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

4

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

6

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24 KEYWORDS: tylosin, sulfonamide resistance, horizontal gene transfer

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 *Enterobacteriaceae*, 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).

51 The first of the three known alternative DHPS genes, *sul1*, is usually found
52 located on the 3' conserved region of a class 1 integron (25) and is frequently
53 identified with this potentially mobile element in the slurry and soil environment (13,
54 22, 29). *Sul2* was first identified on RSF1010 in *Escherichia coli* and has been found
55 on small non-conjugative resistance plasmids (20). The *sul3* gene was identified
56 during a study into sulfonamide resistance in pathogenic *E. coli* isolates in swine from
57 Switzerland (18).

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

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

69 Here we report the prevalence of sulfonamide resistance genes in bacterial
70 isolates from agricultural clay soil, where long-term (2 years) application of slurry
71 from tylosin-fed pigs with experimental addition of sulfachloropyridazine (SCP) and
72 oxytetracycline (OTC) occurred. The study also aimed to investigate whether
73 sulfonamide resistance gene prevalence was due to dissemination of sulfonamide
74 resistance in bacteria from the farm environment (manure) to indigenous soil bacteria.
75

76 **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

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

106 **Bacterial isolation.** Bacterial colonies were randomly picked from non-selective and
107 selective plates containing different concentrations of SCP, OTC and TY and streaked
108 until pure cultures were obtained. The number of isolates obtained from each
109 antibiotic concentration at each time point varied due to differences in resistance of
110 the sampled population (no growth was observed at higher antibiotic concentrations in
111 some samples), and due to loss of viability of some glycerol stocks. To overcome the
112 variable numbers of isolates from each selective media at different time points,
113 comparison of *sul* gene prevalence was made from a sub set of the data. A
114 standardised number of isolates were randomly selected from each antibiotic selective
115 media, SCP, TY or OTC (11, 10 and 8 isolates respectively) for each of the following
116 samples: pig slurry, pre-application year 1, year 1 day, year 1 day 90 and year 1 day
117 289.

118 **DNA extractions.** DNA was extracted using a DNeasy kit (Qiagen, S.A.) according
119 to the manufacturer's instructions from isolates grown in 5 ml Iso-Sensitest broth
120 (Oxoid) o/n at 28 ºC. PCR was performed using 1u *Taq* DNA polymerase and the
121 manufactures buffer (Invitrogen, U.K.), with 4 mM $MgCl_2$. Thirty cycles were
122 performed of: 95 °C/1 min, varying annealing temperatures, according to Table 1 for
123 1 min and 72 °C/1 min. The PCR products were eluted from a 1 % agarose gel
124 (Helena Biosciences, U.K.) using a QIAquick Gel Extraction Kit (Qiagen). All *sul*
125 positive isolates were identified using 16S rDNA sequences of approximately 800 bp
126 in both directions. Sequencing reactions were performed with a terminator cycle
127 sequencing kit (Applied Biosystems, Foster City, CA) as described by the

128 manufacturer, and electrophoresis and readout were performed on an ABI Prism 3100
129 genetic analyzer (Applied Biosystems).
130 **Analysis of DNA sequences.** Resulting DNA sequences were edited using BioEdit
131 (Isis Pharmaceuticals, Inc.) and subsequently analyzed using the BLAST programme,
132 with a sequence similarity ≥ 97% used for species identification (1).
133 **Conjugal transfers.** *Pseudomonas putida* UWC1 (Rif^r) and *Escherichia coli* K-12
134 CV601 (Rif^r Thr^- Leu^- Thi^-) were used as recipients in conjugal transfers which were
135 performed according to Smalla *et al.* (27). The recipients had an MIC to SCP of 0.5
136 μg ml^{-1}. Transconjugants that had been involved in a transfer event were selected for
137 on 50 μg ml^{-1} rifampicin (Sigma) and 8 μg ml^{-1} SCP containing Iso-Sensitest agar
138 plates for 48 hrs at 30 °C. Colonies were screened by PCR to confirm *sul* gene
139 transfer and disregard the possibility of spontaneous mutations. Transfer rates
140 (transconjugants per donor) were calculated according to Binh *et al* (5), where the
141 transfer event equalled, the CFU ml^{-1} transconjugants divided by the CFU ml^{-1}
142 recipients, this rate was then normalised by the number of positive colonies screened
143 by PCR for the gene of interest. The limits on transfer frequencies were set by our
144 ability to detect a single transconjugant cell, but varied with experimental conditions
145 due to differences in initial recipient number.
146 Control plates of donors only were included to investigate rates of rifampicin
147 mutations, these plates were always negative.

