Integron prevalence and diversity in manured soil

Running Title: *intI1* and *intI2* prevalence

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Abstract

Integron abundance and diversity were studied in soil amended with pig slurry. Real-time PCR illustrated a significant increase in class 1 integron prevalence post slurry-application with increased prevalence still evident at 10 months post-application. Culture dependent data revealed 10 genera, including putative human pathogens, carrying class 1 and 2 integrons.

Integrons are genetic elements that integrate or excise mobile cassette genes including those that confer resistance to a wide range of antibiotics (8). Class 1 and 2 integrons are associated with carriage of antibiotic resistance genes in clinically important bacteria and there is increasing evidence of environmental reservoirs of bacteria carrying these integron classes (9, 10, 18, 19). There is concern that the use of veterinary antibiotics selects for antibiotic resistant bacteria which, along with antibiotic residues, enter the wider environment via slurry application. The impact of slurry application on environmental reservoirs of antibiotic resistant bacteria is an important question. This study aimed to investigate the molecular prevalence of class 1 integrons and the diversity of class 1 and 2 integrons in bacteria isolated from pig slurry and from amended clay soils. The study site had a history of long-term application of slurry from tylosin (TY) fed pigs combined with experimental application of slurry containing sulfachloropyridazine (SCP) and oxytetracycline (OTC). Slurry from tylosin fed pigs (100g / ton of feed) was applied to soil annually before the start of the experiment; subsequently two annual experimental applications were also undertaken containing 18.85 mg /L and 2.58 mg /L or SCP and OTC respectively. The slurry was applied to the field at the same rate as normal agricultural practice (45,000 L / ha). Antibiotics were added to model sorption properties in soil and represented real-world concentrations found in pig slurry (4, 5). Slurry was stored

for up to 3 months in a holding tank, containing a mixture of new and older slurry. Soil samples were taken at time points over the two year experimental period (6).

Over 500 isolates from time points pre- and post-slurry application were screened by PCR for *intI1* and *intI2* (6), 14.7 % (n=78) were positive for *intI1* and / or *intI2*, 5.0 % (n=27) carried *intI1* only compared to 8.5 % (n=45) for *intI2* only, with 1.1 % (n=6) of isolates positive for both *intI1* and *intI2*. Integron prevalence in isolates was dependent on selective media used, with numbers of isolates carrying *intI1* being significantly higher under TY selection (9.9 %) compared to OTC (4.8 %), SCP (3.8 %) or no selection (NS) (3.6 %) and *intI2* under OTC selection (24.8 %) compared to TY (5.8 %), SCP (5.6 %) or NS (0.0 %) (chi-square test for comparisons of two proportions). This data suggests that TY and OTC may select or co-select for class 1 and 2 integrons respectively, with TY selection most likely to occur in the pig gut or in the slurry holding tank as it was undetectable in soil cores. Conversely OTC selection may have occurred in the slurry tank or the soil where it persists (12). There was no clear trend in integron prevalence in isolates at sample points after antibiotic amended slurry application in either year of the study (data not shown). Molecular prevalence of *intI1* was determined in soils in year 1 using SYBR Green real-time PCR on triplicate DNA extractions at each time point (UltraClean Soil DNA Kit). PCRs were performed on an Applied Biosystems 7500 Fast System, containing; 20 µl 2X Power SYBR Green PCR Master Mix (Applied Biosystems), 4 µl primer pairs, 0.4 µl Bovine Serum Albumin (10 mg ml^{-1}), 4 µl 1:10 diluted template DNA and 11.6 µl DNA free H_2O. Final concentration of primer pairs were: 0.9 µM for 16S (16), int1f2 (TCGTGCGTCGCCATACA) and int1r2 (GCTTGTTCTACGGCCGTTTGA). Standard curves for absolute quantification were produced from seeded soil inoculated with serial dilutions of *E. coli* SK4903 (IncPβ R751 carrying *intI1*, *qacE*) (17). Molecular prevalence was calculated by dividing target gene abundance by 16S

78 rRNA abundance and multiplying by 100. Corrections were made for 16S rRNA and
79 IncPβ R751 copy number (1, 21). Melting curves were checked for specificity of PCR
80 amplification and template dilution experiments were carried out to check for PCR
81 inhibition.

