
Nalidixic acid	32[c]	*coli*	84.6	0.662	0.289
Ciprofloxacin	1	Both	90.7	0.805	0.227
Ciprofloxacin	1	*jejuni*	89.4	0.778	0.125
Ciprofloxacin	1	*coli*	92.3	0.840	1.000
Erythromycin	4	both	95.8	0.858	1.000
Erythromycin	4	*jejuni*	98.0	0.784	0.500
Erythromycin	16[c]	*coli*	96.2	0.910	0.500
Tetracycline	2[c]	*jejuni*	92.4	0.849	0.375
Tetracycline	2[c]	*coli*	94.2	0.883	1.000
Tetracycline	8[b]	Both	93.2	0.864	0.289
Tetracycline	128[b]	Both	89.0	0.670	0.581
Streptomycin	2[c]	*jejuni*	98.5	0.881	na
Streptomycin	4[c]	*coli*	92.3	0.846	0.625
Gentamicin	1[c]	*jejuni*	100	1.000	1.000
Gentamicin	2[c]	*coli*	100	1.000	1.000
Gentamicin	4[b]	Both	100	1.000	na
**jejuni*: n=67; *coli*: n=53 (n_{total} = 119)					

[a]resistance is classified as MIC > value shown
[b] traditionally applied breakpoints
[c]epidemiological cut-off values developed by EUCAST

<u>Gentamicin</u>
Application of the new EUCAST epidemiological cut-offs for gentamicin of 1 mg/l for *C. jejuni* and 2mg/l for *C. coli*, with resistance classifications of MIC >1 and >2mg/l respectively, did not alter the number of isolates classified as

resistant. Essential agreement is illustrated in Table 9, but as previously stated these findings may have been influenced by the lack of resistant isolates available for inclusion in the panel.

Erythromycin
Species specific cut-off for erythromycin appeared to increase the agreement between laboratories and thus, the confidence in the biological relevance of the cut-offs (Table 9). At 4mg/l, *C. coli* caused some disagreement and once these strains were removed, the level of agreement increased for *C. jejuni*. The new *C. coli* cut-off at 16 mg/l, also improved the probability of getting the same result in both laboratories, suggesting that the confidence in a susceptible or resistant classification at the new cut-off value is increased.

Tetracycline
The level of agreement was very similar for tetracycline, independently of the new cut-off values, with slightly more variation in *C. jejuni* classifications *than* in those obtained for *C. coli* (Table 9). However, the changes were very minor and the new cut-off did not appear to make much difference in test performance.

1.4 Discussion

Strain panel
The isolates tested in this study were selected specifically for test validation purposes and were not representative of the wild type population of *C. jejuni* and *C. coli* from each source. The resistance phenotypes were designed to try and ensure a minimum of 30:70 resistance for each antibiotic for the purpose of assessing test accuracy and should not be interpreted as resistance levels in any underlying population. The ratio was obtained for the majority of antibiotics, but due to a general low prevalence of resistance to kanamycin and gentamicin in *campylobacter* from humans and food animals (Newell, 2001, Randall *et al*, 2003; Thwaites and Frost, 1999) the ratio was not upheld for these antibiotics. This resulted in skewed populations and the sample sizes were not sufficient to accurately interpret the ability of the broth microdilution test to classify resistance to these antimicrobials. The low prevalence of resistance kanamycin and gentamicin is not unique to UK populations; other European countries report little or no resistance to gentamicin (DANMAP 2003; MARAN, 2003) and while data on prevalence of resistance to kanamycin is infrequently collected in other European countries, reports from Australia (Unicomb *et al*, 2006) also indicate a generally low prevalence of resistance to this antimicrobial.

The study strains were not always equally distributed with respect to *Campylobacter* species and population of origin, because of biological associations. Resistance to erythromycin is more common in *C. coli* than *C. jejuni* and is more often found in isolates recovered from pigs than in broilers. These, and other similar dependencies, resulted in a slightly less optimal distribution between all factors.
The panel was collated using resistance profiles obtained at the time of isolation and the lag time to this study varied between 4 and 5 years. Some changes in resistance profiles were observed at re-testing which also contributed to the skewed distribution of Resistant/Susceptible, despite aiming closer to 50:50 at the time of selection. The majority of the changes were probably due to changes in test methodology, because the initial profiles were derived from agar-based tests at the mostly single breakpoints shown in Table 1.

Some isolates with MICs close to the traditional Resistant/Susceptible breakpoints were probably included in the panel. Given the expected and inherent variability of an MIC of one log dilution, such isolates inevitably introduced natural and acceptable variation around the breakpoint, resulting in inaccuracy of the test parameters unless acceptable variation is considered. Where isolates occur with MICs at, or very close to, the breakpoint, categorisation into susceptible and resistant will inevitably present problems because of the inherent nature of MIC determination and the fact, as previously stated, that results may vary by one log dilution when tests are repeated. These observations highlight the difficulties of using breakpoint data to select a panel based on a comprehensive range of resistance phenotypes. Nevertheless, the panel of isolates chosen for inclusion in the panel represented a range of susceptible and resistant isolates and was therefore considered appropriate for validating the test.

