RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Tetracycline Concentrations and Circulation of *tet* Genes in Swine Feeding Operations and Adjacent Environments – **NPB #07-027**

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Industry Summary

Farm animal manure-associated, antibiotic (AB)-resistant microorganisms and genes encoding this resistance are viewed as important sources for the observed increase of AB resistance in humans and animals. The data on relationships between the application of AB in swine farms, and presence and migration of AB resistance genes into the environment are contradictory. A systematic study of their profiles and abundances was conducted in and around three swine farms with different strategy of AB usage.

Three swine farms, with known histories of AB applications, were surveyed in this study. Tetracycline resistance (TCR) genes were used as models. Their presence and concentrations in swine farms and the adjacent environments were evaluated by tracking 16 different groups of TCR genes in corresponding microbial communities. Throughout the year, periodic samples were taken of feces, food, manure lagoons, lagoon-derived irrigation water, irrigated and non-irrigated soils, and adjacent streams and ponds.

TCR genes were observed in all three farms including the AB-free "finisher" branch of one of them. Some of these genes likely originated from animal feed, but most of the TCR genes were selected by farm environments. The profiles of these genes were composed of "transient" and "persistent" TCR genes, and with exception of one gene were quite similar in all three farms. This exceptional gene was exclusively associated with AB usage by farrowing animals and piglets. No TCR genes were observed around the AB-free farm branch. The highest environmental occurrence of TCR genes was observed around the farm with the highest AB usage. Furthermore, at this farm, the concentrations of most persistent and environmentally mobile genes increased during the passage of manure through the system of lagoons. Observed environmental occurrence of these genes was related to spills from the lagoons and irrigation with manure.

Project findings demonstrated that not all AB resistance genes should be monitored in swine farms. The "transient" genes remain in animal feces or the directly receiving them lagoons, and do not pose a threat to the environment. Evidences of the AB usage-dependent proliferation of the "persistent" TCR genes and of their environmental occurrence in proximal soils and water bodies call for the improvement of current protocols for AB administration. To estimate the threat that these genes pose to humans, their longevity and mobility in soils and water bodies should be examined. On the other hand, the observation of a gradual increase in TCR gene concentrations in consequent lagoons calls for a comparative study of different manure-handling practices, such as separate lagoons for manure from AB-treated and untreated animals, and may call for new engineering solutions for manure treatment lagoon systems. Finally, more attention should be paid to the feed that is the source of some persistent AB resistance genes in farms. Potential action here may include the industry-wide quality control and on-site pre-treatment of animal feed.

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III. Scientific Abstract

Three farms varying in antibiotic (AB) usage from permanent to no usage were surveyed for the presence of sixteen tetracycline resistance (TCR) genes including *tet*(A), *A*(P), (B), (C), (D), (E), (G), (K), (L), (M), (O), (Q), (S), (T), (W), and (X) to evaluate the impact of AB usage in farms on the TCR gene profiles, TCR gene persistence, and their environmental occurrence. Animal feces and feed, manure treatment lagoons, irrigated and non-irrigated soils, ponds and creeks were sampled throughout 2007. PCR was used for detecting the above genes with plasmid-based positive controls as references and a ladder for the amplicon size control. TCR genes were observed in all three farms with no apparent difference in their profiles where *tet*(A), *A*(P), (M), (O), (Q), (W), and (X) dominated. No *tet*(K) and *tet*(T) were observed in any farm. *Tet*(B) was found related to chlorotetracycline usage and animal life phase, and was transient being detected either only in animal feces or in the feces and the lagoons directly accepting these feces, and disappearing below the detection limit in consequent lagoons. *Tet*(D) and (L) were also transient in the farms. *TetA*(P), (C), (D), (E), (G), (M), and (O) were detected in animal feed, but their profiles in the feed varied between the farms and even between the houses within the same farm.

Tet(A), (C), (E), (G),(M), (O), (Q), and (X) were persistent in the feces and lagoons, and were found around the farms. The identities of the corresponding genes detected in the lagoons and in adjacent environments were confirmed with sequence of gene-specific amplicons. Their numbers were determined with SYBR Green-based qPCR, normalized per gram or milliliter of the matrix, and to the numbers of the ”housekeeping” 16S rRNA genes. The recovery efficiencies of TCR genes from environmental matrices were determined, and the detection limits for these genes established. Their environmental occurrence around farms was found proportional to the degree of AB usage, and was most prominent in the constantly using AB farm. In the lagoon system of that farm, concentrations of the “persistent” *tet*(M), (O) and (X) genes increased 2-3 orders of magnitude during manure passage through the lagoons, though the concentrations of the “housekeeping” 16S rRNA genes remained stable and, in some cases, decreased. While individual concentrations of these genes varied between 10^5-10^9 /g(ml), their concentrations in the proximal to the farm soil and water bodies varied between 10^1-10^4 /g(ml) with *tet*(A) and (O) being the highest. No environmental occurrence of TCR genes was observed around the AB-free “finisher” branch farm.

It was concluded that (i) except for *tet*(B), there is no apparent correlation between AB usage and TCR gene profiles, (ii) the presence of *tet*(B) is associated with farrowing animals and piglets, and indicates antibiotic usage, (iii) TCR gene profiles are composed of “persistent” and “transient” genes, some of the former originate from animal feed, while the others are likely promoted by farm environment, (iv) persistent genes propagate in manure treatment lagoons, and their environmental occurrence and concentrations correlate to the frequency of AB usage at the farms, number of manure treatment lagoons, and the amount of precipitation in the region.

IV. Introduction

Microbial resistance to antibiotics (AB) is not new, but the number of resistant microorganisms is increasing. The geographic locations affected by drug resistance and the breadth of resistance in single organisms are unprecedented and mounting (1). Because over 84% of the AB produced in the United States are given to animals in animal agriculture (2), the agricultural use of AB is generally viewed as posing a major threat to public health (3). AB use in food animals is associated with substantial benefits. They are applied for therapeutic and prophylactic purposes and used to enhance growth rates and increase feed efficiency (4). Realistically, the current production of food animals cannot be sustained in the absence of AB. Its usage is

permitted (with regulations) even on organic farms (e.g. in the United Kingdom an organically grown animal can receive no more than three courses of treatment per year) (5). Various solutions have been proposed, tested, and implemented to solve the dilemma of AB usage and efficient food animal production including the restriction of human therapeutic AB for animal application, the separation of growth promoting from therapeutic AB, (4) and the replacement of AB with other antibacterial agents such as terpenoids (6).

The risk that agricultural use of antimicrobials poses to the development of resistance in human pathogens remains debatable (7). In particular, high abundance of ampicillin-resistant strains was observed in an organic beef farm (8). Similarly, observations were made in a human population where high prevalence of acquired AB resistance was found unrelated to heavy antimicrobial consumption (9).

In the meantime, a correlation between the spread of AB resistance genes from animal feeding operations and overuse of AB was reported (10). Prevalence of AB-resistant microorganisms in manure from a conventional farm over other microbial habitats was also demonstrated (11). Up to 100% of Enterobacteriaceae isolated from the manure of conventional poultry farms carried extended-spectrum β-lactamase in contrast to 6.6% of human feces isolates (12). There are numerous reports on natural horizontal transfer of AB resistance genes from animal manure-associated bacteria to human-associated and environmental microorganisms (13, 14).

The contribution of swine-feeding operations (SFO) to the migration of AB resistance genes in the environment is contradictory as is the data on relationships between application of AB in these operations, the presence of antibiotic-resistant microorganisms, and their discharge to the environment. In SFOs, the majority of AB are used in feed with their occasional usage through the water and through injections to sick pigs. The chlortetracycline/sulfamethazine/penicillin combination and tylosin are the most frequently used in feed AB in weaners and growers/finishers, respectively (15). Several studies have reported the appearance of AB residues and AB-resistant bacteria in surface and groundwater proximal to SFOs (16, 17). Eighty percent of all Enterobacteriaceae isolated from swine manure at conventional SFOs carried extended-spectrum β-lactamase (12). Ninety-eight percent of airborne bacteria isolated from a SFO (composed of the genera *Enterococcus*, *Staphylococcus*, and *Streptococcus*) exhibited high-level resistance to at least two AB commonly used at SFO,

mainly tetracycline (Tc), erythromycin, and clindamycin (18). The horizontal transfer of tetracycline resistance (TCR) genes from bacteria associated to pigsties and manured soil to *E. coli* and *P. putida* was also proposed based on filter-mating experiments (14).

On the other hand, *Megasphaera elsdenii* strains isolated from the feces of organically raised swine exhibited high levels of TCR and carried *tet*(O), *tet*(W), or *tet* gene mosaic *tet*(OWO) (19). In another study, the percentage of TCR *E. coli* strains isolated from low and high Tc usage SFOs was found to be identical (20). Furthermore, a high percent of vancomycin- and erythromycin-resistant enterococci was observed in a mid-Atlantic pig farm and its environment despite the fact that vancomycin has never been approved for use in livestock in the United States (21). A high population similarity index obtained for vancomycin- and erythromycin-resistant isolates from urban sewage and pig slurry suggests migration of vancomycin- and erythromycin-resistant microorganisms and genes from human-to-swine population rather than vise-versa.

The use of Tc as the key determinant to monitor resistance genes in and around SFOs is relevant due to common use of AB of tetracycline class in swine industry (15-18) and wide diversity among encoding TCR genes (22). There are more than 40 currently known TCR genes that encode ribosomal protection, efflux pump, or enzymatic modification mechanism. Even if TCR gene mosaic varies between and within human and animal populations, particular source-specific trends could be observed. For SFO manure, *tet*(O), *tet*(W), *tet*(Q), *tet*(M), *tetB*(P), *tet*(S), *tet*(T), *tet*(A), and *tet*(33) have been reported (14, 17, 19). *Tet*(O), *tet*(W), *tet*(Q) determinants have often been observed among environmental isolates (22), whereas *tet*(M) was reported to be predominately discharged from a swine-waste lagoon (17). There are indirect evidences that *tet*(M) stability in soil results from *en masse* transfer of carrying the gene transposon Tn*916* from swine-manure microorganisms to the indigenous soil microflora, and not from the survival of its original microbial carriers in soil (23, 24).

