PREVALENCE OF SULFONAMIDE RESISTANCE GENES IN BACTERIAL ISOLATES FROM MANURED UK AGRICULTURAL SOILS AND PIG SLURRY

Running Title: *Sul1*, *sul2* and *sul3* prevalence in bacterial isolates.

Authors: K.G.Byrne-Bailey[1*], W.H.Gaze[1], P.Kay[2†], A.BA.Boxall[2§], P.M.Hawkey[3] and E.M.H.Wellington[1#].

Author affiliation: [1]Department of Biological Sciences, University of Warwick, Gibbet Hill, Coventry, West Midlands, CV4 7AL, U.K.,

[2]Cranfield Centre for EcoChemistry, Shardlow Hall, Shardlow, Derby, Derbyshire, DE72 2GN, U.K.,

[3]Department of Immunity and Infection, University of Birmingham, Birmingham, B15 2TT, UK.

Current addresses: *Department of Plant and Microbiology, University of California, Berkeley, CA 94720, [†]School of Geography, University of Leeds, Leeds, LS2 9JT, U.K., [§]PVMG Central Science Laboratory, Sand Hutton, York, YO41 1LZ, U.K.,

#Corresponding author: Professor E.M.H.Wellington, Department of Biological Sciences, University of Warwick, Gibbet Hill, Coventry, West Midlands, CV4 7AL, U.K., Tel. +44 2476523184, Fax. +44 2476523568, E.M.H.Wellington@warwick.ac.uk.

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Abstract: Prevalence of three sulfonamide resistance genes, *sul1*, *sul2* and *sul3* and sulfachloropyridazine (SCP) resistance was determined in bacteria isolated from UK manured agricultural clay soils and slurry samples, over a two year period. Slurry from tylosin-fed pigs amended with SCP and oxytetracycline (OTC) was used for manuring. *Sul* gene positive isolates were further screened for the presence of class 1 and 2 integrons. Phenotypic resistance to SCP was significantly higher in pig slurry and post application soil than in pre-application soil. Of 531 isolates, 23 % carried *sul1*, 18 % *sul2* and 9 % *sul3* only. Two percent of isolates contained all three *sul* genes. Class 1 and class 2 integrons were identified in 5 % and 11.7 % of *sul* positive isolates. In previous reports, *sul1* was linked to class 1 integrons, but in this study only 8 % of *sul1* positive isolates carried the *intI1* gene. Sulfonamide resistant pathogens were identified in slurry amended soil and soil leachate, including *Shigella flexneri*, *Aerococcus* spp. and *Acinetobacter baumanni*, suggesting a potential environmental reservoir. Sulfonamide resistance in *Psychrobacter*, *Enterococcus* and *Bacillus* spp. is reported for the first time, and this study also provides the first description of the genotype *sul1*, *sul2* and *sul3* outside the *Enterobacteriacae*, and in the soil environment.

Introduction:

Sulfonamides have been widely used to treat bacterial and protozoal infections in clinical and veterinary medicine since their introduction in the 1930's. They act as a structural analogue of ρ-amino-benzoic acid and bind dihydropteroate synthase (DHPS), a catalytic enzyme in the folic acid biosynthesis pathway, resulting in the inhibition of dihydrofolic acid formation (26). Resistance is conferred by mutations in the DHPS gene (*folP*) (30) or from the acquisition of an alternative DHPS gene (*sul*) (18, 20, 29).

The first of the three known alternative DHPS genes, *sul1*, is usually found located on the 3' conserved region of a class 1 integron (25) and is frequently identified with this potentially mobile element in the slurry and soil environment (13, 22, 29). *Sul2* was first identified on RSF1010 in *Escherichia coli* and has been found on small non-conjugative resistance plasmids (20). The *sul3* gene was identified during a study into sulfonamide resistance in pathogenic *E. coli* isolates in swine from Switzerland (18).

The prevalence of each of the sulfonamide resistance genes varies between published studies, depending on environments and bacterial species sampled. The majority of reports relate to *Enterobacteriacae* isolates, specifically *E. coli* and *Salmonella* spp.

Previous investigations have screened for all three *sul* genes, but only Antunes *et al.* (2) in an investigation of *Salmonella enterica* strains, found all three genes. One previous study screened for all three genes in environmental isolates and soil; Heuer and Smalla (14) screened silt and loamy sand soils, known to have lower sorbance properties for antibiotics (especially for tetracyclines and sulfonamides) than clay soils (8, 11). This short term study, suggested that manure from treated pigs enhanced spread of antibiotic resistance in soil bacterial communities (14).

Here we report the prevalence of sulfonamide resistance genes in bacterial isolates from agricultural clay soil, where long-term (2 years) application of slurry from tylosin-fed pigs with experimental addition of sulfachloropyridazine (SCP) and oxytetracycline (OTC) occurred. The study also aimed to investigate whether sulfonamide resistance gene prevalence was due to dissemination of sulfonamide resistance in bacteria from the farm environment (manure) to indigenous soil bacteria.