82 Molecular prevalence of *intI1* was significantly lower in pre-application
83 samples than at day 1, 21, 90 and 289 post-application using a chi-square test for
84 comparisons of two proportions ($p < 0.0001$) (Figure 1). 0.21% of bacteria carried
85 *intI1* in pig slurry spread onto the trial plots (unpublished data). In pre-application
86 soils *intI1* prevalence was 0.0002% which was similar to other unpolluted soils tested
87 (unpublished data), this increased dramatically to 0.01% immediately after
88 application, then decreased slightly to 0.008% at day 21 and then to approximately
89 0.003 and 0.004% at days 90 and 289 respectively. *IntI1* prevalence at later time
90 points were still significantly higher than in pre-application soils indicating that the
91 impact of slurry application on class 1 integron prevalence was still evident after
92 nearly 10 months. This concurs with evidence of *sul1* abundance in manured soils
93 measured using real-time PCR, where a manure effect on abundance was still evident
94 at 61 days post application (11). The discrepancy between pre-application soil (which
95 had a history of tylosin fed pig slurry addition) and post-application soil (slurry also
96 containing SCP and OTC) may be due to additional selective pressure exerted by SCP
97 and OTC. In the present study Enterobacteriaceae spp. carrying *intI1* was present in
98 soil leachate samples 164 days after slurry application, again suggesting that integron
99 positive bacteria, likely to have come from slurry, survived in soil for a considerable
100 length of time.

101 16S rRNA PCR and sequencing (6) revealed the *intI* positive isolates belonged
102 to 10 genera / families (Table 1). The largest number of integrase positive isolates
103 were *Pseudomonas* spp. which were the only integron positive genera present

throughout the year, including in pre-application samples. Gram positive *Bacillus* spp. and *Arthrobacter* spp. were also identified, carrying both *intI1* and *intI2* in pig slurry, soil leachate day 164 and at day 289 post application. *Arthrobacter* and *pseudomonas* spp. have previously been isolated from pigsties (2). *Aerococcus viridians* was only isolated from pig slurry, this species is a pathogen of pigs and humans (15). *Psychrobacter* spp. were isolated from pig slurry, pre-application soil, and day 1 post-application, members of this genus are also opportunistic pathogen of animals and humans. *Acinetobacter* spp., including *A. lwoffi* were repeatedly characterised carrying combinations of the two integrase genes in pig slurry and amended soil at day 1 and 21 but were not isolated at later time points or from pre-application cores; this species is an opportunistic human pathogen that is also found as a commensal in healthy individuals (13). Enterobacteriaceae spp. were isolated at year 1 day 1 and in soil leachate at day 164 (year 1) and *Enterococcus* spp. in soil leachate at day 164 only. The majority of integron bearing genera were isolated post-application, suggesting that they were introduced via slurry application, were already present in the soil and were selected for by antibiotics contained in applied slurry or resulted from HGT between introduced and indigenous bacteria after slurry application. This correlates with the 50 fold increase in *intI1* observed immediately after slurry application. It is clear that some integron positive genera, including *Acinetobacter* spp. were only isolated up to day 21 which correlated with a decrease in molecular prevalence of *intI1* after this time point.

Only six *intI1* positive isolates contained amplifiable variable regions (18), containing *aadA1* (streptomycin / spectinomycin resistance) (GenBank accession: FJ457611), including *Acinetobacter, Aerococcus, Pseudomonas,* Enterobacteriaceae spp and *Arthrobacter arilaitensis* which carried an *intI1Δ1* gene and a 3'-CS including *qacEΔ1* and *sulI* (Fig.2a) (7). The latter class 1 integron variable region had

99% similarity at the nucleotide level to a class 1 integron located on the pTet3 plasmid from *Corynebacterium glutamicum* (20). *aadA* genes were isolated from pig manure in a previous study demonstrating strong selective pressure for streptomycin/ spectinomycin resistance in pig farming (3). In conjugal transfer experiments, conducted as described by Byrne-Bailey *et al.*, (6), the *intI1* and *sul1* genes from *Arthrobacter arilaitensis* transferred into *E. coli* K-12 CV601 at a frequency of 3.71 x 10^{-3} (transconjugants per number of donor cells), and *P. putida* UWC1 at a frequency of 2.98 x 10^{-3} (transconjugants per number of donor cells) indicating the ability of the mobile genetic element bearing the integron to transfer from a Gram positive host into Gram negative recipients.