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148 **MIC determination.** MICs and antibiotic resistance breakpoints were determined on
149 Iso-Sensitest agar plates using an agar dilution method (21), the inoculum (100 µl)
150 was adjusted to 0.4 OD_{600nm} for each isolate to ensure consistency in MIC
151 determination. The antibiotics (Sigma) tested were; streptomycin at a concentration of
152 16 μg ml^{-1}, ampicillin 16 μg ml^{-1}, kanamycin 16 μg ml^{-1}, chloramphenicol 16 μg ml^{-1},
153 tetracycline 8 μg ml^{-1}, trimethoprim 16 μg ml^{-1}, neomycin 8 μg ml^{-1}, nalidixic acid 16

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.

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

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

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

198 **Characterisation of isolates.** All *sul* positive isolates were identified by 16S rDNA
199 typing and the presence of class 1 and 2 integrons determined. Seventeen genera
200 including opportunistic pathogens and indigenous soil bacteria were identified as
201 shown in Table 3. The most prevalent *sul* positive species isolated in this study was
202 *Acinetobacter* spp., which were collected from all soil/slurry samples. *Acinetobacter*
203 sp. were reported to have developed resistance to a large number of antibiotic groups
204 including the sulfonamides, making them a serious problem in hospitals (6, 10, 31).
205 *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

232 isolation of *Psychrobacter* spp. was unusual, commonly isolated from cold marine
233 environments and sediments (7, 9, 24). BLAST analysis (1) of the newly sequenced
234 *Psychrobacter* genomes (www.jgi.doe.gov) using sequences; GenBank accession
235 numbers AM086633 (*sul1*), AY360321 (*sul2*) and AY494779 (*sul3*) did not reveal
236 any sulfonamide resistance genes.
237 Isolate C361, identified with 99 % nucleotide similarity to the 16S rDNA
238 sequence of *Arthrobacter arilaitensis*, was resistant to five antibiotics including
239 streptomycin, chloramphenicol, tetracycline, neomycin and nalidixic acid, as well as a
240 low SCP MIC value of 5 mg l^{-1}.
241 **Class 1 and class 2 integron carriage.** Screening the *sul* positive isolates (n=358)
242 revealed that 5.0 % (n=18) carried class 1 integrons and 11.7 % (n=42) class 2
243 integrons (positive for *intI1* and *intI2* genes respectively). Of 173 *sul* negative
244 isolates 8.7 % (n=15) carried *intI1* and 5.2 % (n=9) carried *intI2* genes. There was no
245 significant difference in *intI1* prevalence between *sul* positive and *sul* negative
246 isolates (Chi-square 0.5, P = 0.5), whereas *intI2* prevalence was significantly higher in
247 *sul* positive isolates (Chi-square 57.6, P < 0.0001). Given the association of *sul1* with
248 class 1 integrons, and the fact that there was no known link between class 2 integrons
249 and *sul* gene carriage, the observed prevalence of *intI1* and *intI2* in *sul* positive and
250 negative populations was surprising. Only 8.1 % (n=11) of *sul1* positive isolates
251 carried the *intI1* gene, whereas 9.4 % (n=12) were observed to carry the *intI2* gene
252 (data not shown). Interestingly, no isolates were positive for both *sul3* and *intI1*
253 (Table 3). Of the *sul2* isolates, 17.9 % (n=18) and 1.1 % (n=2) carried an *intI2* or
254 *intI1* gene respectively (data not shown). A low frequency of *sul1* positive isolates
255 contained *intI1* despite *sul1* having only been found adjacent to *qacEΔ1* in the 3'
256 conserved region of class 1 integrons (3). This indicates that *sul1* is likely to be
257 situated on non class 1 integron mobile elements in most *sul1* positive isolates