Materials and methods:

77 **Field study.** Triplicate clay soil cores were collected at pre-determined time points
78 from an agricultural field, Lincolnshire, UK, which received an application of tylosin-
79 fed pig slurry which had been amended with SCP and OTC at concentrations of 25.58
80 mg l^{-1} and 18.85 mg l^{-1} respectively (8). Time points were; pre-application, year 1
81 day 1 after application, year 1 day 21, year 1 day 90, year 1 day 289, year 1 day 1,
82 year 2 day 90 and year 2 day 240. Pre-application soil cores were used as controls.
83 No tylosin (TY) was detected in the slurry or soil samples preceding or following the
84 slurry applications. SCP was detected in soil leachate at 590 µg l^{-1} at day 7 post
85 application, 64 µg l^{-1} at day 10 and then at low levels _≤ 1µg l^{-1} from day 20. SCP
86 and OTC concentrations (365 to 1691 µg Kg^{-1}) through the soil profile were reported
87 previously (8). Soil leachate samples were chosen from the three highest periods of
88 rainfall and investigated separately, from year 1 day 15, year 2 day 49 and year 2 day
89 164 of the study. For ease of reporting, all results from the soil leachate samples were
90 grouped together. The liquid from each sample was centrifuged and the pellets
91 resuspended in 1ml for serial dilutions which were carried out in triplicate, the
92 original volume was used in calculating numbers of bacteria per sample/ml.
93 **Viable plate counts.** Counts were performed on 3 separate cores from slurry amended
94 UK agricultural soils at 9 time points, samples of pig slurry which had been obtained
95 from a catchment tank below tylosin-fed animals (pig slurry control) and 3 separate
96 pig slurry samples from the slurry tank after antibiotic amendment (pig slurry
97 amended) (8). 1 g of soil from 0-5 cm cores and 1 ml slurry samples were re-
98 suspended in 9 ml of sterile distilled water. Serial dilutions were made and spread
99 onto Iso-Sensitest agar (Oxoid, U.K.) containing different concentrations of SCP
100 (Sigma, U.K.) 5, 10, 25 and 50 µg ml^{-1}, OTC (Sigma) 0.2, 1, 5, 10, 25 and 50 µg ml^{-1},
101 and TY (Sigma) 5, 10, 25, 50 and 100 µg ml^{-1}. All plates contained 100 µg ml^{-1}
102 cycloheximide (Sigma) to inhibit growth of fungi. Plates were incubated o/n and for 5

days aerobically at 28 ºC. Resistance quotients (RQs) were calculated by dividing the mean count from triplicate selective plates by the mean count from triplicate non-selective plates.

Bacterial isolation. Bacterial colonies were randomly picked from non-selective and selective plates containing different concentrations of SCP, OTC and TY and streaked until pure cultures were obtained. The number of isolates obtained from each antibiotic concentration at each time point varied due to differences in resistance of the sampled population (no growth was observed at higher antibiotic concentrations in some samples), and due to loss of viability of some glycerol stocks. To overcome the variable numbers of isolates from each selective media at different time points, comparison of *sul* gene prevalence was made from a sub set of the data. A standardised number of isolates were randomly selected from each antibiotic selective media, SCP, TY or OTC (11, 10 and 8 isolates respectively) for each of the following samples: pig slurry, pre-application year 1, year 1 day, year 1 day 90 and year 1 day 289.

DNA extractions. DNA was extracted using a DNeasy kit (Qiagen, S.A.) according to the manufacturer's instructions from isolates grown in 5 ml Iso-Sensitest broth (Oxoid) o/n at 28 ºC. PCR was performed using 1u *Taq* DNA polymerase and the manufactures buffer (Invitrogen, U.K.), with 4 mM $MgCl_2$. Thirty cycles were performed of: 95 °C/1 min, varying annealing temperatures, according to Table 1 for 1 min and 72 °C/1 min. The PCR products were eluted from a 1 % agarose gel (Helena Biosciences, U.K.) using a QIAquick Gel Extraction Kit (Qiagen). All *sul* positive isolates were identified using 16S rDNA sequences of approximately 800 bp in both directions. Sequencing reactions were performed with a terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) as described by the

manufacturer, and electrophoresis and readout were performed on an ABI Prism 3100 genetic analyzer (Applied Biosystems).

Analysis of DNA sequences. Resulting DNA sequences were edited using BioEdit (Isis Pharmaceuticals, Inc.) and subsequently analyzed using the BLAST programme, with a sequence similarity ≥ 97% used for species identification (1).