Variable regions between *intI2* and *orfX* were amplified in *intI2* positive isolates using primers described by White *et al.* (22) and four class 2 integron types were characterised, 10 failed to amplify, eight gave a 550 bp sequence encoding an *intI2* gene. The third type, which gave a 1560 bp product, found in six isolates was Tn*7* derived encoding *intI2*, a *sat1* gene cassette for streptothricin resistance, an *aadA1* gene cassette and *orfX* (Fig. 2b) (GenBank accession: FJ469574). The largest of the four class 2 integron types was found in 28 isolates, representing indigenous and introduced bacteria, including *Acinetobacter*, *Enterococcus*, *Pseudomonas*, *Psychrobacter* Enterobacteriaceae, *Stenotrophomonas*, *Streptomyces* spp. and uncultured bacterium EBSCPSA-6117, giving a 2300 bp sequence encoding a trimethoprim resistance gene (*dfrA1)*, streptothricin resistance (*sat1*), streptomycin resistance (*aadA1*) and *orfX* (Fig 2c, GenBank accession: FJ492781), an arrangement previously described from *E. coli* isolated from pig faeces (14).

Isolates were tested for resistance against eight antibiotics (6). Isolates bearing class 1 integrons demonstrated resistance to more antibiotics than those carrying class 2 integrons (4.4 as opposed to 3.3 respectively, P = 0.037 ANOVA). One of the

isolates resistant to all eight antibiotics, C506, identified as Enterobacteriaceae spp. was isolated from soil leachate 164 days post slurry application; demonstrating transport of antibiotic resistant bacteria of agricultural origin to water catchments.

This study demonstrates that pig slurry amended soil represents a reservoir of diverse bacterial species carrying class 1 and 2 integrons, with indigenous and introduced bacteria carrying the same integron types. Real-time PCR demonstrated a significant increase in class 1 integrase prevalence after slurry application, a significant effect was still observable at day 289 post-application. The risk of resistance gene transfer from the agricultural environment to the clinic is a matter of controversy. However, it is an accepted fact that farm animals and manure are a source of food and water borne human pathogens. It is clear that the same transfer routes will bring the human population into contact with commensal and pathogenic bacteria carrying antibiotic resistance genes that may be further disseminated within the human bacterial flora.

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FIGURE/TABLE LEGENDS:

Table 1: Summary of *intI* prevalence and sample identification for each species isolated. PS, pig slurry; P, pre application; SL, soil leachate; number denotes days after slurry application.

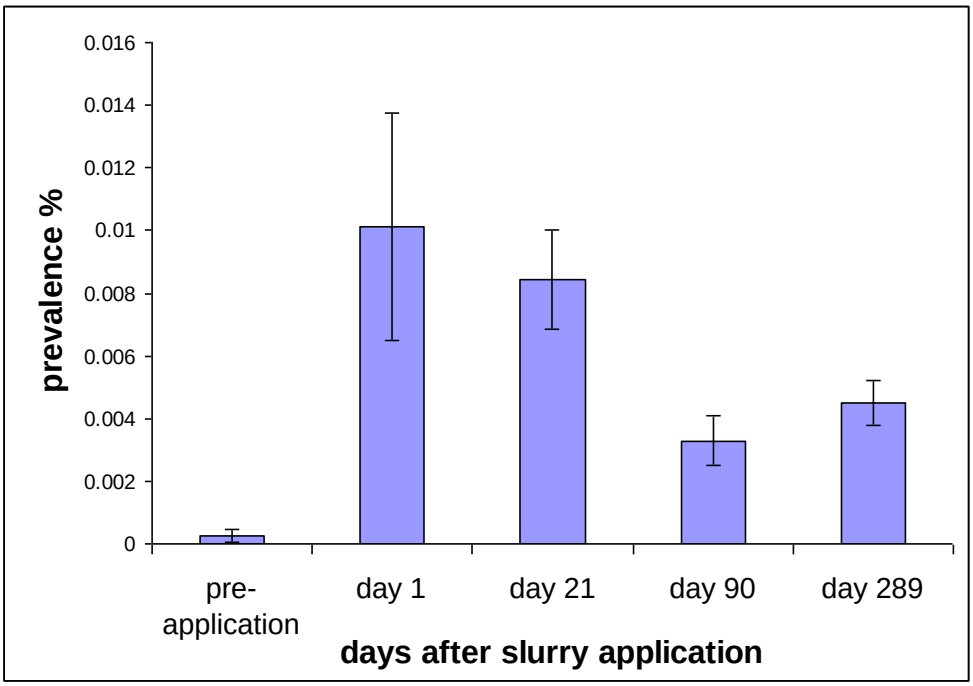
Figure 1. Molecular prevalence of *intI1* in soil amended with pig slurry. Error bars represent standard error of three replicate samples (4 in pre-application), prevalences are statistically different from one another at all time points (Chi-square, $p < 0.0001$).