This issue was most notable for chloramphenicol, where all isolates were susceptible at 16 mg/l, which is very close to the traditional breakpoint of 8 mg/l. When previously tested, it was suspected that resistance to chloramphenicol for 2003 abattoir survey isolates, as detected by the agar breakpoint test, may have been artefactual and related to the proximity of the breakpoint to the wild type campylobacter distribution.

Resistance to ampicillin and tetracycline also appeared to have been similarly overestimated in the original testing of the study panel. Loss of low level (>0.5 mg/l) amoxicillin resistance on storage at -80°C has been reported in the closely related *Helicobacter pylori* and a similar phenomena has been noted with a single isolate of tetracycline-resistant *H. pylori* (AJ Lawson, personal communication).

Broth microdilution test v agar breakpoint
Microdilution methods are recommended for testing antimicrobial susceptibility by the EU ar-CRL (www.CRL-ar.eu). Broth microdilution using antibiotic-containing microtitre plates is now a common approach for susceptibility testing in laboratories that report data to the EFSA harmonised monitoring scheme of antimicrobial resistance. The main advantage of a broth microdilution method, including the one presented in this paper is that it enables monitoring of a range of susceptibilities rather than at a single breakpoint to allow detection of changes in MIC populations over time.

For the purposes of the study the agar dilution breakpoint method was considered the gold standard result to provide the anchor point for comparison of the methods. The validated microdilution test overall produced comparable results to the standard agar breakpoint test. However, for ciprofloxacin and erythromycin it was observed that the broth MIC method was more likely to classify isolates more resistant than the agar test, particularly for Laboratory 1. Inconsistencies between classifications obtained from broth microdilution and agar dilution tests have previously been reported for ciprofloxacin and erythromycin (Luber *et al*, 2003).

Reproducibility
Good agreement between results obtained in the two laboratories using the broth microdilution test, but differences in MICs obtained for ampicillin resulted in differences in classification of resistant/ susceptible status the measured breakpoints. It was notable that in laboratory 1 MIC values of ATCC 33560 (NCTC 11351) which was used as control strain for each test batch showed variation in ampicillin MICs, which ranged from 4 to 16 mg/l. Although, this is within accepted values (manufacturers recommendations; CLSI, 2008), these observations suggested intrinsic variation of MICs was present, when using the broth microdilution test repeatedly. Intra-strain variation in ampicillin MIC values derived by the broth microdilution test were also observed in the repeatability testing of our study. These findings may be indicative of the variability of beta-lactamase resistance mechanisms for campylobacters (Tajada *et al*, 1996), which include intrinsic resistance to penicillin G and narrow spectrum cephalosporins conferred by penicillin binding proteins (Tajada *et al*, 1996) and production of B-lactamase conferred by a class D B-lactmase gene blaOXA-61 (Taylor and Courvalin, 1988; Alfredson and Korolik, 2005). Alternatively, observations of systematic overestimation in one laboratory compared with another may indicate minor differences in test conditions that warrant further investigated to ensure optimal harmonisation. Ampicillin was included in our study as this antimicrobial is commonly used in human and veterinary medicine, but ß-lactamases are not indicated for treatment of human Campylobacteriosis. However, this antimicrobial is not included as a required antimicrobial by EFSA Working group (2008) and has not yet been included in the EQAS scheme.

Erythromycin
In contrast, erythromycin is of great importance to human health being the drug of choice for treating human Campylobacteriosis. This emphasises the importance of reliability of tests results. Nevertheless, MIC results for strains can be classified as acceptable with a variation of up to two MIC steps from the expected MIC value in inter-laboratory ring trials (www.crl-ar.eu/146-presentations.htm). This tolerance appears necessary to accommodate differences in methods used between laboratories. This level of variability was also observed in our study, although the inter-laboratory differences observed for erythromycin at the lowest breakpoints were mostly within the acceptable range of 1 doubling dilution and were typically consistent between laboratories. The observed differences for this antimicrobial may be attributable to minor technical differences between the test laboratories relating to end point interpretations.

Tetracycline
Tetracycline MICs were found to vary by up to 4 doubling dilutions for individual isolates and this would have resulted in a difference in Resistant/Susceptible classifications at breakpoints of 8 and 128 mg/l. The tetracycline intra-strain variation was not always laboratory-specific, but it was more often encountered in one laboratory. Intra-strain variation for tetracycline has not been reported in other studies and was unexpected. We have no obvious explanation for this observation. All strains were clearly labelled and the copy of the strains sent to each laboratory originated from same agar plates. However tetracycline is on the outside edge of the Sensititre^{TM} UKVLC2 plate where the indented column numbers are. This may have resulted in the adhesive film being less firmly attached, thus allowing the well to dry out and warrants further investigation.