Conjugal transfers. *Pseudomonas putida* UWC1 (Rif^r) and *Escherichia coli* K-12 CV601 (Rif^r Thr^- Leu^- Thi^-) were used as recipients in conjugal transfers which were performed according to Smalla *et al.* (27). The recipients had an MIC to SCP of 0.5 μg ml^{-1}. Transconjugants that had been involved in a transfer event were selected for on 50 μg ml^{-1} rifampicin (Sigma) and 8 μg ml^{-1} SCP containing Iso-Sensitest agar plates for 48 hrs at 30 °C. Colonies were screened by PCR to confirm *sul* gene transfer and disregard the possibility of spontaneous mutations. Transfer rates (transconjugants per donor) were calculated according to Binh *et al* (5), where the transfer event equalled, the CFU ml^{-1} transconjugants divided by the CFU ml^{-1} recipients, this rate was then normalised by the number of positive colonies screened by PCR for the gene of interest. The limits on transfer frequencies were set by our ability to detect a single transconjugant cell, but varied with experimental conditions due to differences in initial recipient number.

Control plates of donors only were included to investigate rates of rifampicin mutations, these plates were always negative.

MIC determination. MICs and antibiotic resistance breakpoints were determined on Iso-Sensitest agar plates using an agar dilution method (21), the inoculum (100 μl) was adjusted to 0.4 OD_{600nm} for each isolate to ensure consistency in MIC determination. The antibiotics (Sigma) tested were; streptomycin at a concentration of 16 μg ml^{-1}, ampicillin 16 μg ml^{-1}, kanamycin 16 μg ml^{-1}, chloramphenicol 16 μg ml^{-1}, tetracycline 8 μg ml^{-1}, trimethoprim 16 μg ml^{-1}, neomycin 8 μg ml^{-1}, nalidixic acid 16

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154 μg ml^{-1}. Breakpoints were selected on the basis of identifying likely clinically
155 relevant mechanisms of resistance. MIC determinations for SCP were performed
156 using the following concentrations; 0, 1, 2.5, 5, 25, 50 and 100 μg ml^{-1}.
157 **Statistical analysis.** Resistance quotients and prevalence were compared using a Chi-
158 square test for the comparison of two proportions (from independent samples).
159 Statistical analyses were performed using MedCalc for Windows, version 9.3.7.0
160 (MedCalc Software, Mariakerke, Belgium).
161
162 **RESULTS and DISCUSSION:**
163 **Antibiotic resistance.** RQs calculated from SCP plate counts are shown for each
164 sample in Figure 1. Using a Chi-square test for the comparison of two proportions
165 (from independent samples), RQs were compared for culturable bacteria between
166 samples and at different time points. SCP resistance was significantly higher in pig
167 slurry than in pre-application soil with selection of 10, 25 and 50 μg ml^{-1} ($p< 0.0001$).
168 Resistance was also significantly higher at day 1 post application than in pre-
169 application soils ($p< 0.0001$) and remained higher at day 289 ($p<0.0001$). This was
170 not the case in day 21 and day 90 samples, possibly due to patchy distribution of
171 slurry and uneven retention of antibiotic residues. Clay soils are characterised by a
172 network of cracks and fissures that allow localised mobilisation of rainfall, dissolved
173 compounds and suspended particles. The resistance observed at day 289 can not be
174 attributed to continued selective pressure exerted by SCP after slurry application as
175 SCP was quickly washed out of the soil due to its high mobility and low K_d (16),
176 where K_d is the sorption coefficient (28). Thus persistence of resistant phenotype is
177 likely to be due to survival of bacteria carrying resistance determinants or transfer of
178 resistance gene to indigenous bacteria rather than selective pressure exerted by SCP in
179 the environment.

***sul* gene prevalence.** All 531 bacterial isolates collected in this study were screened by PCR for *sul1*, *sul2* and *sul3*. The most common genotypes were those of single genes, *sul1* had the highest prevalence, followed by *sul2* and subsequently *sul3* (Table 2. In total 67 % (n=358) of isolates collected were PCR positive for one or more sulfonamide resistance genes and 17.5 % (n=93) of these carried combinations of the three genes, *sul2* and *sul3* being the most frequent. The genotype of *sul1*, *sul2* and *sul3* in occurred in 2.3 % (n = 12) of isolates.

All genotypes were present in all samples with the exception of the *sul2* + *sul3* genotype, which appeared to originate from the amended slurry and was only present in isolates collected from amended soil in year 1 post-application, appearing again in isolates from year 2 after a second slurry application. Pre-application soil cores displayed a high number of *sul* containing isolates (Table 2), possibly because of repeated pig slurry application over the previous decade. Whilst these previous slurry applications were not known to have included sulfonamides, they may have been used therapeutically.

When *sul* gene prevalence was compared over time in a subset of the data including bacteria isolated on the same selective media there was no significant difference in prevalence when all *sul* genes were considered together (Fig. 2).