The presence of AB and heavy metals can support AB resistance genes in environmental microorganisms, especially when they are organized into gene cassettes and integrons. In the latter case, the presence of a single selective agent can support the entire integron. In soil, Tc was detected in the range of several hundred micrograms per kilogram some months after manure application (25). Tc concentrations observed in U.S.

streams are much lower and range between 0.05-0.69 micrograms per liter (26). No information on Tc content in animal food was found.

Available information on AB resistance in SFO and their surrounding environments is either derived from a single sampling event (17), or limited to a single group of microorganisms (12, 19, 20), and is contradictive and incomplete (12, 18-21). Based on this information, it is hard to make conclusions on the correlations between AB usage at SFOs and the presence and profiles of AB resistance genes, and their occurrence in proximal to farms soils and water bodies; therefore, on the contribution of SFOs to the spreading of AB resistance through the environment. There are several scenarios possible: (i) the manure in a SFO is a reservoir for antibiotic resistance genes selected and preserved due to a heavy antibiotic load causing a significant loading of antibiotic resistance genes to the environment, (ii) SFO manure is a reservoir for antibiotic resistance genes selected and preserved due to the heavy antibiotic load, but is NOT causing *en masse* transfer of antibiotic resistance genes; instead, some genes selectively migrate to the environment, (iii) there is no strong correlation between the antibiotics load to swine population and the abundance of antibiotic resistance genes in SFO, (iv) antibiotic resistance genes come to SFO *via* food and water, and SFO provide a suitable environment for their preservation and multiplication due to heavy antibiotic usage, or (v) trace concentrations of antibiotics contained in animal food and water *per se* are sufficient to preserve these genes in SFO, whereas similar antibiotic concentrations facilitate gene transfer and their support in the environment. The real picture most likely is composed of various combinations of these and other currently not foreseen scenarios, and that real picture needs to be carefully evaluated. This project evaluated the impact of AB usage in farms on the TCR gene profiles, TCR gene persistence, and their occurrence in proximal to farms environments.

V. Objectives

V.1. To evaluate and compare community-based *tet* gene profiles in three diversified upon antibiotic usage SFO and their adjacent environments.

V.2. To quantify *tet* genes commonly observed in SFO manure treatment lagoons, feed, and the environment.

V.3. To determine the profiles and concentrations of tetracycline antibiotics in SFO manure treatment lagoons, feed, and the environment.

VI. Materials and Methods

Three farms diversified upon the AB-usage strategy were surveyed in this study (Table 1). As seen in the Table, the farms varied in the AB-usage strategy, in numbers of manure treatment lagoons, their construction; but were similar in size and manure application technique and regimes. Wildlife was present at all three farms, and the third farm had grazing cattle on the pasture for 6 months as an additional potential vector for TCR genes. The first farm hosted nursery/starter and farrow/gestation animals. The nursery/starter pigs were treated with chlorotetracycline (CTC) for 7 days after delivery in the dosage recommended by the manufacturer. All the manure was collected into a single lagoon that was used for irrigation 5-6 times a year at the lowest for all the farms loading *per* square kilometer. There were two ponds within 130 and 500 m of the lagoon. No direct above-ground connection existed between the lagoon and these ponds.

The second farm hosted starter and finisher animals, and their manure was collected into separate lagoons. No antibiotics were directly used on this farm but because the starter pigs came to the farm pre-treated with CTC, only the finisher branch of this farm was considered as truly AB-free. The liquid from the starter lagoon and the finisher Lagoon 2 was used for irrigation 5- 6 times a year. There was a pond between the starter and the finisher Lagoon 2, and a creek within 200 m of both finisher lagoons. No direct above-ground connection existed between the lagoon, the adjacent pond and creek.

The third farm was the most complex and hosted animals at all the stages: nursery, farrow, and finisher. CTC and Denagard were used for nursery pigs within the first four weeks. Three times a year, the gestation sows isolated in the house connected to the Lagoon 3 were treated with the same antibiotics three times a week for a three-week period. The manure was collected into a system made up of seven lagoons, three of which received the manure only from isolated houses while the other four were sequential, and received manure from all the houses and the above three lagoons. Eventually, all the manure was collected into Lagoon 7, the final lagoon of the system which was used for irrigation 6 times a year. There were two ponds within 100-120 m of

Lagoon 4 and Lagoon 2, respectively; and a creek within 1150 m of the farm. No direct above-ground connection existed between the lagoons, ponds, and creek.

Table 1. Farms surveyed in the study (CTC = chlorotetracycline)

	Farm 1	Farm 2	Farm 3
Antibiotic Usage	CTC for 7 days upon arrival	None	CTC and Denagard® for 4 weeks upon arrival. Gestation sows receive 3 times a year
Total Swine Population	**3850**	**8000**	**5600**
Nursery/Starter	2000	2500	400
Farrow/Gestation	1850	0	2500
Finisher	0	5500	2700
Number of Lagoons	1	3	7
Lagoon Parameters	8000 m^2 ×2.5 m, clay lining	2000 m^2 × 5 m (average), clay lining	2400-14000 m^2 × 1.25 m, clay lining
Field Irrigation Source	Lagoon	Starter Lagoon Finisher Lagoon 2	Lagoon 7
Manure Application	Low pressure central pivot, down to the ground noses, 5-6 times/year, 6800 m^3/km^2 each (average)	Solid phase irrigation through sprinklers to the ground, 5-6 times/year, 24000 m^3/km^2 each (average).	Central pivot, 6 times/year, 28000 m^3/km^2 each (average).
Other Potential Vectors	Wildlife	Wildlife	Wildlife and cows (6 month/year)

Animal feces, lagoon slurries and sediments, irrigated and non-irrigated soils, ponds and creeks water and sediments, and animal feed (more than 70 points for each sampling event) were sampled in May, August-September, and December of 2007; and analyzed for sixteen TCR genes. The samples were stored in ice, transported to the P.I's. laboratory, and within 48 hours community DNA was isolated from them using soil and fecal DNA purification kits (Mo Bio, Solana Beach, CA). The DNA was stored at–20^{o}C until analyzed for TCR determinants by PCR using primers shown in Table 2. Twenty µl of PCR mixtures were prepared with Promega's PCR core system (Promega Corp., Madison, WI) and contained 0.25 µg of template DNA (determined by A260 using a NanoDrop spectrophotometer (NanoDrop®, Inc.), 200µm of each nucleotide mix, PCR reaction buffer w/1.5 mM $MgCl_2$, 50 nmol of each primer (synthesized by Sigma Genosys, The Woodlands, TX), and 1 unit of Taq DNA polymerase. The final volume was adjusted with nuclease-free water. The reactions were performed in a mini-cycler with hot top PTC 0150 (MJ Research Inc., Waltham, MA) using optimal PCR-cycling conditions. The PCR analyses were performed in triplicate, and products (amplicons) were analyzed for the expected size by agarose gel electrophoresis, stained in ethidium bromide, plasmid-based positive controls were used as references with a ladder for the amplicon size control. The gels were documented with Biochemi Bio Imaging system with Lab Works 4.6 software. The PCR products were eluted from gel, purified, and sent for sequencing to test the identity of SFO- and environmental *tet* genes of the same class.

Gene quantitation was performed with SYBR Green-based qPCR on the Mini Opticon System (BioRad) using the primers shown in Table 2. Their numbers were normalized per gram or milliliter of the matrix, and to the numbers of the "housekeeping" 16S rRNA genes. The recovery efficiencies of TCR genes from environmental matrices were determined, and the detection limits for these genes established.

Table 2. Primer sets used in this study.

Primer Pair	Sequence	Amplicon Size	Ref.
*tet*A	5'-GCTACATCCTGCTTGCCTTC-3' 5'-CATAGATCGCCGTGAAGAGG-3'	210	Ng(27)
*tet*A(P)	5'-CTTGGATTGCGGAAGAAGAG-3' 5'-ATATGCCCATTTAACCACGC-3'	676	Ng
*tet*B	5'-TTGGTTAGGGGCAAGTTTTG-3' 5'-GTAATGGGCCAATAACACCG-3'	659	Ng
*tet*C	5'-CTTGAGAGCCTTCAACCCAG-3' 5'-ATGGTCGTCATCTACCTGCC-3'	418	Ng
*tet*D	5'-AAACCATTACGGCATTCTGC-3' 5'-GACCGGATACACCATCCATC-3'	787	Ng
*tet*E	5'-AAACCACATCCTCCATACGC-3' 5'-AAATAGGCCACAACCGTCAG-3'	278	Ng
*tet*G	5'-GCTCGGTGGTATCTCTGCTC-3' 5'-AGCAACAGAATCGGGAACAC-3'	468	Ng
*tet*K	5'-TCGATAGGAACAGCAGTA-3' 5'-CAGCAGATCCTACTCCTT-3'	169	Ng
*tet*L	5'-TCGTTAGCGTGCTGTCATTC-3' 5'-GTATCCCACCAATGTAGCCG-3'	267	Ng
*tet*M	5'-GTGGACAAAGGTACAACGAG-3' 5'-CGGTAAAGTTCGTCACACAC-3'	406	Ng
*tet*O	5'-AACTTAGGCATTCTGGCTCAC-3' 5'-TCCCACTGTTCCATATCGTCA-3'	515	Ng
*tet*Q	5'-TTATACTTCCTCCGGCATCG-3' 5'-ATCGGTTCGAGAATGTCCAC-3'	904	Ng
*tet*S	5'-CATAGACAAGCCGTTGACC-3' 5'-ATGTTTTTGGAACGCCAGAG-3'	667	Ng
*tet*T	5'-AAGGTTTATTATATAAAAGTG-3' 5'-AGGTGTATCTATGATATTTAC-3'	169	Aminov(28)
*tet*W	5'-GAGAGCCTGCTATATGCCAGC-3' 5'-GGGCGTATCCACAATGTTAAC-3'	168	Aminov
*tet*X	5'-CAATAATTGGTGGTGGACCC-3' 5'-TTCTTACCTTGGACATCCCG-3'	468	Ng
16S rDNA	5'-C(C/T)AACT(T/C/A)CGTGCCAGCAGCC-3' 5'-GACGTC(A/G)TCCCC(A/C)CCTTCCTC-3'	688	Courtois(29)

Soil analysis for C and N contents was performed on the Flash Elemental Analyzer by UGA. The rainfall data were obtained from http://www.ncdc.noaa.gov/oa/land.html. For the first sampling (May 2007), the rainfall was calculated for three preceding months.