Figure 2: (a) Schematic of the class 1 integron fragment sequenced from *Arthrobacter arilaitensis* (isolate C361). (b) Diagrammatic representation of class 2 integron PCR fragments, approximately 1500 bp in length, sequenced from a number of *intI2* positive isolates. (c) Class 2 structure as Fig. 2b, approximately 2500 bp in length but this integron structure had the additional insertion of a *dfrA1* gene cassette.

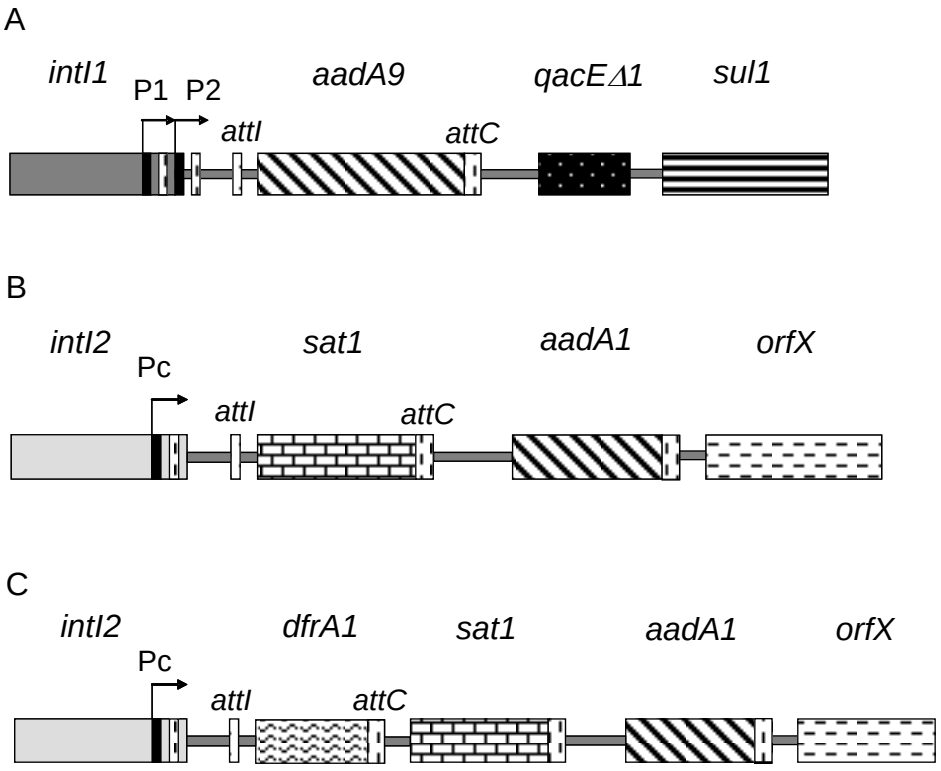
Table 1.

Genus as identified by 16S rRNA sequencing	Numbers (percentage) of *intI* positive isolates	*intI* genotype, number isolates and day isolated after slurry application		
Acinetobacter	21 (26.9))	*intI1*	(3)	(1, 21)
		intI2	(16)	(PS, 21)
Aerococcus	2 (2.6)	*intI1*	(1)	(PS)
		intI2	(1)	(PS)
Arthrobacter	2 (2.6)	*intI1*	(2)	(289)
Bacillus	7 (9.0)	*intI1*	(2)	(SL 164, 289)
		intI2	(5)	(PS, SL 164)
		intI1+intI2	(1)	(PS, SL 164)
Enterococcus	1 (1.3)	*intI1+intI2*	(1)	(SL 164)
Pseudomonas	34 (43.6)	*intI1*	(19)	(P, 1, 21, 90)
		intI2	(14)	(P, 1, 21, 90, 240)
		intI1+intI2	(2)	(21)
Psychrobacter	6 (7.7)	*intI2*	(5)	(PS, P, 1)
		intI1+intI2	(1)	(PS)
Enterobacteriaceae	2 (2.6)	*intI2*	(1)	(1)
		intI1+intI2	(1)	(SL 164)
Stenotrophomonas	1 (1.3)	*intI2*	(1)	(21)
Streptomyces	1 (1.3)	*intI2*	(1)	(1)
Unknown	1 (1.3)	*intI2*	(1)	(21)

Figure 1.


0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
prevalence %
pre-application
day 1
day 21
day 90
day 289
days after slurry application

321 **Figure 2.**



322