Characterisation of isolates. All *sul* positive isolates were identified by 16S rDNA typing and the presence of class 1 and 2 integrons determined. Seventeen genera including opportunistic pathogens and indigenous soil bacteria were identified as shown in Table 3. The most prevalent *sul* positive species isolated in this study was *Acinetobacter* spp., which were collected from all soil/slurry samples. *Acinetobacter* sp. were reported to have developed resistance to a large number of antibiotic groups including the sulfonamides, making them a serious problem in hospitals (6, 10, 31). *A. baumanni* and other species of *Acinetobacter* have previously been identified in

206 diverse environments, now including an agricultural soil environment, a potential
207 'hot-spot' of gene acquisition from the vast gene pool found in soil and rhizosphere
208 bacteria (4, 17, 19, 23). This is the first report of *sul3* in *Acinetobacter*. Table 3
209 demonstrates that *Acinetobacter* spp. carrying *sul* genes were present in the soil for up
210 to a year after slurry application, as were *Arthrobacter, Bacillus, Carnobacterium* and
211 *Pseudomonas* spp.. *Agrobacterium* and *Stenotrophomonas* spp. carrying *sul* genes
212 were detected at 90 days post slurry application. In contrast, *Aerococcus,*
213 *Brevibacterium, Comamonas, Corynebacterium, Planococcus, Providencia,*
214 *Psychrobacter* and *Wiesella* spp. were only present in pig slurry or amended soil
215 immediately after slurry application; these bacteria are therefore more likely to enter
216 the human population via the food chain than by environmental transport routes.
217 *Enterococcus* and *Shigella* spp. were only found in pig slurry and soil leachate
218 samples, suggesting that they were quickly washed out of the soil into ground water
219 and drainage systems by rain.

220 Table 4 displays a number of representative isolates from the main genotypes
221 and all isolates containing the three *sul* genes. A surviving enteric isolate and human
222 pathogen, identified as a *Shigella flexneri* (C506) with 96 % similarity, was isolated
223 from a soil leachate sample, and contained *sul2* and *intI1* with a multiple resistance
224 phenotype. The *sul2* and *sul3* genotype was found in *Acinetobacter lwoffi,*
225 *Enterococcus sulfureus* and *Aerococcus viridans* isolates, all pig associated pathogens
226 / commensals entering the soil through the slurry applications. Isolates PGS21 and
227 PGS22 from the antibiotic amended slurry were both identified as *Aerococcus*
228 *viridians* with 99 % nucleotide similarity (16S rDNA, 800 bp), and a number of
229 *Psychrobacter* spp. were also identified (Table 3). The *Psychrobacter* spp. isolates
230 were resistant to tetracycline, streptomycin, chloramphenicol, trimethoprim and
231 nalidixic acid, with calculated SCP MIC values of between 5 and 16 mg l^{-1}. The

isolation of *Psychrobacter* spp. was unusual, commonly isolated from cold marine environments and sediments (7, 9, 24). BLAST analysis (1) of the newly sequenced *Psychrobacter* genomes (www.jgi.doe.gov) using sequences; GenBank accession numbers AM086633 (*sul1*), AY360321 (*sul2*) and AY494779 (*sul3*) did not reveal any sulfonamide resistance genes.

Isolate C361, identified with 99 % nucleotide similarity to the 16S rDNA sequence of *Arthrobacter arilaitensis*, was resistant to five antibiotics including streptomycin, chloramphenicol, tetracycline, neomycin and nalidixic acid, as well as a low SCP MIC value of 5 mg l^{-1}.

Class 1 and class 2 integron carriage. Screening the *sul* positive isolates (n=358) revealed that 5.0 % (n=18) carried class 1 integrons and 11.7 % (n=42) class 2 integrons (positive for *intI1* and *intI2* genes respectively). Of 173 *sul* negative isolates 8.7 % (n=15) carried *intI1* and 5.2 % (n=9) carried *intI2* genes. There was no significant difference in *intI1* prevalence between *sul* positive and *sul* negative isolates (Chi-square 0.5, P = 0.5), whereas *intI2* prevalence was significantly higher in *sul* positive isolates (Chi-square 57.6, P < 0.0001). Given the association of *sul1* with class 1 integrons, and the fact that there was no known link between class 2 integrons and *sul* gene carriage, the observed prevalence of *intI1* and *intI2* in *sul* positive and negative populations was surprising. Only 8.1 % (n=11) of *sul1* positive isolates carried the *intI1* gene, whereas 9.4 % (n=12) were observed to carry the *intI2* gene (data not shown). Interestingly, no isolates were positive for both *sul3* and *intI1* (Table 3). Of the *sul2* isolates, 17.9 % (n=18) and 1.1 % (n=2) carried an *intI2* or *intI1* gene respectively (data not shown). A low frequency of *sul1* positive isolates contained *intI1* despite *sul1* having only been found adjacent to *qacEΔ1* in the 3' conserved region of class 1 integrons (3). This indicates that *sul1* is likely to be situated on non class 1 integron mobile elements in most *sul1* positive isolates

identified in this study. Only one other published investigation has reported the prevalence of class 1 integrons in sulfonamide resistant isolates from the environment, but involved a brief temporal study in a different soil type (14).