258 identified in this study. Only one other published investigation has reported the
259 prevalence of class 1 integrons in sulfonamide resistant isolates from the environment,
260 but involved a brief temporal study in a different soil type (14).
261 **Isolates encoding the three known *sul* genes.** Twelve isolates positive for the 3 *sul*
262 genes were identified by 16S rDNA typing as members of the genera *Psychrobacter*,
263 *Acinetobacter* and *Bacillus* (Table 4). Of these, 10 isolates were cultured from
264 agricultural soils which had undergone long-term application of slurry from tylosin-
265 fed pigs. Two isolates, *Acinetobacter lwoffi* (C15) and *Psychrobacter ikaite* (C20)
266 were recovered from the antibiotic amended slurry. The 12 isolates were negative for
267 *intI1*, but one, *Psychrobacter ikaite* (C713), contained *intI2*. The 12 isolates displayed
268 multiple antibiotic resistance phenotypes to between three and eight antibiotics;
269 including nalidixic acid, tetracycline, trimethoprim, and neomycin (Table 4). MIC
270 tests indicated that the presence of the three sulfonamide resistance genes conferred
271 only low resistance of between 5-8 mg l^{-1} SCP.
272 **Conjugal transfers.** Conjugal transfers were performed with 11 of the 12 strains that
273 contained simultaneously; *sul1*, *sul2* and *sul3*, and *P. putida* or *E. coli* recipients
274 (isolate C36 failed to grow). The transfer rates of these three genes are shown in Table
275 5. It was observed that in most isolates, *sul1* and *sul2* were transferred at different
276 rates indicating their presence on different mobile elements. The exception, to *sul1*
277 and *sul2* separate transfers, was an *Acinetobacter* sp. (C141) from which both genes
278 transferred at a frequency of 3.44 x 10^{-3} transconjugants per donor cell into *P. putida*
279 recipients, an equal transfer rate suggesting that the two genes are physically linked.
280 *Sul3* was not observed to transfer into any of the recipients used in this study. In all
281 cases where genes transferred into *P. putida*, transfer also took place into *E. coli* but at
282 a lower frequency. Absence of transfer in a number of isolates may have been due to
283 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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References:

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J Mol Biol **215:**403-10.
2. **Antunes, P., J. Machado, J. C. Sousa, and L. Peixe.** 2005. Dissemination of Sulfonamide Resistance Genes (*sul*1, *sul*2, and *sul*3) in Portuguese *Salmonella enterica* Strains and Relation with Integrons. Antimicrob Agents Chemother **49:**836-839.
3. **Bennett, P. M.** 1999. Integrons and gene cassettes: a genetic construction kit for bacteria. J Antimicrob Chemother **43:**1-4.
4. **Bento, F. M., F. A. Camargo, B. C. Okeke, and W. T. Frankenberger.** 2005. Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. Microbial Research **160**.
5. **Binh, C. T., H. Heuer, M. Kaupenjohann, and K. Smalla.** 2008. Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. FEMS Microbiol Ecol.
6. **Böerlin, P., S. Eugster, F. Gaschen, R. Straub, and P. Schawalder.** 2001. Transmission of opportunistic pathogens in a veterinary teaching hospital. Vet Microbiol **82:**347-359.
7. **Bowman, J. P., S. A. McCammon, M. V. Brown, D. S. Nichols, and T. A. McMeekin.** 1997. Diversity and association of psychrophilic bacteria in Antarctic sea ice. Appl Environ Microbiol **63:**3068-78.
8. **Boxall, A. B., P. Blackwell, R. Cavallo, P. Kay, and J. Tolls.** 2002. The sorption and transport of a sulphonamide antibiotic in soil systems. Toxicol Lett **131:**19-28.
9. **Cavanagh, J., J. J. Austin, and K. Sanderson.** 1996. Novel Psychrobacter species from Antarctic ornithogenic soils. Int J Syst Bacteriol **46:**841-8.