Conclusion

The broth microdilution method for the determination of antimicrobial susceptibility of veterinary and human C. jejuni *and* C. coli *isolates was validated and found suitable for replacing the previous test of agar breakpoint determination. We found no evidence that the broth microdilution test was unacceptably inaccurate or in serious disagreement with the agar breakpoint test. The broth microdilution test also appeared to be fairly reproducible between laboratories for the majority of antimicrobials and had high levels of accordance even exceeding the traditional agar dilution break-point test. Nevertheless, some inter-laboratory and intra-strain variability was observed and even though it did not affect our conclusion further work could be done to assess the origin of this variation.*

Objective 02. Investigate wild type distributions obtained for *C. jejuni* and *C. coli* and evaluate suitability of epidemiological cut-off points recommended by the EU Antimicrobial Resistance CRL for GB isolates from a variety of hosts.

In this objective the validated broth microdilution test was used to test a randomly selected panel of retrospectively collected test isolates of unknown susceptibility to investigate MIC distributions of *C. jejuni* and *C. coli* populations recovered from different hosts. To ensure an isolate panel would comprise different sources from the same time period, survey isolates from the retail meat survey 2004-2005 which were available for study were chosen.

2.1 Methods

2.1.1 Strain selection

A total of 100 human and 100 retail meat *C. jejuni* isolates submitted to GEZI in 2004 were randomly selected from the GEZI databases of isolates received between 2004 and 2006. From the same database 50 *C. coli* strains from each study source was also selected. Prior to random selection of the human panel any multiple isolates from the same patient were removed. The retail meats were categorised according to species of origin; poultry (chicken and turkey), beef, pig and mutton/lamb. Any isolates from unspecified meat types were excluded prior to the randomisation process.

2.1.2 Cultivation and distribution of isolates

The isolates were cultivated in Laboratory 2 and submitted to Laboratory 1 on transport swabs (TS5-2, Technical Service Consultants, Heywood, UK) for antimicrobial susceptibility testing using the validated Campylobacter Sensititre^{TM} plate UKVLC2. This system of submission of isolates for susceptibility testing is the same as that used in the EU (FZ2025) and GB (OZ0613) broiler surveys and was consistent with previous abattoir level surveys. None of the selected isolates had been previously tested for susceptibility to antimicrobials. The MICs of antimicrobials for the isolates were determined using the microdilution test validated in Objective 1. Control isolates NCTC 11351 and NCTC 11366 were used as previously described. The susceptibility data were imported into an Excel spreadsheet (Microsoft Excel 2000; Microsoft Corp) and used to derive tables and graphical illustrations of the spread of MIC values obtained (Appendix 3).

2.2 Results

2.2.1 *C. jejuni* MIC distributions

MIC distributions for the randomly selected human (n=99) and retail meat (n=100) *C. jejuni* isolates for each antimicrobial are shown as histograms in Appendix 3, Fig A3.1. Table A3.1 illustrates MIC values comprising EUCAST wild type distributions after combining human and retail meat isolates, where designated. The final panel of retail meat isolates mainly comprised whole and portioned chicken (83%), with isolates from lamb (7%), beef (5%), turkey (4%) and duck (1%) also included.

The MIC distributions obtained for the human and retail meat isolates appeared remarkably similar to the data available for *C. jejuni* isolates in the reference database on the EUCAST website (http://www.eucast.org) for seven of the antimicrobials tested here for which there is comparative data (Appendix 3, Table A3.1).

Erythromycin

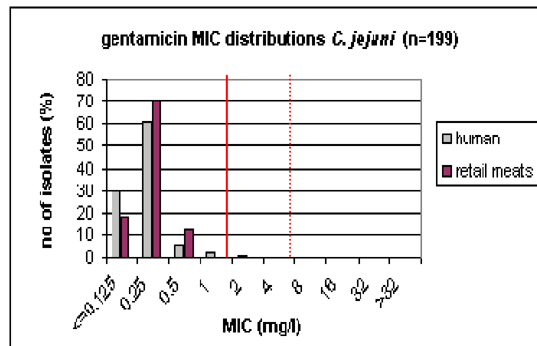
The range of erythromycin MIC distributions obtained for human and retail meat isolate sets were very similar, although the human isolates had a slightly higher mode value (2 mg/l) than observed for the retail meat set (1 mg/l) (Appendix 3, Fig A3.1). When combined, both the range and resulting shape of the histogram was strikingly similar to that obtained from the EUCAST reference database, which is based on 1473 observations (www.EUCAST.org; search 23/03/2009). Interestingly, both datasets included a small number of isolates yielding MIC value just above the EU cut-off of 4 mg/l. In our study these isolates were of human origin. The EUCAST and traditional GB cut-off values are the same for this antimicrobial and, apart from the aforementioned isolates, our data showed good correlation with these designated values. However, at the new BSAC breakpoint of 0.5 mg/l for this antimicrobial, only 14.1% of the 199 *C. jejuni* MIC values would be classified as susceptible, although a higher proportion (24.9%) of those in the EUCAST reference database would be similarly classified. Only one of the 199 study isolates yielded erythromycin MIC values of between 8 and 64, but 7 had MIC values of >=128. The later isolates are considered naturally resistant.