Isolates encoding the three known *sul* genes. Twelve isolates positive for the 3 *sul* genes were identified by 16S rDNA typing as members of the genera *Psychrobacter*, *Acinetobacter* and *Bacillus* (Table 4). Of these, 10 isolates were cultured from agricultural soils which had undergone long-term application of slurry from tylosin-fed pigs. Two isolates, *Acinetobacter lwoffi* (C15) and *Psychrobacter ikaite* (C20) were recovered from the antibiotic amended slurry. The 12 isolates were negative for *intI1*, but one, *Psychrobacter ikaite* (C713), contained *intI2*. The 12 isolates displayed multiple antibiotic resistance phenotypes to between three and eight antibiotics; including nalidixic acid, tetracycline, trimethoprim, and neomycin (Table 4). MIC tests indicated that the presence of the three sulfonamide resistance genes conferred only low resistance of between 5-8 mg l^{-1} SCP.

Conjugal transfers. Conjugal transfers were performed with 11 of the 12 strains that contained simultaneously; *sul1*, *sul2* and *sul3*, and *P. putida* or *E. coli* recipients (isolate C36 failed to grow). The transfer rates of these three genes are shown in Table 5. It was observed that in most isolates, *sul1* and *sul2* were transferred at different rates indicating their presence on different mobile elements. The exception, to *sul1* and *sul2* separate transfers, was an *Acinetobacter* sp. (C141) from which both genes transferred at a frequency of 3.44×10^{-3} transconjugants per donor cell into *P. putida* recipients, an equal transfer rate suggesting that the two genes are physically linked. *Sul3* was not observed to transfer into any of the recipients used in this study. In all cases where genes transferred into *P. putida*, transfer also took place into *E. coli* but at a lower frequency. Absence of transfer in a number of isolates may have been due to the carriage of *sul* genes on non-conjugative plasmids or on the chromosome, whereas

in the *Bacillus* sp. (C328), failure may have been due to the presence of a Gram positive specific mobile element.

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FIGURE/TABLE LEGENDS:

Fig 1. SCP RQ values for soil and slurry samples collected over year 1; 10 μg ml^{-1}**,** horizontal lines; 25 μg ml^{-1}, diagonal lines; 50 μg ml^{-1}, stippled bars.

Figure 2. Prevalence of *sul* gene bearing bacteria, isolated on TY (diamonds), SCP (solid), OTC (horizontal lines) and all selective plates (stippled). TY (11 isolates at each time point), SCP (10) and OTC (8).

Table 1: Primer sequences used for PCR amplification and sequencing

Table 2: Summary of total numbers of isolates collected per sample, in total over the study and numbers positive for each *sul* genotype.

Table 3. Summary of prevalence and total number of *sul* positive bacterial species isolated, their *sul* genotype and sample from which they were collected

Table 4: Characterisation of a number of cultured bacterial isolates encoding different *sul* genotypes, their 16S rDNA identification, source, multiple antibiotic resistance phenotype profile, SCP MIC (mg l^{-1}) and presence of the integrase genes *intI1* or *intI2*. PS: pig slurry; 1,P: soil cores from year 1 before slurry application, 1,1: soil cores from year 1, day 1 time point; 1,289: year 1, day 289; 2,1: soil cores from year 2, day 1 time point, SL: soil leachate.

Table 5: Conjugal transfer rates of the *sul1*, *sul2* and *sul3* genes from the 12 bacterial host isolates carrying the three known *sul* genes into either *E. coli* K-12 CV601 or *P. putida* UWC1 recipient.

Figure 1

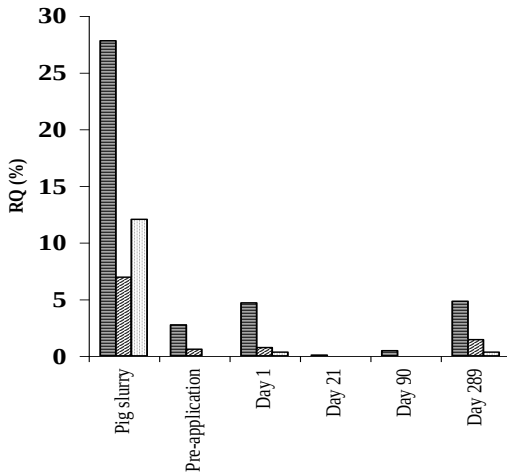
RQ (%)
30
25
20
15
10
5
0
Pig slurry
Pre-application
Day 1
Day 21
Day 90
Day 289

Figure 2.