322 10. **Chastre, J.** 2003. Infections due to *Acinetobacter baumannii* in the ICU.
323 Semin Respir Crit Care Med **24:**69-78.
324 11. **De Liguoro, M., V. Cibin, F. Capolongo, B. Halling-Sørensen, and C.**
325 **Montesissa.** 2003. Use of oxytetracycline and tylosin in intensive calf
326 farming: evaluation of transfer to manure and soil. Chemosphere **52:**203-12.
327 12. **Edwards, U., T. Rogall, H. Blocker, M. Emde, and E. C. Böttger.** 1989.
328 Isolation and direct complete nucleotide determination of entire genes.
329 Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res
330 **17:**7843-53.
331 13. **Guerra, B., E. Junker, A. Schröeter, B. Malorny, S. Lehmann, and R.**
332 **Helmuth.** 2003. Phenotypic and genotypic characterization of antimicrobial
333 resistance in German *Escherichia coli* isolates from cattle, swine and poultry. J
334 Antimicrob Chemother **52:**489-92.
335 14. **Heuer, H., and K. Smalla.** 2007. Manure and sulfadiazine synergistically
336 increased bacterial antibiotic resistance in soil over at least two months.
337 Environmental Microbiology **9:**657.
338 15. **Kazama, H., H. Hamashima, M. Sasatsu, and T. Arai.** 1998. Distribution of
339 the antiseptic-resistance gene *qacE delta 1* in gram- positive bacteria. FEMS
340 Microbiol Lett **165:**295-9.
341 16. **Loke, M. L., J. Tjornelund, and B. Halling-Sorensen.** 2002. Determination
342 of the distribution coefficient (log Kd) of oxytetracycline, tylosin A,
343 olaquindox and metronidazole in manure. Chemosphere **48:**351-61.
344 17. **Messi, P., E. Guerrieri, and M. Bondi.** 2005. Antibiotic resistance and
345 antibacterial activity in heterotrophic bacteria of mineral water origin. Sci
346 Total Environ **346:**213-9.
347 18. **Perreten, V., and P. Böerlin.** 2003. A new sulfonamide resistance gene (*sul*3)
348 in *Escherichia coli* is widespread in the pig population of Switzerland.
349 Antimicrob Agents Chemother **47:**1169-72.
350 19. **Petersen, A., L. Guardabassi, A. Dalsgäard, and J. E. Olsen.** 2000. Class I
351 integrons containing a *dhfrI* trimethoprim resistance gene cassette in aquatic
352 *Acinetobacter* spp. FEMS Microbiol Lett **182:**73-6.
353 20. **Radström, P., and G. Swedberg.** 1988. RSF1010 and a conjugative plasmid
354 contain *sul*II, one of two known genes for plasmid-borne sulfonamide
355 resistance dihydropteroate synthase. Antimicrob Agents Chemother **32:**1684-
356 92.
357 21. **Reynolds, R., J. Shackcloth, D. Felmingham, and A. MacGowan.** 2003.
358 Antimicrobial susceptibility of lower respiratory tract pathogens in Great
359 Britain and Ireland 1999-2001 related to demographic and geographical
360 factors: the BSAC Respiratory Resistance Surveillance Programme. J
361 Antimicrob Chemother **52:**931-43.
362 22. **Rosser, S. J., and H. K. Young.** 1999. Identification and characterization of
363 class 1 integrons in bacteria from an aquatic environment. J Antimicrob
364 Chemother **44:**11-8.
365 23. **Sarma, P. M., D. Bhattacharya, S. Krishnan, and B. Lal.** 2005. Assessment
366 of intra-species diversity among strains of *Acinetobacter baumannii* isolated
367 from sites contaminated with petroleum hydrocarbons. Can J Microbiol **50**.
368 24. **Shivaji, S., G. S. Reddy, K. Suresh, P. Gupta, S. Chintalapati, P.**
369 **Schumann, E. Stackebrandt, and G. I. Matsumoto.** 2005. *Psychrobacter*
370 *vallis* sp. nov. and *Psychrobacter aquaticus* sp. nov., from Antarctica. Int J
371 Syst Evol Microbiol **55:**757-62.

25. **Sköld, O.** 1976. R-factor-mediated resistance to sulfonamides by a plasmid-borne, drug-resistant dihydropteroate synthase. Antimicrob Agents Chemother **9:**49-54.
26. **Sköld, O.** 2000. Sulfonamide resistance: mechanisms and trends. Drug Resist Updat **3:**155-160.
27. **Smalla, K., H. Heuer, A. Götz, D. Niemeyer, E. Krogerrecklenfort, and E. Tietze.** 2000. Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids. Appl Environ Microbiol **66:**4854-62.
28. **Stuer-Lauridsen, F., M. Birkved, L. P. Hansen, H. C. Lutzhoft, and B. Halling-Sorensen.** 2000. Environmental risk assessment of human pharmaceuticals in Denmark after normal therapeutic use. Chemosphere **40:**783-93.
29. **Sundström, L., P. Rädström, G. Swedberg, and O. Sköld.** 1988. Site-specific recombination promotes linkage between trimethoprim- and sulfonamide resistance genes. Sequence characterization of *dhfrV* and *sulI* and a recombination active locus of Tn21. Mol Gen Genet **213:**191-201.
30. **Swedberg, G., C. Fermer, and O. Sköld.** 1993. Point mutations in the dihydropteroate synthase gene causing sulfonamide resistance. Adv Exp Med Biol **338:**555-8.
31. **Van Looveren, M., and H. Goossens.** 2004. Antimicrobial resistance of *Acinetobacter* spp. in Europe. Clin Microbiol Infect **10:**684-704.
32. **White, P. A., C. J. McIver, and W. D. Rawlinson.** 2001. Integrons and gene cassettes in the enterobacteriaceae. Antimicrob Agents Chemother **45:**2658-61.