Gentamicin

The MIC distributions for gentamicin (Fig. 4) yielded a steep curve with the majority of isolates yielding an MIC value of 0.25 mg/l. Although the EUCAST dataset shows a wider spread of MIC values the observed differences may in part be due to the relatively small number of isolates tested in this study, although the population size required for submission to EUCAST (100) was exceeded. In addition, 24% of isolates had MIC values to gentamicin of ≤0.125 mg/l and may have resulted in truncation of the true wild type distribution. Another

explanation may be the limited origins of the panel isolates studied here, which were derived from human and retail meat isolates during a single year (2004).

Fig 4. Gentamicin MIC distributions for *C. jejuni*. The solid red vertical line represents the cut-off for resistance recommended by EUCAST for *C. jejuni*. Dashed red line represents traditional breakpoint.

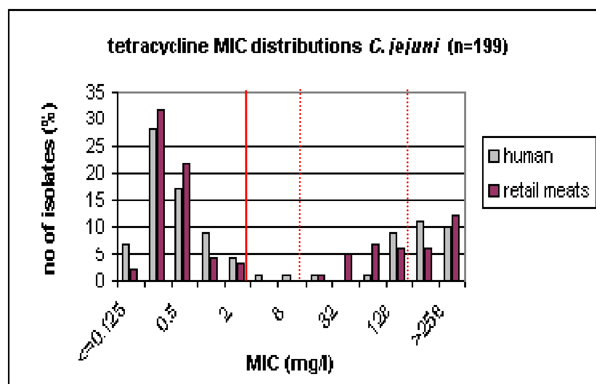


Ciprofloxacin and nalidixic acid
The histogram generated for ciprofloxacin had a bimodal distribution with an clear differentiation between naturally resistant and susceptible populations (Appendix 3, Fig A3.1). Interestingly, a greater number of isolates yielded high MIC values in human population (36%) than found in retail meats. The wild type distribution and shape of the histogram obtained with our data corresponded very well with that available from the EUCAST reference database, which is based on 800 observations. The MIC distributions confirm that the EU epidemiological cut-off value, which classifies resistance at MIC values of >1 mg/l, also fits with our data. The EUCAST cut-off recommended for nalidixic acid for *C. jejuni,* again matches the traditional breakpoint and fitted well with our data from human isolates (Appendix 3, Fig. A3.1). However, the MIC distribution of retail meat isolates appeared to suggest some overlap between susceptible and resistant populations (Appendix 3, Fig A3.1).

Streptomycin
The MIC range and mode value for the susceptible population was very similar for human and retail meat isolates (Appendix 3, Fig A3.1). The MIC distributions obtained also correlated well with the EUCAST reference database. However, three isolates yielded streptomycin MIC values ranging from 4-8 mg/l, which is just above the EUCAST cut-off and may comprise intrinsically resilient isolates. Our data clearly shows that 9 of the 199 (4.5%) isolates tested have MICs of $\geq$ 32 mg/l, comprising the naturally resistant population.

Tetracycline
For tetracycline a bimodal distribution of MIC values was obtained for both human and retail meat isolates, suggesting susceptible and naturally resistant populations (Fig. 5). The majority of the wild type isolates had MIC values of 0.25 or 0.5 mg/l.

Fig 5. Tetracycline MIC distributions for *C. jejuni* from humans and retail meats

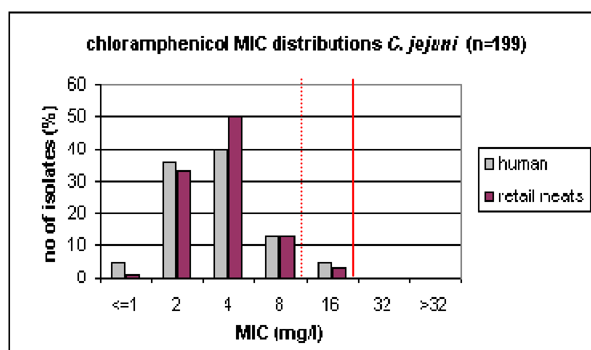


In contrast, 60% of isolates included in the EUCAST wild type distribution database for this antimicrobial have an MIC of 0.25, with only 1.4 and 4.1% of isolates yielding MIC values of 0.125 and 0.5, respectively. A small proportion of the human isolates had MIC values of 4 or 8 mg/l (n= 2). The EUCAST MIC distribution for this antimicrobial is also populated at MIC values of 4 and 8 mg/l, with 3.7% of the total isolates yielding these MIC values and are thus classified as resistant.