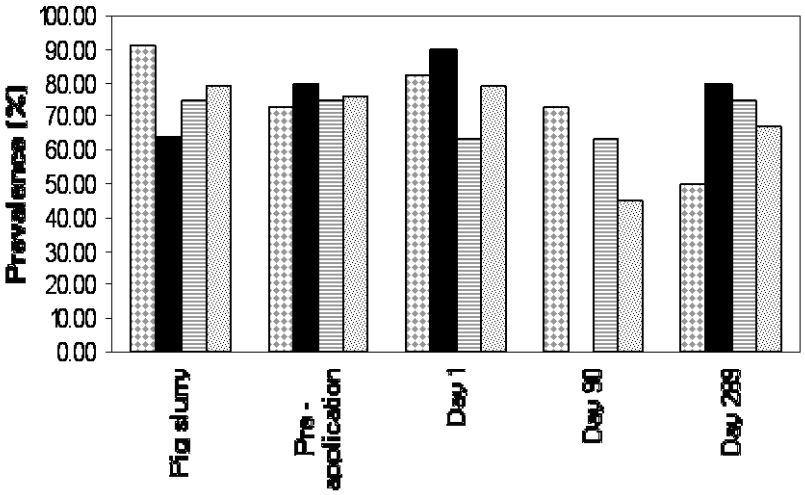
Prevalence (%)
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Pig slurry
Pre - application
Day 1
Day 90
Day 289

Table 1:

Gene	Primer	Sequence (5' to 3')	Annealing temp (ºC)	Reference
16S rDNA (8-1522 bp)	pA pH	AGA GTT TGA TCC TGG CTC AG AAG GAG GTG ATC CAG CCG CA	62	(12)
sul1	sul1bF sul1bR	CTT CGA TGA GAG CCG GCG GC GCA AGG CGG AAA CCC GCG CC	63	(29)
sul2	sul2F sul2R	TCG TCA ACA TAA CCT CGG ACA G GTT GCG TTT GAT ACC GGC AC	60	V.Enne
sul3	sul3F sul3R	GAG CAA GAT TTT TGG AAT CG CAT CTG CAG CTA ACC TAG GGC TTT GGA	51	(18)
intI1	intA intB	ACA GGG CAA GCT TAG TAA AGC C CTC GCT AGA ACT TTT GGA AA	67	(22)
intI2	int2F int2R	CAC GGA TAT GCG ACA AAA AGG T GTA GCA AAC GAG TGA CGA AAT G	58.5	(32)
qacE	KazamF1 KazamR1	GGGAATTCGCCCTACACAACAAATTGGGAGA TACTCGAGTTAGTGGGCACTTGCTTTGG	50	(15)
qacEΔ1	KazamF2 KazamR2	GGGAATTCGCCCTACACAACAAATTGGGAGA GCTGCAGCTGCGGTACCACTGCCACAA	60	(15)

Table 2

Sample site	Number of isolates with *sul* genotypes								Number of isolates analysed from each sample
	***sul* +ve**	***sul1***	***sul2***	***sul3***	***sul1* +*sul2***	***sul1* +*sul3***	***sul2* +*sul3***	***sul1* +*sul2* +*sul3***	
PS (amended)	52	7	18	10	4	0	11	2	**78**
PS (control)	4	1	1	2	0	0	0	0	**18**
Pre-app.(soil)	53	21	13	7	6	0	0	6	**79**
1,1 (soil)	53	6	14	12	7	1	12	1	**71**
1,21 (soil)	9	6	1	0	2	0	0	0	**20**
1,90 (soil)	15	13	0	0	1	0	1	0	**39**
1, 289 (soil)	23	6	8	6	3	0	0	0	**41**
2,1 (soil)	37	6	21	3	3	0	4	0	**45**
2,21 (soil)	35	13	7	6	3	1	3	2	**39**
2,90 (soil)	36	17	4	1	6	0	7	1	**41**
2,240 (soil)	19	16	1	1	0	0	1	0	**29**
Soil leachate	22	8	7	2	1	2	2	0	**31**
Total	**358**	**120**	**95**	**50**	**36**	**4**	**41**	**12**	**531**

Where sample sites were; PS (amended): pig slurry amended with 25.58 mg l^{-1} SCP and 18.85 mg l^{-1} OTC; PS (control): unamended pig slurry; Pre-app (soil): soil cores from year 1 before slurry application, 1,1 (soil): soil cores from year 1, day 1 time point; 1,289 (soil): year 1, day 289; 2,1 (soil): soil cores from year 2, day 1 time point, Soil leachate: three combined samples collected over the sample period from large rainfall events.