VII. Results

VII.1 Objective 1. To evaluate and compare community-based *tet* gene profiles in three diversified upon antibiotic usage SFO and their adjacent environments.

Thirteen *tet* genes were observed in Farm 1 (Table 3). Most permanently observed genes in the lagoon were *tet*(C), (M), (O), (Q), and (W). Most permanently observed genes in animal feces were *tet*(M), (O), (Q), and (W). Obviously, there was a consistency between the profiles of these most permanent genes, suggesting that most permanently observed TCR genes in lagoons originated from animal feces. Such genes were designated as "persistent".

On the other hand, there were two genes, *tet*(B) and *tet*(D) that were at least once observed in feces of CTC-treated animals but have never been detected in the lagoon or in the adjacent environments. These genes were designated as "transient". None of the "transient" genes was observed in the feed. Finally, some genes, such as *tet*(E), (K), and (T) have never been detected in any sampling; and, therefore were not associated with tetracycline resistance in this farm.

Tet(A), (G), and (Q) were detected not only in feces and the lagoon but in the water of either one of two ponds, one of which was located as far as 500 m from the farm. In addition to feces and the lagoon, *tet*(G) was discovered in the "nursery" feed along with *tet*A(P). None of the TCR genes was detected in the irrigated soil. All three environmentally-observed genes were consistently detected in the lagoon and in the animal feces but were not among the most persistent genes.

Table 3. Tetracycline resistance genes observed in Farm 1

Location	tetA	tetA(P)	tetB	tetC	tetD	tetE	tetG	tetK	tetL	tetM	tetO	tetQ	tetS	tetT	tetW	tetX
Lagoon Slurry	2,3	1,2		1,2,3			1,2			1,2,3	1,2,3	2,3	1		1,2,3	3
Lagoon Sediment	2	2		2					2	2	2	2			2	
Pond Water	2															
Pond Sediment																
Irrigated Soil																
Partially Irrigated Soil 25m																
Non-Irrigated Soil 500m																
Pond Water 500m							3					3				
Pond Sediment 500m																
Nursery Fecal		1,2	3	3			1,2		1,3	1,2,3	1,2,3	1,2,3	3		1,2,3	1,3
Farrow Fecal	1,3	1,2,3	1,3	3	1		1,2,3		1	1,2,3	1,2,3	1,2,3	1		1,2,3	1,3
Gestation Fecal		1,2	2	3	1		3		1,2,3	1,2,3	1,2,3	1,2,3	1,2,3		1,2,3	1,3
Nursery Feed*		3					3									
Farrow Feed*																
Gestation Feed*																
Lagoon Soil Spillway 10m *																
Lagoon Soil Spillway 15m *																

1: Observed in May sampling
2: Observed in Aug sampling
3: Observed in Dec sampling
*: Only one sampling (Dec 2007) was performed

High similarity in the amplicon sequences of *tet*(A), (G), and (Q) observed in Farm 1 and adjacent environments indicated close relationships between corresponding group of genes. It was concluded that environmentally observed genes likely originated from the farm.

Fourteen TCR genes were observed in three samplings in Farm 2 (Table 4). Most permanently observed TCR genes in the "starter" lagoon were *tet*(C), (Q), and (W), while in the feces they were *tet*(C), (Q), (W), and (X). Again, a high fidelity among the profiles of the feces-related and waste lagoon-related TCR genes was observed. Even *tet*(X) being not the most persistent TCR gene in the lagoon, was observed there in two of three samplings. None of these "persistent" genes was detected in the feed, but *tet*(M) and *tet*(O) frequently observed in the feces and in the "starter" lagoon were also found in the feed. In contrast, *tet*(E) detected in both the "starter" and "finisher" lagoons was also found in the feed of both houses but never in swine feces.

Similarly to Farm 1, *tet*(B) revealed its "transient" character being observed only in the "starter" feces of Farm 2; and another "transient" TCR gene of Farm 1, *tet*(D), was never observed in the "starter" branch of Farm 2. *Tet*(K) and *tet*(T) were not observed here either.

Tet(A) was the only gene detected in adjacent soil. After one week of soil irrigation with manure, *tet*(A) was simultaneously found in the irrigated soil and "starter" lagoon slurry. This gene was not detected in the "dead zone" of the sprinkler (3m around the stand), thus soil-borne *tet*(A) likely originated from the lagoon. This observation was confirmed by high similarity in the amplicon sequences of both the lagoon- and soil-borne genes.

Table 4. Tetracycline resistance genes observed in Farm 2

Location	*tet*A	*tet*A(P)	*tet*B	*tet*C	*tet*D	*tet*E	*tet*G	*tet*K	*tet*L	*tet*M	*tet*O	*tet*Q	*tet*S	*tet*T	*tet*W	*tet*X
Starter Lagoon Slurry	1,2	1		1,2,3		2,3	3		3	1,3	1,3	1,2,3	1		1,2,3	1,3
Starter Lagoon Sediment	2										2				2	
Starter Lagoon Creek Sediment																
Starter Lagoon Pond Sediment																
Starter Irrigated 3m Sprinkler																
Starter Irrigated 20m Sprinkler	1															
Finisher Lagoon 1 Slurry	1,2	1,2,3		1,2,3	2	2	3		3	1,2,3	1,2,3	3			1,2,3	1,2,3
Finisher Lagoon 1 Sediment	1			1,2,3			3			1,2,3	3		1		1,2,3	1,2,3
Finisher Lagoon 2 Slurry	1,2	1,2,3		1,2,3	2	2,3	3		3	1,2,3	1,2,3	1,3	1		1,2,3	1,2,3
Finisher Lagoon 2 Sediment										2						
Orchard Irrigated Soil 1wk																
Orchard Irrigated Soil 1wk 18m																
Orchard Irrigated Soil 3wks																
Pond Water																
Pond Sediment																
Non-Irrigated Soil Blackberry																
Finisher Fecal		1,2,3		3	2		3		1,2,3	1,2,3	1,2,3	1,3	1		1,2,3	1,2,3
Starter Fecal	3	2	3	1,2,3			3		2,3	2,3	1,2,3	2,3	1		1,2,3	1,2,3
Starter Lagoon Pond Water																
Starter Lagoon Creek Water																
Finisher Creek Sediment																
*Finisher Creek Water																
Starter Feed*						3				3	3					
Finisher Feed*					3	3										
Starter Lagoon Spillway Sedim																
Finisher Lagoon Spillway Sedim																

1: Observed in May sampling
2: Observed in Aug sampling
3: Observed in Dec sampling, *: Only one sampling (Dec. 2007) was performed.

Most permanently observed in the "finisher" lagoons TCR genes were *tet*A(P), (C), (M), (O), (W), and (X); hence their profile was substantially different from the "starter" lagoon. In "finisher" feces, *tet*A(P), (L), (M), (O), (W), and (X) were most permanently observed. Except that *tet*(L) replaced *tet*(C) in the latter, the profiles of "persistent" genes in the feces and lagoons are identical. No *tet*(B) was observed in the AB-free "finisher" branch. As in the "starter" branch of this Farm and the Farm 1, no *tet*(K) and *tet*(T) were detected. *Tet*(D) and *tet*(E) were found in the feed, with the latter detected in the lagoon but never in the feces. No TCR genes were observed in soils and waters around the "finisher" branch.

Thirteen TCR genes were observed in Farm 3 (Table 5a, 5b). This was the most complex farm composed of houses hosting animals at all stages, and the manure treatment system composed of seven lagoons, three of which received the manure only from isolated houses while the other four were sequential and received manure from all the houses and the above three lagoons. Due to this complexity, it was hard to determine the most persistent TCR genes there. Overall, *tet*A(P), (G), (M), (O), (W), and (X) were most common for all the lagoons; while feces revealed *tet*A(P), (G), (W), and (X) as most widely observed. The profiles of these genes varied between the lagoons and were not quite consistent with the profiles of incoming feces. In particular, constantly observed in all the lagoons *tet*(O) was randomly detected in the feces. *Tet*(B) again revealed its transient nature and its connection to CTC treatment. This gene was detected only in the feces of CTC-treated animals and directly received them Lagoon 3. One more gene, *tet*(L), was found as "transient" in Farm 3. Similarly to the "starter" branch of Farm 1, *tet*(D) was not observed in Farm 3.

Table 5a. Tetracycline resistance genes observed in the waste lagoons of Farm 3.