Chloramphenicol
The MIC distribution data from both human and retail meat isolates for chloramphenicol was similar, with the majority of isolates yielding MIC values in the range of 2-4 mg/l (Fig. 6). The data also matched closely with that

of the EUCAST reference database. Our findings suggest that the epidemiological cut-off value proposed and recently applied in the CRL EQAS scheme for resistance to this antimicrobial of >16 fits well with our data.

Fig 6. Chloramphenicol MIC distributions for *C. jejuni* from humans and retail meats



Ampicillin

Only the data obtained for ampicillin differed considerably from the wild type distributions available for this antibiotic from the EUCAST database. Our data indicates mode MIC values of 8 mg/l for both human and retail meat populations, while the majority of isolates in the EUCAST data have MIC values ranging from 1 to 4 mg/l. Moreover, the distribution of the MICs from our retail meat study population appears slightly positively skewed, with a tail of resistant isolates (Appendix 3, Fig.A3.1). Although not included in the EQAS scheme for the EU ar-CRL (www.CRL-ar.eu) EUCAST has indicated an epidemiological cut-off such that R > 8 mg/l was is consistent with originally applied breakpoint in GB. Interestingly, the EUCAST data for this antimicrobial also shows 17% of isolates with MICs of 16 or 32 mg/l. Our MIC distributions showed a small second peak (Appendix 3, Fig.A3.1) which is attributable to naturally resistant isolates.

Kanamycin and Imipenem

There is no EUCAST data for kanamycin or imipenem. The distribution of MIC values obtained for kanamycin was similar for isolates of human and retail meat origins (Appendix 3, Fig A3.1), although the shape of the curves produced by the histograms was slightly different. Our data confirms that the breakpoint of 16 includes the wild type distribution for this *Campylobacter* species and is appropriate, at least for the populations studied. MIC values for imipenem ranged from 0.016 to 1 mg/l (Appendix 3, Fig A3.1).

2.2.2 Prevalence of resistance in *C. jejuni* according to EUCAST epidemiological cut-off values

Prevalence of resistance to the 9 antimicrobials for the human and retail meat isolates are shown in Table 10. Of the 99 *C. jejuni* isolates of human origin 33 (33%) resistant to ciprofloxacin, while 36 (36%) were resistant to nalidixic acid. Thus 3 isolates were resistant to nalidixic acid but not ciprofloxacin. A lower proportion of retail meats were resistant to ciprofloxacin (12%) or nalidixic acid (14%). Ciprofloxacin resistance in UK *Campylobacter* isolates has previously been associated with foreign travel. This may account for the high proportion of resistance in humans rather than from the largely indigenous meat sources. The prevalence of resistance to erythromycin was low in both populations (Table 10). Of the other groups included in the EU panel, only one isolate was resistant to gentamicin, while 34% and 37% of human and retail meat isolates, respectively, were resistant to tetracycline (>2 mg/l). Unsurprisingly there was no resistance to chloramphenicol, although 4% of all isolates would have been were classified as resistant at the traditional HPA breakpoint of 8mg/l. Thirty three percent of isolates were resistant to more than 1 class of antimicrobial.

Table 10. Resistance to antimicrobials by Sensititre broth microdilution for 199 randomly selected *C. jejuni* isolates collected from humans and retail meats

Antimicrobial agent	R>(mg/l)	% resistance humans	% resistance retail meats
Ampicillin	NA / 8 and 32	NA / 35.4 and 28	NA / 48.0 and 30.0
Chloramphenicol	16	0	0 (3)
Nalidixic acid	16	36.4	14.0
Ciprofloxacin	1	33.3	12.0
Erythromycin	2	1	0
Tetracycline	2 / 8 and 128	34.3 / 32 and 22	37.0 / 37 and 18
Kanamycin	NA / 16	NA / 3.0	2.0
Streptomycin	2	5.0	7.0
Gentamycin	1	1.0	0
Imipenem	None	NA	NA

2.2.3 *C. coli* MIC distributions

MIC distributions for the randomly selected human (n=49) and retail meat (n=49) *C. jejuni* isolates for each antimicrobial are shown as histograms in Appendix 3, Fig A3.2. Table A3.2 illustrates MIC values obtained, showing EUCAST wild type distributions, where designated. The final panel of retail meat isolates mainly comprised whole and portioned chicken (n=44 (89.8%)), with isolates from lamb (n=2 (4.1%)), pig (n=1 (2.0%)) and turkey (n=2 (4.1%)) also included.

<u>Erythromycin</u>

The majority of isolates from both human and retail meat study panels yielded MIC values of between was 0.5 and 2 mg/l (80.6%) and was not markedly different from that obtained from the *C. jejuni* panel (Fig 7; Appendix 3, Table A3.2). The mode observed for EUCAST wild type distributions for this species was 4 mg/l, 1-2 MIC steps above the values obtained for our study, but EUCAST reference database is based on 1580 observations from 18 independent sources, which may have contributed to these differences in our findings. A single isolate yielded MIC values of 8 mg/l and there was a small number of naturally resistant isolates with MIC values of ≥128 mg/l.

