RESEARCH REPORT
PORK SAFETY

Title: Survey of swine isolates for the presence/absence of resistance integrons
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Abstract

A survey of the antibiotic resistance genes present in environmental and animal associated samples was conducted. Both culturable organisms and the genetic pool were sampled by standard microbiological methods and direct PCR respectively. Organisms resistant to a number of different antibiotics were recovered from both environmental and animal associated samples. In addition, sequences were recovered directly from environmental samples without culturing. The antibiotic resistance genes identified were associated with integrons found to be homologous to beta-lactamase, aminoglycoside transferase and chloramphenicol acyl transferase genes. Some genes recovered especially from *E. coli* had significant homology to resistance genes found in *Salmonella* DT104, suggesting that intragenus transfer had occurred. The mechanism promoting this transfer is not clear from the limited study conducted to date.

Introduction

Bacteria become resistant to antibiotics through a number of different mechanisms (4, 6). In some cases, resistance develops to a single antibiotic, while in other situations resistance to several antibiotics evolves in seemingly a single event. Antibiotic resistance may involve degradation of the antibiotic, modification of the target of the antibiotic, reduction in antibiotic transport into the cell, or activation of pumps which remove the antibiotic from the cell. In multidrug resistance, the last mechanism is composed of a number of pumps that exist to remove antibiotics from the cell. Pump-based mechanisms can evolve to confer increased levels of resistance with time (4). Resistance conferred by these pumps is usually not as high as when resistance develops to a single antibiotic usually through a non-pump-based mechanism. Recent evidence for *Salmonella* DT104, however, argues against the assumption of a pump-based mechanism and in favor of an integron-based mechanism.

Included among the most studied genes involved in MDR in Gram (-) bacteria are *emr*RAB, *mar*RAB, *sox*RS and *acr*RAB. MarA and SoxS regulate for example *acr*. There is significant sequence homology among chromosomally encoded systems that confer antibiotic resistance (6).

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Recently Sandvang *et al* (9) reported the discovery of integrons in *Salmonella* DT104 that partially account for the penta-resistance pattern. Integrons are mobile genetic elements that can carry a number of different genes including those coding for antibiotic resistance (1). The Sandvang study was limited to 8 strains and genes coding for a β-lactamase (*pse*) and aminoglycoside resistance (*ant*) were discovered. These results do not account for resistance to tetracycline and perhaps chloramphenicol. Therefore, although integrons can account for a readily transferable resistance, they cannot account for simultaneous MDR development. On the other hand, a simultaneous appearance of MDR in *Salmonella* DT104 has not been observed as compared to a seemingly coordinate appearance.

Antibiotic resistance in bacteria associated with swine has been reported. Primary attention appears to be focused on enteric bacteria and phenotypic screening has been carried out. Resistance has been surveyed in distinct genus and species and no wide spread microbial flora surveys have been conducted. Resistance to ampicillin, tetracycline and sulphonamide has been reported in *Salmonella* recovered from swine (10). In addition antibiotic resistance in *E. coli* recovered from commercial swine operations appears to be significant (5). For example, on a finished product level there are reports that a significant number of *Salmonella* recovered from swine carcasses are resistant to one or more antibiotics including penicillin, trimethoprim, ampicillin and tetracycline (2). There is also evidence for the transfer of antibiotic resistance plasmids from a bacteria flora associated with swine to an *E. coli* recipient strain (7). The limitation in interpreting this study is the use of plasmid borne resistance genes which are far easier to transfer as compared to chromosomal markers. Transfer from biotype-specific bacteria associated with farmers as well as swine occurs at a significantly higher level as compared to bacteria from other biotypes (8).

Examining antibiotic resistance genes on integrons.

We have screened *Salmonella* DT104 and have sequenced the antibiotic resistance genes from a two integrons comparing them to non-DT104 strains. An 1133-bp PCR product was found in many strains and this codes for the *pse* resistance, while a 1008-bp fragment codes for the *ant* resistance Figure 1.

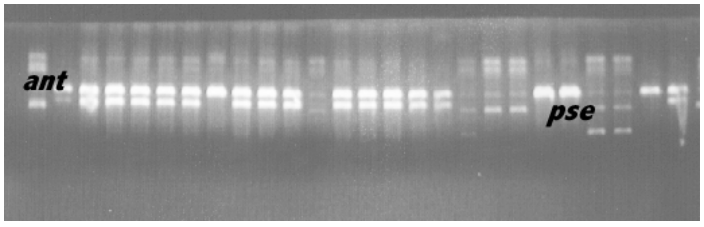

Figure 1. Gel electrophoresis of PCR products derived from a generic set of integron primers. The larger 1.2-kb product codes for the pse resistance while the smaller 1.0-kb product codes for the ant resistance.