Location	*tet*A	*tet*A(P)	*tet*B	*tet*C	*tet*D	*tet*E	*tet*G	*tet*K	*tet*L	*tet*M	*tet*O	*tet*Q	*tet*S	*tet*T	*tet*W	*tet*X
Lagoon 1 Slurry	1,2	1,2,3		1,3		3	2,3			1,2,3	1,2,3	2,3	1,3		1,2	2,3
Lagoon 1 Sediment	3			3			3			2,3	3	3			2,3	3
Lagoon 2 Slurry	1,2,3	1,2		1,3			1,2,3		1	1,2,3	1,2,3	2,3	1		1,2,3	3
Lagoon 2 Sediment	1			1,3			1,3		1	1,3	3		1			3
Lagoon 3 Slurry	1,2,3		1	1,3			1,2,3		1	1,2,3	1,2,3	3	1		1,2,3	3
Lagoon 3 Sediment			3				2			1,2	2	2				
Lagoon 4 Slurry	1,2,3			1,3			1,2,3			1,2,3	1,2,3		1,3		1,2,3	1,2,3
Lagoon 4 Sediment	1,2,3			1,3			1,3			1,3	3		1		3	3
Lagoon 5 Slurry	1,2,3	1,2		1,3		3	1,2,3			1,2,3	1,2,3	2,3	1		1,2,3	1,2,3
Lagoon 5 Sediment	2						2			2	2	2				
Lagoon 6 Slurry	1,3			1,3		3	1,2,			1,2	1,2,3	2,3			1,2,3	3
Lagoon 6 Sediment							2			2	2	2				
Lagoon 7 Slurry	1	1		1,3			1,2,3			1,2,3	1,2,3	2,3	1		1,2,3	1,2,3
Lagoon 7 Sediment																

1: Observed in May sampling
2: Observed in Aug sampling
3: Observed in Dec sampling
*: Only one sampling was performed (Dec 2007)

Table 5b. Tetracycline resistance genes observed in and around Farm 3.

Location	*tet*A	*tet*A(P)	*tet*B	*tet*C	*tet*D	*tet*E	*tet*G	*tet*K	*tet*L	*tet*M	*tet*O	*tet*Q	*tet*S	*tet*T	*tet*W	*tet*X
Entry Pond Water	3						2									3
Entry Pond Sediment																
Finisher Pond Water																
Finisher Pond Sediment																
Creek Water 1.13 km											1					
Creek Sediment 1.13 km																
Irrigated Soil																
Non-Irrigated Soil																
Nursery Fecal to lagoon 1	2	2,3					1,2		2,3	2,3	1	2,3	1		1,2,3	1,2,3
Sow Fecal to lagoon 3		1,3	1				1,2,3		1,3	1,2	1	2,3	1		1,2,3	1,2,3
Lactation Fecal to lagoon 1	3	1,2							1,3	3	1	2	1		1,2,3	1,3
Finisher Fecal to lagoon 2	1,3	1,2,3	3	1		3	1,2		1,3	2,3	1	3	1		1,2,3	1,3
Nursery/Farrowing Fecal lag 4	2	2	3				2,3		3	2,3	2,3	2,3			2,3	3
Nursery Feed to lagoon 1*																
Sow Feed to lagoon 3*																
Lactation Feed to lagoon 1*																
Finisher Feed to lagoon 2*																
Nursery/Farrowing Feed to lag 4				3			3				3					
Spillway 1 by lagoon 2 sediment*																
Spillway 2 by lagoon 1 sediment*																
Spillway 2 by lagoon 1 water*											3					
Spillway 3 by lagoon 4 sediment*						3										
Spillway 3 by lagoon 4 water*						3	3			3	3					

1: Observed in May sampling
2: Observed in Aug sampling
3: Observed in Dec sampling
*: Only one sampling was performed (Dec 2007)

Tet(C), (G), and (O) were found in the nursery/farrowing feed. While *tet*(G) was consistently detected in feces and lagoons, *tet*(O) was constantly present in the lagoons but randomly detected in the feces, and *tet*(C) being also frequent in the lagoons was detected in feces just once.

Five TCR genes, *tet*(A), (E), (G), (M), (O), and (X) occurred in environmental matrices proximal to this Farm. While *tet*(A), (G), (M), and (X) likely originated from animal feces by the way of the lagoons, *tet*(O) and *tet*(E) may have different origins.

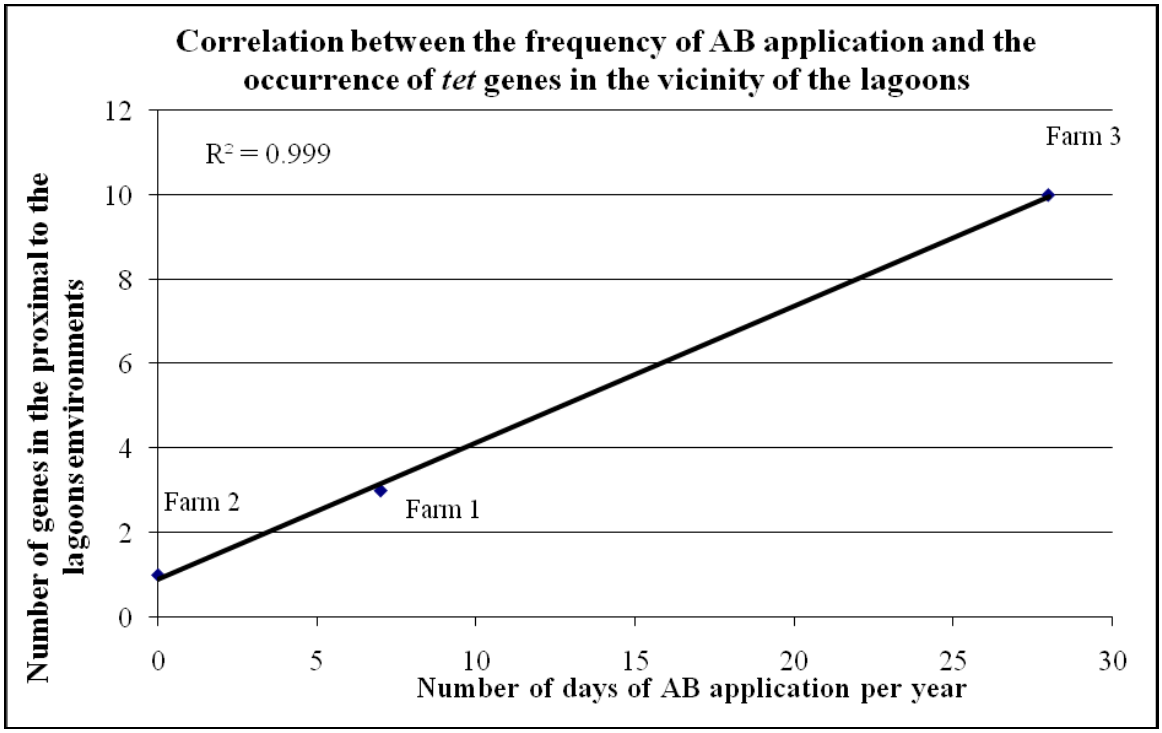

Fig. 1. AB application and environmental occurrence of TCR genes in the vicinity of farms.

We attempted to correlate the environmental occurrence of TCR genes around farms to various parameters, including frequency of AB usage, rainfall, number of lagoons, number of animals, and amount of manure used for irrigation at each farm. The observed correlations are presented in Fig. 1-5.

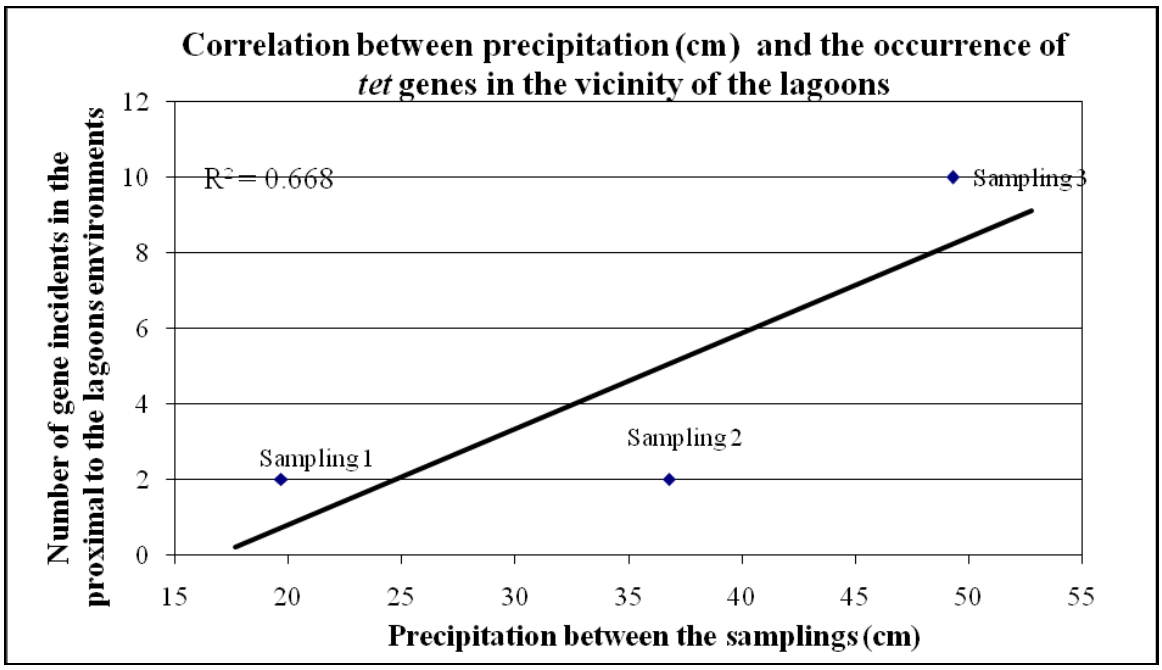

Fig. 2. Rainfall amount and environmental occurrence (all farms) of TCR genes in the vicinity of farms.