399 **FIGURE/TABLE LEGENDS:**

400 **Fig 1**. SCP RQ values for soil and slurry samples collected over year 1; 10 μg ml^{-1}**,**

401 horizontal lines; 25 μg ml^{-1}, diagonal lines; 50 μg ml^{-1}, stippled bars.

402 **Figure 2**. Prevalence of *sul* gene bearing bacteria, isolated on TY (diamonds), SCP

403 (solid), OTC (horizontal lines) and all selective plates (stippled). TY (11 isolates at

404 each time point), SCP (10) and OTC (8).

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406 **Table 1:** Primer sequences used for PCR amplification and sequencing

407 **Table 2:** Summary of total numbers of isolates collected per sample, in total over the

408 study and numbers positive for each *sul* genotype.

409 **Table 3.** Summary of prevalence and total number of *sul* positive bacterial species

410 isolated, their *sul* genotype and sample from which they were collected

411 **Table 4:** Characterisation of a number of cultured bacterial isolates encoding different

412 *sul* genotypes, their 16S rDNA identification, source, multiple antibiotic resistance

413 phenotype profile, SCP MIC (mg l^{-1}) and presence of the integrase genes *intI1* or

414 *intI2*. PS: pig slurry; 1,P: soil cores from year 1 before slurry application, 1,1: soil

415 cores from year 1, day 1 time point; 1,289: year 1, day 289; 2,1: soil cores from year

416 2, day 1 time point, SL: soil leachate.

417 **Table 5:** Conjugal transfer rates of the *sul1*, *sul2* and *sul3* genes from the 12 bacterial

418 host isolates carrying the three known *sul* genes into either *E. coli* K-12 CV601 or *P.*

419 *putida* UWC1 recipient.

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425 **Figure 1**

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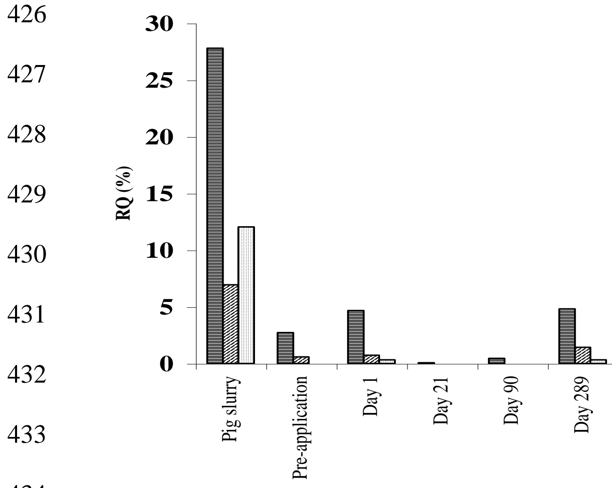

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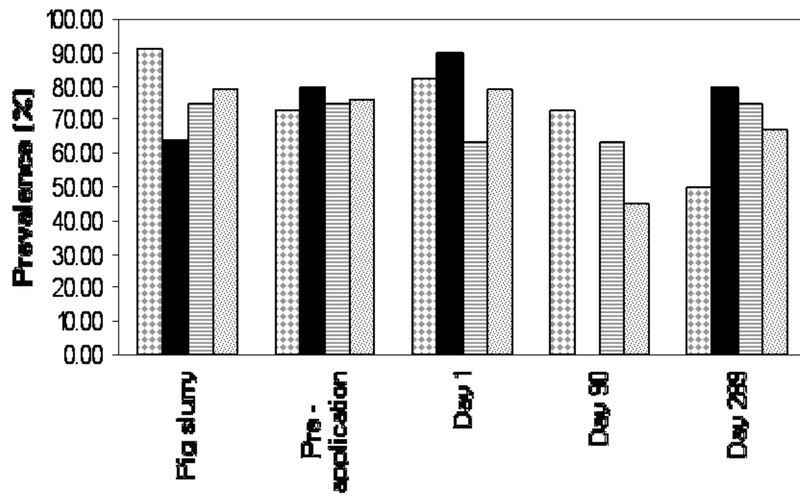
451 **Figure 2.**



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468 **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)

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482 **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**

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

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496 **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

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

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544 **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

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546 [a] % nucleotide similarity of approximately 800 bp 16S rDNA sequence to bacterial strains submitted to databases and searched using the BLAST programme (1). [b]
547 *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
548 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
549 acid 16 µg ml^{-1}. NG, no growth. Soil core sample; first number = year, second number = day. Preapp= pre-application.

550 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

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

552 [b] this isolate carried an *intI2* gene

553 ND; not detected.

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