Table 3

Genus as identified by 16S DNA	Numbers (percentage) of *sul* positive isolates	Sample isolated from	*sul* genotype	Percentage (numbers) of *sul* positive isolates
Acinetobacter	127 (35.7)	PS,PSC,all soil samples,SL	*sul1* *sul2* *sul3* *sul1+sul2* *sul1+sul3* *sul2+sul3* *sul1+sul2+sul3*	20 30 31 13 3 21 7
Aerococcus	10 (2.8)	PS,1.1	*sul1* *sul2*	2 8
Agrobacterium	2 (0.6)	2.90	*sul2+sul3*	2
Arthrobacter	16 (4.5)	PSC,PS, 1P, 1.90, 1.289, 2.21	*sul1* *sul2* *sul3* *sul1+sul2*	6 4 1 5
Bacillus	29 (8.2)	PSC,1P, 1.289, 2.21, 2.240	*sul1* *sul2* *sul3* *sul1+sul2* *sul2+sul3* *sul1+sul2+sul3*	7 8 9 1 3 1
Brevibacterium	1 (0.3)	PS, PSC	*sul2+sul3*	1
Carnobacterium	3 (0.8)	1,289	*sul1*	3
Comamonas	3 (0.8)	2.1	*sul2*	3
Corynebacterium	3 (0.8)	2.1	*sul2*	3
Enterococcus	10 (2.8)	PS, PSC	*sul2* *sul3* *sul2+sul3*	4 3 3
Planococcus	1 (0.3)	PSC	*sul1+sul2*	1
Providencia	3 (0.8)	2.1	*sul3*	3
Pseudomonas	88 (24.7)	PS, all soil samples, 1P, 1.1, 1.21, 2.90	*sul1* *sul2* *sul3* *sul1+sul2* *sul2+sul3*	64 12 3 5 4
Psychrobacter	51 (14.3)	PS, 1P, 1.1	*sul1* *sul2* *sul1+sul2* *sul1+sul3* *sul2+sul3* *sul1+sul2+sul3*	13 18 10 1 5 4
Shigella	3 (0.8)	SL	*sul2*	3
Stenotrophomonas	5 (1.4)	2.21, 2.90	*sul1* *sul2* *sul3* *sul2+sul3*	1 1 1 2
Weisella	3 (0.8)	PSC	*sul1+sul2*	3

Where sample sites were; PS: pig slurry amended with 25.58 mg l^{-1} SCP and 18.85 mg l^{-1} OTC; PSC: unamended pig slurry; 1P: soil cores from year 1 before slurry application, 1.1 : soil cores from year 1, day 1 time point; 1.289: year 1, day 289; 2.1: soil cores from year 2, day 1 time point; SL: soil leachate, three combined samples collected over the sample period from large rainfall events.

Table 4

Isolate	Bacterial source	16S rDNA identification	% BLAST[a] similarity	*sul* gene	Class of integron[b]	Antibiotic resistance phenotype of isolates[c]	SCP MIC (mg l^{-1})
C237	1,Preapp (soil)	*Bacillus sphaericus*	98	*1*	2	Sm^R Tmp^R Nal^R	1
C422	2,21 (soil)	*Stenotrophomonas maltophilia*	98	*1*	2	Sm^R	1
C131	2,240 (soil)	*Pseudomonas fluorescens*	97	*1*	-	Tet^R	32
C3	pig slurry	*Acinetobacter lwoffi*	99	*1*	2	NG	1
PGS22	pig slurry	*Aerococcus viridians*	99	*2*	1*	Sm^R Cm^R Tet^R Nm^R Nal^R	8
C506	soil leachate	*Shigella flexneri*	99	*2*	1*	Sm^R Cm^R Tet^R Tmp^R Nm^R Nal^R Km^R Amp^R	8
C701	1,Preapp (soil)	*Pseudomonas lini*	99	*2*	2	Sm^R Amp^R Tmp^R Nal^R	1
C439	2,90 (soil)	*Acinetobacter sp. N2*	97	*2*	2	Sm^R Tet^R Tmp^R Nal^R	16
C5	pig slurry	*Pseudomonas borealis*	99	*3*	2	Cm^R Tet^R	32
PGS48	pig slurry	*Enterococcus hirae*	97	*3*	-	Sm^R Tet^R	4
C167	2,1 (soil)	*Providencia stuart2*	97	*3*	-	Sm^R Tet^R	6
C2	pig slurry	*Acinetobacter lwoffi*	97	*3*	-	Sm^R Cm^R Tet^R Tmp^R Nal^R	5
C231	1,21 (soil)	*Pseudomonas putida*	97	*1+2*	1*	Amp^R Cm^R Tet^R Nm^R Nal^R	8
C361	1,289 (soil)	*Arthrobacter arilaitensis*	99	*1+2*	1*	Sm^R Cm^R Tet^R Nm^R Nal^R	5
PGS49	pig slurry	*Acinetobacter sp. An9*	99	*1+2*	2	Sm^R Nal^R	16
C410	1,1 (soil)	*Psychrobacter ikaite*	98	*1+2*	2	Tet^R	16
PGS47	pig slurry	*Acinetobacter lwoffi*	97	*2+3*	2	Sm^R Nal^R	16
PGS61	pig slurry	*Enterococcus sulfureus*	97	*2+3*	-	Sm^R Tet^R	4
PGS21	pig slurry	*Aerococcus viridans*	99	*2+3*	2	Cm^R Tet^R	32
C15	pig slurry	*Acinetobacter lwoffi*	99	*1+2+3*	2	Sm^R Cm^R Tet^R Tmp^R Nm^R Nal^R	5
C20	pig slurry	*Psychrobacter ikaite*	98	*1+2+3*	-	Sm^R Cm^R Tet^R Tmp^R Nal^R	5
C711	1, Preapp (soil)	*Psychrobacter sp. DY9-2*	97	*1+2+3*	-	Sm^R Tet^R Tmp^R Nal^R	8
C712	1, Preapp (soil)	*Psychrobacter frigidicola*	96	*1+2+3*	-	Sm^R Tet^R Tmp^R Nal^R	8
C713	1, Preapp (soil)	*Psychrobacter ikaite*	97	*1+2+3*	2	Sm^R Cm^R Tet^R Tmp^R Nal^R	8
C35	1, Preapp (soil)	*Acinetobacter calcoaceticus*	97	*1+2+3*	-	Sm^R Cm^R Tet^R Tmp^R Nal^R	8
C36	1, Preapp (soil)	*Acinetobacter calcoaceticus*	97	*1+2+3*	-	Sm^R Cm^R Tet^R Tmp^R Nal^R	8
C37	1, Preapp (soil)	*Acinetobacter lwoffi*	97	*1+2+3*	-	Sm^R Cm^R Tet^R Tmp^R Nal^R	8
C44	1,1 (soil)	*Acinetobacter rhizosphaerae*	98	*1+2+3*	-	Sm^R Cm^R Tet^R Tmp^R Nm^R Nal^R	5
C141	2,21 (soil)	*Acinetobacter lwoffi*	98	*1+2+3*	-	Sm^R Km^R Cm^R Tet^R Tmp^R Nm^R Nal^R Amp^R	6
C328	2,21 (soil)	*Bacillus psychrodurans*	98	*1+2+3*	-	Sm^R Km^R Cm^R Tet^R Nm^R Nal^R	8
C442	2,90 (soil)	*Acinetobacter baumanni*	99	*1+2+3*	-	Cm^R Tet^R Nal^R	8