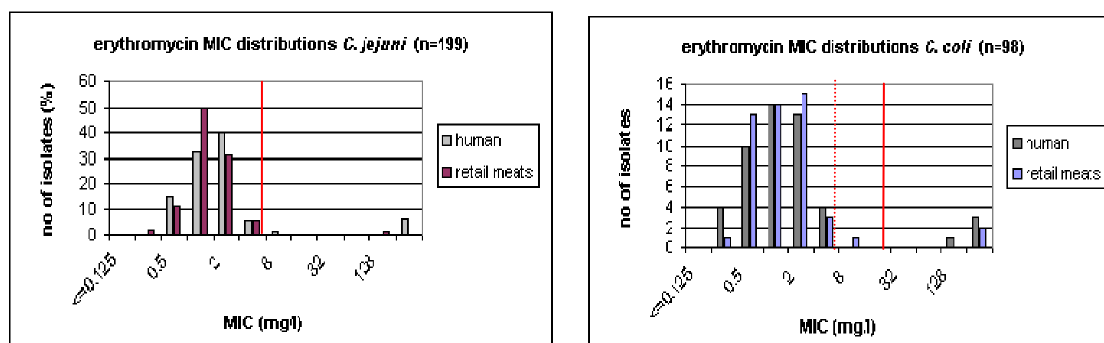


Fig 7. Erythromycin MIC distributions for *C. jejuni* and *C. coli* study populations

<u>Gentamicin</u>

A mode MIC value of 0.25 mg/l was obtained for both human and meat isolate panels (Appendix 3, Fig A3.2). This compares well with reference data available from EUCAST (0.5 mg/l) and all isolates yielded MIC values within the wild type distribution proposed by EUCAST, albeit that our concentration range for this antimicrobial was truncated as previously described. However, none of our study isolates yielded MIC values of 2 mg/l or higher and, with the exception of a lack of naturally resistant isolates, the range of MIC values was not markedly different to that obtained from the *C. jejuni* panel.

<u>Ciprofloxacin and nalidixic acid</u>

As previously reported for *C. jejuni* a bimodal distribution was observed for ciprofloxacin (Appendix 3, Fig A3.2), including susceptible and naturally resistant populations for both humans and retail meat panels. Two isolates yielded MIC values which fell below the wild type distribution proposed by EUCAST, although isolates with similar MIC values are included in the reference database, so the finding is not unexpected. As expected from shared epidemiological cut-off values the mode and range of MIC values obtained were not notably different from those observed for *C. jejuni*. For nalidixic acid the distribution of MIC values was very similar to that observed for EUCAST data. Although no human isolates yielded MIC values just below at the *C. coli* EUCAST cut-off of 32 mg/l, four retail meat isolates yielded this MIC value and would have been classified as resistant according to EUCAST, but susceptible at the traditional HPA breakpoint.

Streptomycin
The data obtained for streptomycin showed modal MIC value of 1 mg/l for both human and retail meat panels, which was 1 MIC step lower than compared with the EUCAST reference database (Appendix 3, Fig A3.2). None of our study isolates yielded MIC values of 4mg/l, close to the value which is used to classify *C. coli* isolates as resistant (R>4 mg/l). Moreover, the data also correlated well with that obtained from the *C. jejuni* panel, indicating that for the small *C. coli* population sampled here all wild type isolates would have been classified as susceptible at the *C. jejuni* cut-off value. A small number of naturally resistant isolates were observed of both human and retail meat origins.

Tetracycline
The data obtained for this antimicrobial was similar to that obtained for *C. jejuni* ,as expected by corresponding epidemiological cut-off values (Appendix 3, Tables A3.1 and A3.2). As previously described for the *C. jejuni* panel, use of the EU cut-off value of 2 mg/l, left a small number of isolates with MIC values of 4mg/l classified as resistant.

Kanamycin and Imipenem
For kanamycin the majority of *C. coli* isolates yielded MIC values of 4-8 mg/l (88.8%). The traditionally applied cut-off value of 16 mg/l (R>16 mg/l) appears appropriate for isolates belonging to this *Campylobacter* species, in the absence of appointed EUCAST values. The mode MIC value (0.25 mg/l) for both human and retail meat isolates (68.4%) (Fig. 8) for imipenem was 1 MIC step higher than that observed for *C. jejuni*, while the range also differed with no *C. coli* isolates having MIC values of <0.064 mg/l. These findings indicate that the *C. coli* population may be intrinsically less susceptible to this antimicrobial than *C. jejuni*.