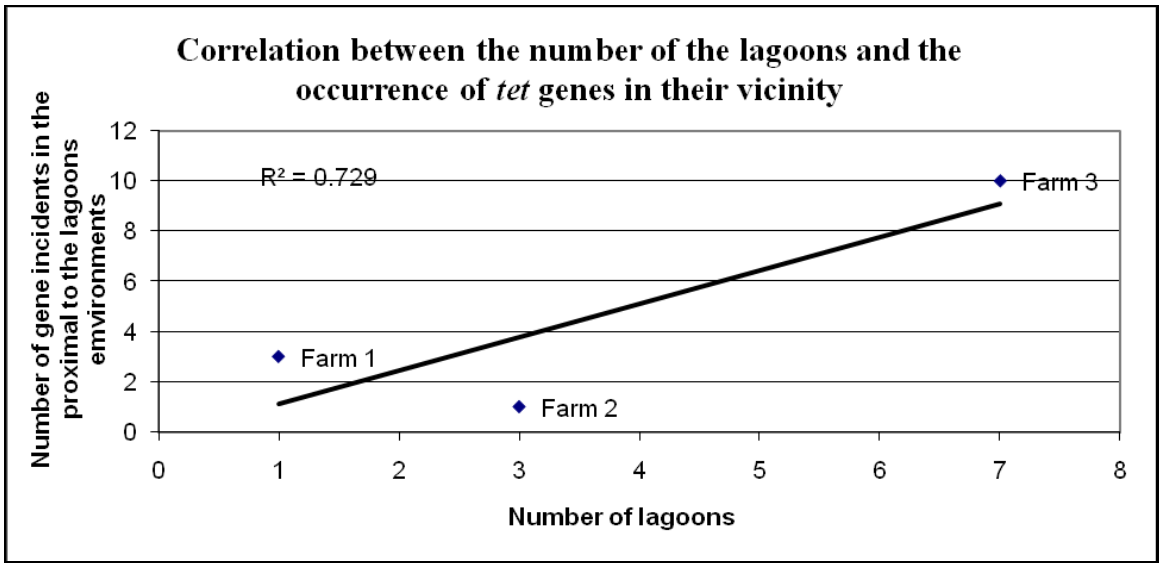

Fig. 3. Number of the lagoons and environmental occurrence of TCR genes in the vicinity of farms

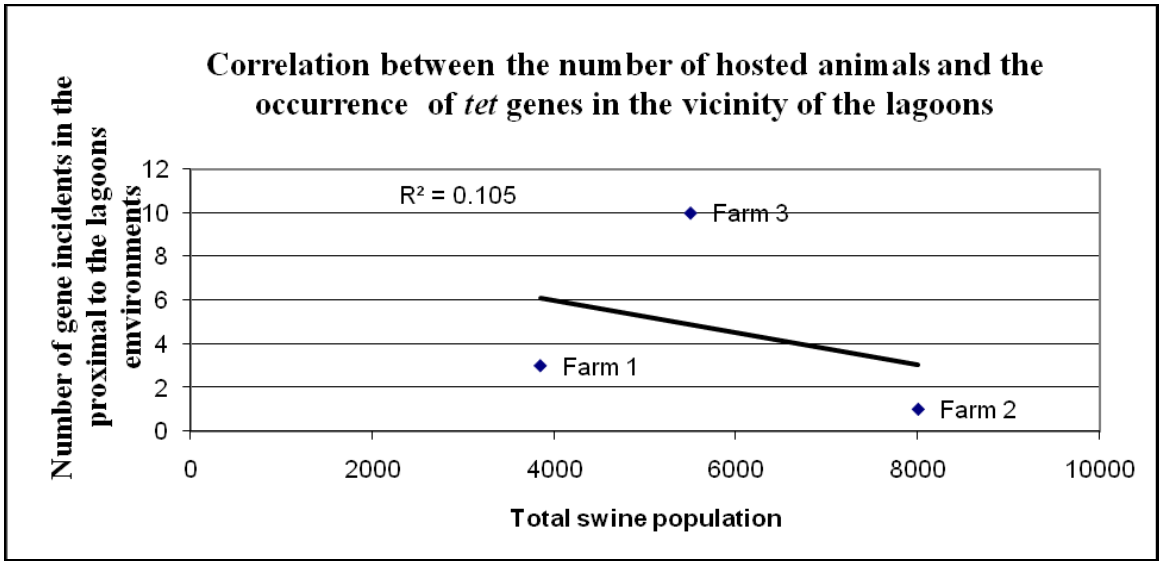

Fig. 4. Number of animals and environmental occurrence of TCR genes in the vicinity of farms.

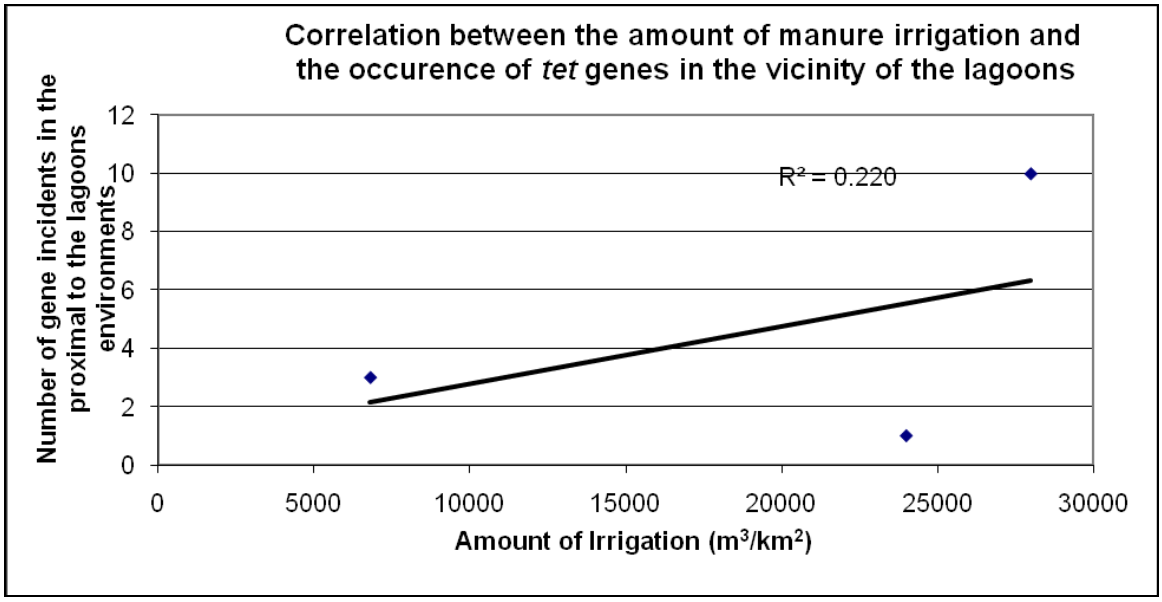

Fig. 5. Amount of manure used for irrigation (per one irrigation event) and environmental occurrence of TCR genes in the vicinity of farms.

We also assessed the presence of manure in irrigated soils *via* comparing nitrogen and carbon contents in irrigated and non-irrigated soils at each farm (Table 6). The analysis was performed during low-rainfall season when irrigation was applied at all three farms. Only Farm 2 revealed higher nitrogen concentration in irrigated

soil suggesting a substantial presence of manure. This irrigated soil also contained the only TCR gene observed in the farm vicinity, tet(A), detected there one week after irrigation when the soil was collected for both TCR gene and nitrogen-carbon analyses.

Table 6. Nitrogen and carbon contents in irrigated and non-irrigated soils.

Soils	Nitrogen (%)	Carbon (%)	N/C ratio
Farm 1, non-irrigated	0.045±0.003	0.797±0.013	0.050
Farm 1, irrigated	0.037±0.001	0.561±0.022	0.066
Farm 2, non-irrigated	0.046±0.001	0.699±0.027	0.066
Farm 2, irrigated	0.167±0.005	2.718±0.193	0.061
Farm 3, non-irrigated	0.153±0.007	1.576±0.001	0.097
Farm 3, irrigated	0.148±0.001	1.839±0.041	0.080

The data shown in Fig 1-5 demonstrated the highest correlation between AB usage and the incidents of environmental occurrence of TCR genes in the vicinity of farms, following with lower but still significant correlations of the latter parameter to the number of lagoons, and to the amount of rainfall in the region. No correlation between the occurrence of TCR genes and number of hosted animals or amount of manure used for irrigation was observed. As suggested by nitrogen and carbon contents in irrigated and non-irrigated soils (Table 6), irrigated soils from Farm 1 and 3 not revealed a significant presence of manure; and where its presence was evident (Farm 2), the environmental occurrence of TCR genes was the lowest.

VII. 2. Objective 2. To quantify *tet* genes commonly observed in SFO manure treatment lagoons, feed, and the environment.

Tet(A), (G), (M), (O), and (X) were found persistent in the feces and lagoons and discovered in proximal to the farms environments. These TCR genes were consequently quantified in feed, feces, lagoons, and these environments. To estimate the TCR gene abundances, their copy numbers were normalized to the copy numbers of 16S rRNA ('housekeeping") genes detected in the same matrices. The data on *tet*(A) concentrations and abundances in the farms and proximal to them environments are given in Table 7.