[a] % nucleotide similarity of approximately 800 bp 16S rDNA sequence to bacterial strains submitted to databases and searched using the BLAST programme (1). [b] *qacEΔ1/qacE* screened for in all *intI1* positive isolates. * *qacEΔ1* detected. [c]Antibiotic resistance breakpoints: SmR: resistance to streptomycin 16 µg ml^{-1}, Amp^R: ampicillin 16 µg ml^{-1}, Km^R: kanamycin 16 µg ml^{-1}, Cm^R: chloramphenicol 16 µg ml^{-1}, Tet^R: tetracycline 8 µg ml^{-1}, Tmp^R: trimethoprim 16 µg ml^{-1}, Nm^R: neomycin 8 µg ml^{-1}, Nal^R: nalidixic acid 16 µg ml^{-1}. NG, no growth. Soil core sample; first number = year, second number = day. Preapp= pre-application.

Table 5

Donor isolate[a]	Transfer rates of *sul* genes for recipients *E. coli* and *P. putida* (Number of transconjugants per donor)					
	sul1		***sul2***		***sul3***	
	E. coli	***P. putida***	***E.coli***	***P. putida***	***E. coli***	***P. putida***
P. sp. DY9-2 (C711)	$4.3x10^{-4}$	$7.3x10^{-3}$	ND	ND	ND	ND
P. frigidicola (C712)	ND	ND	ND	ND	ND	ND
P. Ikaite[b] (C713)	$6.5x10^{-5}$	$1.9x10^{-3}$	ND	ND	ND	ND
A. lwoffi (C15)	$2.6x10^{-4}$	$7.5x10^{-3}$	$7.2x10^{-5}$	$2.5x10^{-3}$	ND	ND
P. ikaite (C20)	ND	ND	ND	ND	ND	ND
A. calcoaceticus (C35)	$9.5x10^{-4}$	$1.1x10^{-3}$	$7.5x10^{-4}$	$1.9x10^{-3}$	ND	ND
A. lwoffi (C37)	ND	ND	ND	ND	ND	ND
A. rhizosphaera (C44)	$9.0x10^{-4}$	$2.4x10^{-2}$	$2.5x10^{-4}$	$7.1x10^{-2}$	ND	ND
A. lwoffi (C141)	$4.2x10^{-4}$	$3.4x10^{-3}$	$4.2x10^{-4}$	$3.4x10^{-3}$	ND	ND
B. psychrodurans (C328)	ND	ND	ND	ND	ND	ND
A. baumanni (C442)	$3.5x10^{-4}$	$1.7x10^{-3}$	ND	ND	ND	ND

[a]isolate *A. calcoaceticus* (C36) failed to grow when tested for transfer.

[b] this isolate carried an *intI2* gene

ND; not detected.