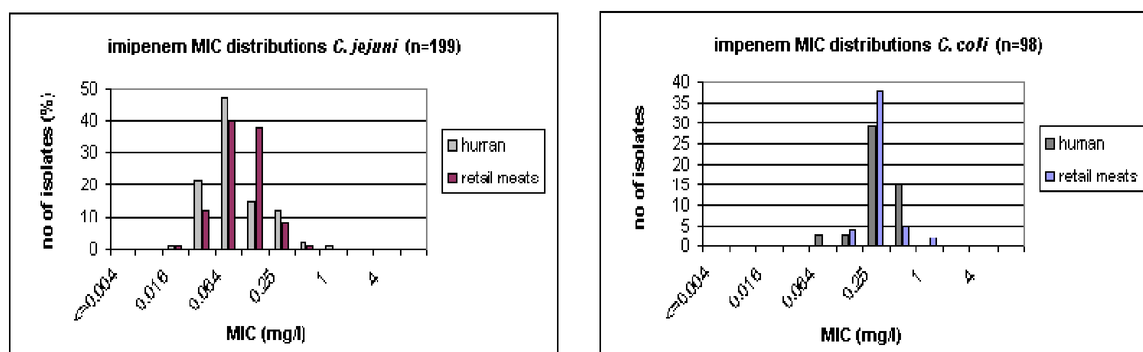


Fig. 8. Imipenem MIC distributions for *C. jejuni* and *C. coli*

2.2.4. Prevalence of resistance in *C. coli* according to EUCAST epidemiological cut-off values
The prevalence of resistance to the test antimicrobials using EUCAST cut-off values, where available is shown in Table 11. Resistance to ciprofloxacin was higher amongst retail meat isolates than those from humans and generally corresponded with resistance to nalidixic acid. Prevalence of resistance to erythomycin in both *C. coli* study populations was higher than observed for *C. jejuni* , with similar findings obtained for streptomycin. Although prevalence of tetracycline resistance for human *C. coli* isolates was similar to that obtained for *C. jejuni*, resistance amongst retail meat *C. coli* isolates was higher than obtained from *C. jejuni*, despite the preponderance of chicken in both populations. No resistance to gentamicin was observed and only a single isolate was resistant to chloramphenicol. Thirty-three percent of isolates were resistant to more than one class of antimicrobial.

Table 11. Resistance to antimicrobials by Sensititre broth microdilution for 98 randomly selected *C. coli* isolates collected from humans and retail meats

		% resistance	
Antimicrobial agent	R>(mg/l)	humans	retail meats
Ampicillin	8 / 32	20.4 / 10.2	18.4 / 16.3
Chloramphenicol	16	1	0
Nalidixic acid	32	26.5	34.7
Ciprofloxacin	1	20.4	34.7
Erythromycin	16	8.2	4.1
Tetracycline	2	34.7	51
Kanamycin	NA / 16	NA / 0	NA/0
Streptomycin	4	6.1	14.3
Gentamicin	2	0	0
Imipenem	None	NA	NA

2.2.5 Discussion

In our MIC distribution analyses no *C. coli* isolates yielded erythromycin MIC values of 4-16 mg/l thus classification of resistance was the same for the population sampled irrespective of whether the *C. jejuni* or higher *C. coli* breakpoints were applied. Surprisingly, a single *C. jejuni* isolate had an MIC value just above the cut-off (at 8 mg/l) This isolate was also highly resistant to ampicillin (MIC 128 mg/l) so association with efflux mechanisms is not suggested and it may be appropriate to identify whether any of the known 23S rRNA mutations are present in this isolate. Our findings, albeit based on relatively small sample sizes, indicates that the separate *C. coli* EUCAST epidemiological cut-off value breakpoint value, which classifies resistance in at >16 mg/l did not affect prevalence of resistance to this antimicrobial compared with the traditional value of >4 mg/l, which is consistent with the EUCAST value for *C. jejuni*. Interestingly, a higher proportion of *C. coli* isolates were classified as susceptible than those belonging to *C. jejuni* at the new BSAC breakpoint of 0.5 mg/l. Our findings suggest that this breakpoint is inconsistent with wild type MIC distributions of either *C. jejuni* or *C. coli* populations from our study and this observation is supported by the EUCAST reference database.

Gentamicin MIC data obtained for *C. jejuni* and *C. coli* correlated well, with no strains yielding MIC values above 1 mg/l. Although the study population was relatively small for *C. coli*, the *C. jejuni* cut-off value of 1 mg/l, which would classify resistance as >1mg/l, fitted well with our data for both species. Analysis of further randomly selected populations which could include the data now available from the broiler and pig abattoir-level surveys warrants further investigation. Although the proportions of naturally tetracycline resistant isolates was higher in *C. coli* the wild type distributions were similar between the two *Campylobacter* species. A small proportion of isolates belonging to both *Campylobacter* species yielded MIC values of 4-8 mg/l, thereby while classified as resistant according to EUCAST cut-off values, but would have been susceptible at traditional GB breakpoint of 8 mg/l. Tetracycline resistance is typically encoded by carriage of the *tet*O gene and previous work in our laboratories has shown that *tet*O is not generally associated with isolates yielding MIC values below 32 mg/l (A Ridley, Final report VM2105). While other mechanisms that lead to reduced susceptibility (e.g. efflux) may be at play this would be typified by reduced susceptibility to a range of antimicrobials and this was not detected in the majority of the affected isolates.