As seen in the Table, *tet*(A) concentrations and abundances varied between the farms and the environments. For example, if in Farm 2 *tet*(A) was not that numerous in the feces (10^4 range), its concentration substantially increased in the lagoon (10^6 range) but again decreased in irrigated soil (10^3 range); and the *tet*(A)/16S rDNA ratio reflected corresponding changes in its abundance in bacterial communities. In contrast, the abundance of this gene in Farm 3 was high in the feces, lower in the receiving them

Table 7. Concentration and abundance of *tet*(A) in and around Farms

Environment	***Tet*(A)/mL(g)**	**16S rDNA/mL/g**	***Tet*(A)/16S rDNA**
F1 Lagoon liquid	1.16×10^{6}	2.61×10^{10}	$(4.45\pm3.4\%)\times10^{-5}$
F1 Lagoon sediment	5.25×10^{4}	8.24×10^{11}	$(6.37\pm1.0\%)\times10^{-8}$
F1 Pond water	4.35×10^{2}	1.53×10^{3}	$(2.86\pm17.2\%)\times10^{-1}$
F1 Pond sediment	7.37×10^{4}	1.86×10^{6}	$(3.96\pm17.2.0\%)\times10^{-2}$
F2 Starter fecal	2.07×10^{4}	4.54×10^{10}	$(9.21\pm17.3\%)\times10^{-7}$
F2 Lagoon liquid	6.72×10^{6}	5.45×10^{10}	$(1.23\pm38.8\%)\times10^{-4}$
F2 Lagoon sediment	4.57×10^{6}	3.79×10^{11}	$(1.21\pm15.0\%)\times10^{-5}$
F2 Irrigated soil	8.39×10^{3}	5.15×10^{7}	$(1.63\pm25.1\%)\times10^{-4}$
F3 Nursery fecal	1.12×10^{9}	1.53×10^{11}	$(7.32\pm38.7\%)\times10^{-3}$
F3 Lagoon 4 liquid	3.90×10^{5}	1.60×10^{11}	$(2.43\pm73.7\%)\times10^{-6}$
F3 Lagoon 4 sedim.	7.30×10^{3}	7.95×10^{9}	$(9.19\pm39.3\%)\times10^{-7}$
F3 Entry pond water	1.87×10^{3}	1.03×10^{8}	$(1.82\pm23.1\%)\times10^{-5}$
F3 Entry pond sedim.	7.61×10^{4}	5.42×10^{7}	$(1.40\pm10.2\%)\times10^{-3}$

lagoon, but substantially increased in the entry pond, especially its sediment. The higher presence of *tet*(A) in the nursery feces (receiving Lagoon 4) of Farm 3 was likely caused by frequent treatments of animals with CTC, while in the "starter" house of Farm 2 CTC occurred at residual after-pretreatment concentrations. Overall, the environmental concentration of this gene was high, and the *tet*(A)/16S rDNA reached 10^{-1} range that was the highest observed for any TCR gene in the study. This ratio was observed in the pond of CTC-using Farm 1.

Tet(G), being persistent in most farm environments, was found in the areas impacted by spills from Farm 3 lagoons, and in the ponds around Farm 2 and Farm 3, albeit its concentration in these objects varied within 30-40 copies per milliliter or gram, and its abundance there was low, between 1.85×10^{-8} and 6.7×10^{-6} per 16S rRNA gene.

In most cases, an increase in the concentrations and abundances of TCR genes increased in the process of their passage with manure through manure treatment lagoons. The fate of *tet*(M), *tet*(O), and *tet*(X) in the manure treatment lagoon system of Farm 3 illustrates the above statement. For a better understanding of the data obtained, an aerial overview of the manure treatment lagoon system for Farm 3 is included (Fig 6).

Dynamics of *tet*(M) concentration is shown in Fig. 7. This figure demonstrates approximately a 100-fold increase in its concentration during manure passage from Lagoon 1 to Lagoon 7. This increase can not be attributed solely to the very high concentration of *tet*(M) observed in Lagoon 4 because its concentrations in consequent Lagoons 5 and 7 were relatively low. In the meantime, the concentration of 16S rDNA in these lagoons remained relatively constant except Lagoon 5 where some drop in its concentration was observed. In rainy December, *tet*(M) was detected in soils impacted by spillways from Lagoon 4, which coincided with the fact that *tet*(M) highest concentration and abundance were measured in this lagoon. It also coincided with the fact that Lagoon 4 received feces from CTC-treated animals.

Figure 6. The outline of Farm 3.

The same tendency for the augmentation with the passage of fecal material through the lagoons was observed for *tet*(X) (Figure 8). Again, an increase of this gene concentration and abundance between Lagoon 1 and 7 was detected.

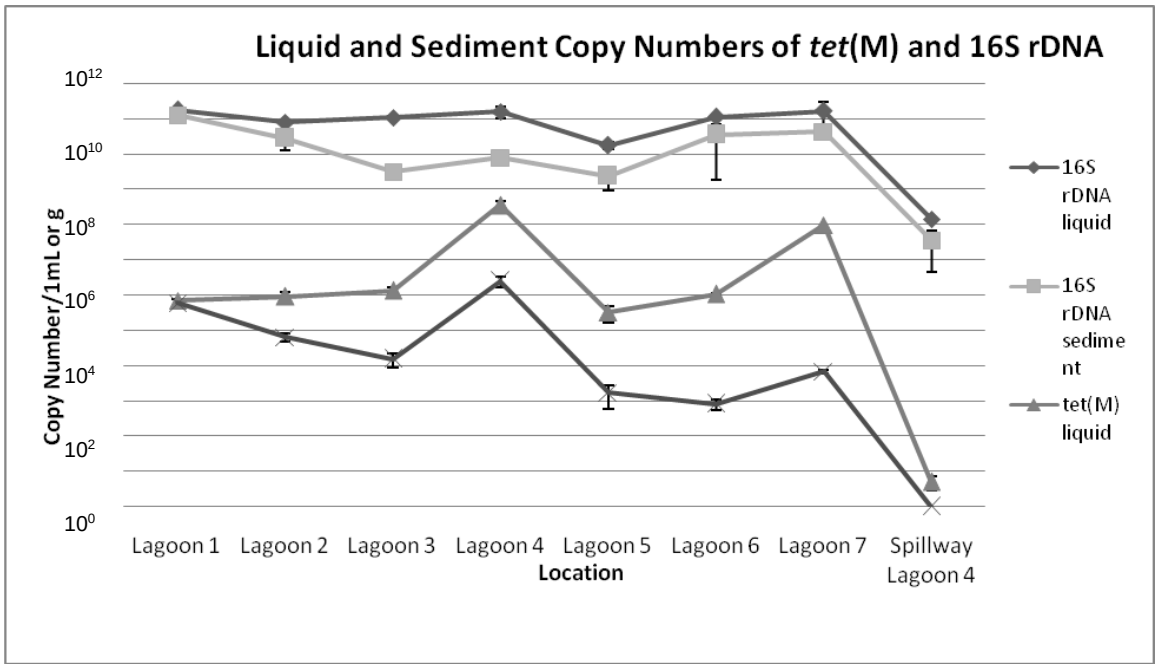

Figure 7. Concentrations (copy numbers per milliliter or gram) of *tet*(M) and 16S rDNA in Farm 3.

The highest *tet*(X) concentration was observed in Lagoon 3 receiving feces from CTC-treated sows. However, this surge in *tet*(X) presence can not be a single explanation for its high concentration in the last Lagoon 7 due to the fact that Lagoon 5 directly receiving wastes from Lagoon 3 demonstrated a relatively low presence of *tet*(X). The presence of *tet*(X) in the Entry Pond was also detected but no spillways from the lagoon system to the pond were observed.

Tet(O) was observed in Lagoons 1 and 4 (Fig. 9). A significant environmental occurrence of this gene around the lagoon system was also discovered. Its concentration reached up to $5x10^3$ copies per gram of matrices impacted by spills from the lagoons. This gene was environmentally present in the pond located from as far as 1300 m from the lagoons. In contrast to most TCR genes, the *tet*(O) concentration in lagoon sediment was almost equal to its concentration in the liquid phase, while its concentration in sediments of adjacent to these lagoons spill-impacted creeks prevailed over its concentration in the aquatic phase.

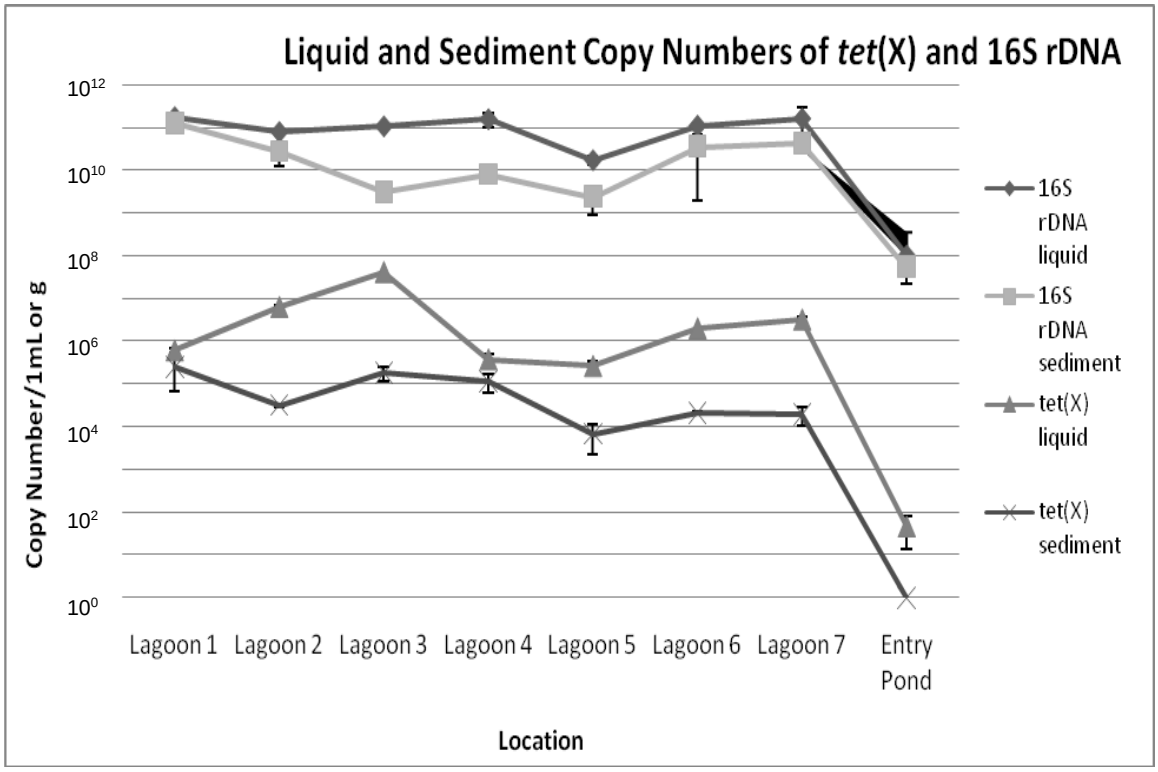

Figure 8. Concentrations (copy numbers per milliliter or gram) of *tet*(X) and 16S rDNA in Farm 3.

The feed in the Nursery/Farrowing house might be the origin of *tet*(O) (Table 5b), this gene might be supported by CTC usage in this house and consequently released in Lagoon 4.

Concentrations and abundances of the "transient" TCR genes in animal feces and the receiving lagoons (Table 8) were also determined. The concentrations and abundances of these genes in animal feces were not that different from the corresponding values for the "persistent" TCR genes, and varied mostly within the similar to the persistent genes orders of magnitude. These data indicate that the concentration of TCR genes in animal feces does not necessarily explain their persistence in the lagoon system and their occurrence in proximal to the lagoons environments. As will be later discussed, other factors may play a part in determining the fate of TCR genes in SFO lagoons and adjacent environments.

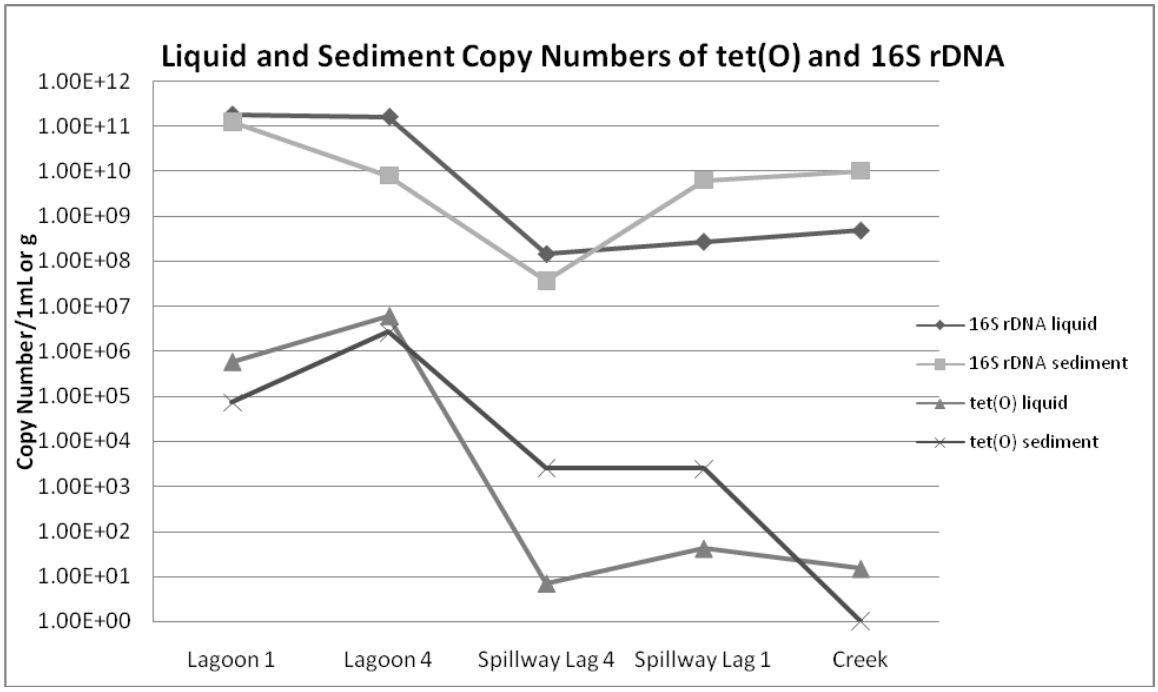

Figure 9. Concentrations (copy numbers per milliliter or gram) of *tet*(O) and 16S rDNA in Farm 3.

Table 8. Concentrations and abundances of the “transient” TCR genes in Farms.

		tet(B)			*tet*(D)			*tet*(L)		
		tet Gene Copy Number	16S rDNA Copy Number	Ratio	*tet* Gene Copy Number	16S rDNA Copy Number	Ratio	*tet* Gene Copy Number	16S rDNA Copy Number	Ratio
Farm 1	Starter Fecal	1.18×10^{6}	4.54×10^{10}	$(2.59 \pm 38.50\%) \times 10^{-5}$	ND			ND		
Farm 2	Farrow Fecal	1.41×10^{4}	4.98×10^{12}	$(2.83 \pm 7.34\%) \times 10^{-9}$	4.44×10^{3}	4.98×10^{12}	$(8.92 \pm 36.86\%) \times 10^{-10}$	$1.46 \times 10^{+9}$	4.98×10^{12}	$(5.56 \pm 7.42\%) \times 10^{-4}$
	Nursery Fecal	5.55×10^{4}	6.30×10^{12}	$(8.81 \pm 87.73\%) \times 10^{-9}$	ND			$9.54 \times 10^{+5}$	6.30×10^{12}	$(2.69 \pm 4.12\%) \times 10^{-7}$
	Gestation Fecal	ND			3.93×10^{5}	1.80×10^{10}	$(3.43 \pm 27.45\%) \times 10^{-5}$	ND		
	Lagoon Liquid	ND			ND			$1.63 \times 10^{+4}$	2.15×10^{10}	$(7.56 \pm 85.23\%) \times 10^{-7}$
	Lagoon Sediment	ND			ND			$6.21 \times 10^{+2}$	8.24×10^{11}	$(1.80 \pm 8.40\%) \times 10^{-8}$
Farm 3	Nursery Fecal	ND			ND			$6.92 \times 10^{+7}$	1.53×10^{11}	$(4.52 \pm 85.40\%) \times 10^{-4}$
	Sow Fecal	1.26×10^{10}	1.03×10^{11}	$(1.22 \pm 95.11\%) \times 10^{-1}$	ND			$7.34 \times 10^{+8}$	1.03×10^{11}	$(7.13 \pm 26.20\%) \times 10^{-3}$
	Lactation Fecal	ND			ND			$4.16 \times 10^{+6}$	1.80×10^{10}	$(2.31 \pm 60.62\%) \times 10^{-4}$
	Finisher Fecal	1.10×10^{7}	8.44×10^{11}	$(1.30 \pm 46.02\%) \times 10^{-5}$	ND			$1.97 \times 10^{+8}$	8.44×10^{11}	$(2.33 \pm 102.27\%) \times 10^{-4}$
	Nursery/Farrowing Fecal	1.07×10^{5}	5.50×10^{10}	$(1.94 \pm 47.94\%) \times 10^{-6}$	ND			$2.76 \times 10^{+8}$	5.50×10^{10}	$(5.03 \pm 46.08\%) \times 10^{-3}$
	Lagoon 2 Liquid	ND			ND			$1.01 \times 10^{+5}$	8.04×10^{10}	$(1.25 \pm 9.23\%) \times 10^{-6}$
	Lagoon 2 Sediment	ND			ND			$1.18 \times 10^{+5}$	2.78×10^{10}	$(5.62 \pm 7.54\%) \times 10^{-6}$
	Lagoon 3 Liquid	5.19×10^{3}	1.03×10^{11}	$(5.04 \pm 22.79\%) \times 10^{-8}$	ND			$8.78 \times 10^{+4}$	1.09×10^{11}	$(8.05 \pm 19.57\%) \times 10^{-7}$
	Lagoon 3 Sediment	4.50×10^{6}	3.10×10^{9}	$(1.45 \pm 99.82\%) \times 10^{-3}$	ND			$1.74 \times 10^{+4}$	3.10×10^{9}	$(4.24 \pm 2.85\%) \times 10^{-6}$

VII.3. Objective 3. To determine the profiles and concentrations of tetracycline antibiotics in SFO manure treatment lagoons, feed, and the environment.

Modifications to the Proposal were made after a phone conference between Dr. Wagstrom, Dr. Reeves, and the P.I. on 03/21/07. Most notable, the diversity of *tet* genes and their spreading among various environments were decided to be the main priority of this study while direct tetracycline analysis was seen as not relevant. Consequently, five more *tet* genes (increasing their total number to 16 genes) and additional sampling points (more than 70 in total) were included in the study, and tetracycline analysis was removed from the project.

VIII. Discussion

VIII.1 Objective 1. To evaluate and compare community-based *tet* gene profiles in three diversified upon antibiotic usage SFO and their adjacent environments.

In this part, most important point of discussion is the relationship between AB usage, the TCR gene profiles in the farms, and their occurrence and profiles in proximal to these farms environments. The TCR gene profiles for all three farms are compared in Table 9.

Table 9. Summary of tetracycline gene profiles of Farms 1-3.

Farms	tet A	tet A(P)	tet B	tet C	tet D	tet E	tet G	tet K	tet L	tet M	tet O	tet Q	tet S	tet T	tet W	tet X
Farm 1	+++	+++	+++	+++	+		+++		+++	+++	+++	+++	+++		+++	++
Farm 2	+++	+++	+	+++	++	++	+		+++	+++	+++	+++	+		+++	+++
Farm 3	+++	+++	++	++		+	+++		+++	+++	+++	+++	++		+++	+++

Legends: (+), (++), (+++): observed in one, two, and three samplings, respectively.