The EUCAST epidemiological cut-off for chloramphenicol which classifies resistance at >16 mg/l fitted the MIC distribution data from both *C. jejuni* and *C. coli* panels investigated here slightly better than the traditionally applied GB breakpoint of 8mg/l. Our findings indicate that the adoption of this cut-off value should avoid any likelihood of artefactual over-estimation of resistance to this antimicrobial.

MIC distributions obtained for ampicillin for both *C. jejuni* and *C. coli* in this study differed from those of the EUCAST reference database. The mode MIC value differed by 1 MIC dilution step between *C. jejuni* (8 mg/l) and *C. jejuni* (16 mg/l), which is consistent within the data for this antimicrobial present in the EUCAST reference database, there appears to be no clear distinction between wild type and naturally resistant populations. It is therefore likely that our observed tail towards higher MIC values is composed of wild type and slightly resistant isolates, which is makes determination of an epidemiological cut-off for this antimicrobial more challenging. This observed reduced susceptibility of such isolates may reflect the widespread usage of the drug in the UK which has been attributed to the high ampicillin resistance (at 37%) in *E.coli* from UTI in the UK, reported by the ECO-SENS project (Kahlmeter et al, 2003), which was the fifth highest in Europe.

03. Produce recommendations for joint reporting of human and animal campylobacter antimicrobial resistance surveillance which facilitates comparison with data produced in other EU countries

The study outcomes yielded the following recommendations and outcomes:

1. An SOP for broth microdilution test for *C. jejuni*/*coli* as a specific National Reference Method. The broth microdilution method using standard susceptibility testing plate should be used in combination with standardised protocol (attached) to ensure compatibility with EU countries.
2. The same microaerobic conditions (time and gas mixtures) and incubation times were used by each laboratory for the loaded Sensititre plates in the study, so any inter-laboratory differences relating to these should have been minimal. Standardisation of procedures in all test laboratories submitting data on human and food animal isolate susceptibility to the EU to minimise differences in interpretation relating to end point.
3. Maintenance and enhancement of validation. A validated assay needs constant monitoring of repeatability and accuracy to ensure reliability of performance in test laboratories. Proficiency testing as part of the EU-ar CRL EQAS scheme ring trial should be mandatory for laboratories using the broth microdilution test for food and human isolates from national studies. It is recommended that appointed human/food/veterinary NRLs participate annually to this, or similar scheme, as this would facilitate networking between laboratories in the UK and with other European laboratories who contribute susceptibility data to EFSA.

4. As the standard control strain *C. jejuni* (ATCC 33560) has MICs below designated cut-off values for the majority of antimicrobials and the range proposed for ampicillin for *C. coli* strain ATCC 33559 is 4-16 mg/l it would be appropriate to include additional control isolates to include one multi-resistant strain of *C. jejuni* and of *C. coli* . These could be from the current EQAS scheme; for example *C. jejuni* strain C 2.6 appears to be highly suitable, with resistance to ciprofloxacin, nalidixic acid, erythromycin, gentamicin, streptomycin and tetracycline. However, no data is provided for ampicillin, although this may have been determined by the originating laboratory. Suggestions for suitable isolates for *C. coli* include EQAS reference C3.4, resistant to ciprofloxacin, nalidixic acid, streptomycin and tetracycline, although this strain has an expected MIC value for erythromycin of 16, making it just susceptible according to the EU cut-off.
5. Generally for *C. jejuni* our wild type distribution data fitted well with EUCAST epidemiological cut-off values, including that for clinically relevant antimicrobials ciprofloxacin and erythromycin. Application of the new BSAC breakpoint of 0.5 would have resulted in few isolates being classified as susceptible.
6. Although EUCAST *C. coli* -specific breakpoints have been adopted by EFSA for erythromycin, gentamicin and streptomycin our data showed very similar MIC distributions to those obtained for *C. jejuni*. This observation warrants further investigation by analysis of broiler and other similar randomly selected population's susceptibility tested using this method.
7. The EUCAST cut-off value for chloramphenicol, classifying R>16 mg/l, supported the data obtained from objectives 1 and 2 of this project better than the traditional GB breakpoint of 8 mg/l. However, there appears to be no clear distinction between S and R populations for tetracycline and ampicillin.
8. The results provide data on the MIC distribution for imipenem which may be used clinically in the treatment of invasive campylobacter infections in humans. Although imipenem is not required in EU surveillance monitoring this data may be of clinical relevance for assessment of treatment in a limited number of human infections and will therefore be passed to BSAC.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

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