This table reveals a high similarity in the TCR gene profiles of all three farms. With exception of one gene, no relationships between TCR gene profiles in the feces and lagoons and AB usage were observed. Neither farm revealed the presence of *tet*(K) and *tet*(T) that were earlier detected as associated with poultry farms (Barkovskii, personal communication). Evidently, these genes (or their hosts) are not indicative of SFO contribution to the environmental pool of TCR genes. Two groups of genes were revealed as stated earlier:

these that were constantly observed in feces, lagoons, and other analyzed matrices (the persistent); and those that were detected in feces and directly received them lagoons but not in the consequent lagoons or adjacent environments (the transient). Disappearance of the latter in manure lagoons might be caused by a negative selection imposed by these environments towards the hosts of these genes, or by insufficient horizontal transfer of these genes due to incompatibility of their carriers to most bacteria in the lagoon treatment systems. As far as we know, this is the first report on the existence of "transient" genes in SFO. Apparently, this group of genes does not pose a threat to the environment. The only gene indicative of antibiotic usage was *tet*(B), also a "transient" gene, detected only in feces and in the first immediately receiving them lagoon. This gene was apparently associated with microflora of farrowing animals and piglets. The latter observation does not contradict to the fact that these animals most often received AB including CTC with their feed.

The presence of many TCR genes in animal feed was also observed. To what extent this presence impacts the gene profiles in lagoons needs to be studied further. The fact that some TCR genes observed in proximal to the farms environments were common in the animal feed suggests that the role of feed as a potential source of these genes to the environment could be higher than previously expected.

The presence of some genes (e.g., *tet*(S) and (G) in the Farm 2, *tet*(E) in Farm 3) varied between samplings, but the most persistent TCR genes did not reveal seasonal variations. Those most persistent genes (e.g., A, A(P), M, Q, W, X) were shared by all three farms, and were rather attributive of dominating in SFO TCR gene profile than indicative of a particular swine farm.

Among the "persistent" genes, *tet*(A), (E), (G), (M), (O), and (X) were found present in proximal to the farms environments; thus those are the genes that should be monitored in SFO. Their environmental occurrence most strongly correlated to the AB usage in the farms ($R^2 = 0.999$, Fig. 1), and to a lesser extent to the number of manure treatment lagoons ($R^2 = 0.729$, Fig. 3) and the amount of precipitation in the area ($R^2 = 0.668$, Fig. 2). No correlation was observed between the environmental occurrence of these genes and the number of hosted by farm animals ($R^2 = 0.105$, Fig. 4) or the amount of manure used for irrigation ($R^2 = 0.220$, Fig. 5). The highest number of TCR gene environmental incidents was observed in Farm 3, the one with the most frequent AB (including CTC) usage. The concentrations and abundances of those genes in Farm 3 (see the

second part of the Discussion) were the highest in the feces of CTC-treated animals and directly receiving those lagoons. In Farm 2 that was AB-free, only *tet*(A) was observed environmentally present. This gene was found in soil irrigated with the manure of "starter" branch of the Farm that did not use AB directly but received AB-pre-treated piglets, and also revealed the presence of TCR genes in piglets' feed. No environmental occurrence of TCR genes was observed for the "finisher" branch of Farm 2 where animals had no contact to AB.

We considered a potential impact of other varied between the farms parameters (Table 1) on the occurrence of TCR genes around them. These factors included (i) lagoon system construction, (ii) irrigation method and regime, and (iii) other than swine animal vectors. The lagoon systems of all three farms varied upon the numbers of the lagoons, and these numbers correlated to the frequency of environmentally present TCR genes. These lagoons varied in size and depth (therefore, in surface/volume ratios) but all had clay as the lining material. The impact that this ratio may have on environmental incidents of TCR genes around farms was not evaluated in this study and may require additional investigation. Irrigation regimes were similar between all three farms. The first and third farm irrigated land *via* central pivot, albeit the first used nose-to-ground technology, and the third did not. The second farm used solid-phase irrigation method, therefore resembling the first. At this point, no impact of irrigation method on the environmental occurrence of TCR genes could be implied, and this subject also might need a special investigation. The farms also varied on potential animal vectors of TCR genes. Only wildlife could potentially contribute to the observed TCR genes in farms 1 and 2, whereas there were also grazing cows for 6 month/year at the third farm. Table 5b demonstrates that most of the environmentally occurring TCR genes there were detected in the areas visibly impacted by spills (overflow) of the lagoon system. This likely explains a high correlation between the environmental presence of these genes and the amount of precipitation (Fig. 3). The irrigation, as applied, was rather moderate as suggested by the amount of nitrogen in irrigated and non-irrigated soils and by N/C ratios there. No grazing animals were observed in spillways and receiving them adjacent creeks. Other environment where TCR genes were commonly observed was the entry pond (Fig. 5 and Table 5b). In this area, where grazing animals were frequently observed, their impact on the environmental presence of TCR genes might be relevant. Since no comparative studies of the TCR gene environmental occurrence and the presence of other than swine animal

vectors were conducted, no conclusion on the role of the latter in the TCR gene mobility in the environment could be made at this point. This topic requires a separate study that could be performed later. Animal feces and receiving those sites should be compared in that study to appropriate control sites. Farm 3 having grazing animals would be a good site to conduct such a study.

Another important factor that needs to be evaluated is the persistence of TCR genes in the environment following introduction with a spill from the lagoons or from manure application. The PI suggests a systematic study of this problem that should include TCR gene analyses in the animal houses and lagoons, and their monitoring before/after irrigation and rainfall events. Farm 3 with its complex and multifaceted setting would be a good site to conduct such a study.

We concluded that: (i) moderate AB usage does not cause emerging new TCR genes but increases their presence in the environment, (ii) environmental occurrence of TCR genes is also proportional to the numbers of manure treatment lagoons and to he amount of precipitation in the region, (iii) not all genes observed in SFO are equally relevant for monitoring the SFO impact on migration of TCR and other ABR genes in the environment, (iv) *tet*(B) is a potential marker distinguishing AB-free from AB-using farms, (v) animal feed contains TCR genes that can potentially proliferate in waste lagoons and migrate from the lagoons to the environment, (vi) impact that manure application method may have on the presence of TCR genes the environment and their longevity there needed to be evaluated, (vii) other than swine animal vectors for these genes and their contribution to environmental presence of TCR genes needed to be evaluated.

VIII.1 Objective 2. To quantify *tet* genes commonly observed in SFO waste lagoons, feed, and the environment.

qPCR analyses revealed high copy numbers of TCR genes in animal feces and manure treatment lagoons reaching 10^{10} and 10^{9} copies per gram and milliliter, respectively. Being lower than in lagoons, environmental TCR gene concentrations nevertheless reached as high as 10^{4} per gram or milliliter. In the lagoons, TCR gene concentrations were usually higher in the liquid phase, while in creeks, ponds, and rivers they were higher in sediments. This distribution most likely reflects the nutritional and compositional states of the lagoons and

environmental bodies of water. In the first, the liquid phase contained many particles that can be used for microbial attachment as well as substantial amounts of dissolved nutrients. In the second, most particles and nutrients were accumulated by sediments. The TCR gene/16S rDNA ratio indicating the abundance of TCR bacteria in corresponding bacterial communities varied between the lagoons. In multi-lagoon manure treatment system, this ratio increased between the first and the last lagoons of the system indicating proliferation of corresponding TCR genes. This proliferation has never been reported before and needs to be studied in detail. There are two most likely reasons for this proliferation; intensive horizontal transfer of TCR genes in waste lagoons, and preferential survival of TCR gene-possessing bacteria. Unexpectedly, in same cases this ratio was even higher for environmentally occurring genes, e.g. reaching 10^{-1} for *tet*(A) in pond water of Farm 1. Because the numbers of TCR and 16S rRNA genes vary between different bacteria or even in the same bacteria at different growth stages, there is no way to precisely calculate the percentage of bacterial cells carrying *tet*(A); however, this ratio indicates that about 1/10 of bacterial cells carried *tet*(A).

Concentrations of the "transient" genes were also calculated. Unexpectedly, their concentrations in feces were quite high and similar to the "persistent" genes. For example, *tet*(B) concentration in sow feces of Farm 3 reached 10^{10}, therefore was even higher then *tet*(A) concentration there. In the meantime, *tet*(B) did not get beyond Lagoon 3 that directly received the sow feces. Similar behavior demonstrated another "transient" gene, *tet*(L). Most likely, either the lagoon environment was not supportive to their bacterial hosts, or their molecular carriers were not suitable for extensive horizontal transfer in that environment. Detailed study of the diversity between the "persistent" and "transient" bacterial hosts and molecular carriers of these genes are needed to understand molecular and physiological mechanisms of TCR genes proliferation in manure treatment lagoons. We concluded that: (i) manure treatment lagoons contain high numbers of TCR bacteria, (ii) the abundance of the "persistent" genes usually increases during manure passage through the lagoons, and (iii) copy numbers and abundances of the "transient" genes in animal feces are similar to those of the "persistent" genes, therefore their disappearance during manure treatment is caused by the nature of their bacterial hosts and molecular carriers.

Project results can benefit pork producers in following ways: (i) they indicate what AB resistance genes should be monitored in swine farms to assure their compliance with the environment, (ii) they offer *tet*(B) gene

as indicative of AB usage, (iii) observed proliferation of TCR genes in manure treatment lagoon environment calls for a comparative study of different manure-handling practices, for the necessity of separate lagoons for manure from AB-treated and untreated animals, and may be for new engineering solutions for manure treatment lagoon systems. Finally, more attention should be paid to the feed that is the source of some most persistent AB resistance genes in farms. Potential action may include the industry-wide quality control and on-site pre-treatment of animal feed.

Results obtained in this project were reported at (i) Annual Meeting of South Eastern Branch of American Society for Microbiology, Huntsville, AL., November 8-10, 2007, (ii) the II International Conference on Environmental, Industrial, and Applied Microbiology (BioMicroWorld-2007), Seville, Spain, November 27-Dec.1, 2007, (iii) 108th General Annual Meeting of American Society for Microbiology, Boston, MA, June 1-5, 2008, and (iv) at 12th Symposium of International Society for Microbial Ecology, Cairns, Australia, August 17th-22nd, 2008. The ASM presentation received travel award for the presenting student. Two manuscripts derived from the study are currently in preparation.

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