A total of 30 DT104 and 3 non-DT104 strains were analyzed of which 16 had integrons apparently coding for both resistances. Several strains that are resistant to all five antibiotics lack the *pse* integron while one strain that is sensitive also lacks the integron but appears to contain the *ant* integron. These assays were carried out using PCR primers and it is unclear at this stage whether heterogeneity in the binding sites accounts for the false negative results**.**

We have sequenced several *pse* and *ant* genes from DT104 and non-DT104 strains. Within the DT104 strains all of the *pse* sequences are identical and the non-DT104 strains examined to date do not contain the *pse* integron. The *ant* are similar within the DT104 and significantly different in the non-DT104 strains. We have also sequenced the regions flanking these integrons and in the two strains examined discovered that in strain 951 474 the integron lies between the carbonic anhydrase gene and a DNA invertase gene, while in strain 940 973 it lies between the *smv*A and *nar*K genes.

Objectives

1. Isolate at least 50 independent strains of bacteria associated with swine through initial genetic screening for integron sequences
2. Identify these organisms to the genus and species level by 16S rRNA sequencing
3. Characterize the antibiotic resistance of these isolates
4. Determine the nucleotide sequence of the antibiotic resistance genes carried by these integrons and compare them to sequences found in bacteria, which are of concern in food safety.

Procedures

Isolation of bacteria associated with swine and initial genetic screening for integron sequences.

We have worked with Cornell University swine farm and collected mixed populations from animals, feed and the general environment. Although not a rigorous survey, it was relatively easy to schedule and complete within the context of the short term of the grant. Isolates were collected using swabs and either directly examined by PCR or cultured on agar medium containing the various antibiotics including ampicillin, tetracycline and kanamycin.

Swab samples were collected on two different occasions from animals of different ages, testing both rectums and noses as well as feces. These swabs were then transported back to the laboratory in Trypticase Soy Broth (TSB) and incubated for 10-16 hrs. The cultures were then streaked onto Luria Agar contain either ampicillin (amp), tetracycline (tet), kanamycin (kan) or chloramphenicol (cam). Growth is scored as a (+) for agar that had visible colonies appearing after 48 hours at 30^{o}C or 37^{o}C. These antibiotics were selected based upon the resistance typically found in *Salmonella* Typhimurium DT104. Individual isolates were purified by restreaking onto a new set of antibiotic containing agar. DNA was purified from each of these isolates and then subjected to PCR using the integron primers.

Genus and species level identification by 16S rRNA sequencing

An unequivocal identification of the microorganism is important to place it within the context of its likely ecology, in terms of its potential for being a donor or its potential for being an animal or human pathogen. 16S rRNA represents a simple direct method for determining speciation. 16S rRNA sequencing was carried out by the *Laboratory for Molecular Typing*. Briefly, a set of generic PCR primers that amplify an internal 350 bp fragment from the 16S rRNA gene were used and the PCR product purified. The sequence of the fragment was then determined by direct sequence analysis as described below.

Characterization the antibiotic resistance profile

We characterized the antibiotic resistance profile of the speciated isolates recovered from various swine-associated samples. Our collaborators at the Diagnostic Laboratory-College of Veterinary Medicine determined the antibiotic resistance profile. The resistance profile was assessed using a Sensititer panel of more than 10 different antibiotics that are typically used in veterinary applications. A quantitative and qualitative pattern of resistance was obtained for various isolates.

Nucleotide sequence of integrons and homology searches

The nucleotide sequence for the resistance genes was determined using direct PCR sequencing and also sequencing of cloned fragments. The sequence of the PCR products or the cloned fragments was determined by direct cycle sequencing. The PCR product were purified and sequenced with dye terminator chemistry (Applied Biosystems) and AmpliTaq Gold. The sequencing products were resolved on an ABI 377 DNA Sequencer (available through the BioResource facility at Cornell University). Internal sequences extending beyond what can be resolved priming from the distal PCR primers were determined using internal primers. Nucleotide sequences were analyzed using DNASTAR and homology searches via WWW GeneBank carried out.

Riboprinting

Riboprinting was carried out at the Laboratory for Molecular Typing. For details, please see www.riboprinter.cornell.edu

Results

Isolate at least 50 independent strains of bacteria associated with swine through initial genetic screening for integron sequences

A screening program was initiated to recover antibiotic resistant microorganisms from swine. A cooperative effort was established with Dr. Mary Smith (Cornell University) who is the attending veterinarian for the swine farm at the university. Environmental swab samples were collected from individual animals and growth on different antibiotics including ampicillin (amp), tetracycline (tet), kanamycin (km) and chloramphenicol (cam) tested.

The size of the PCR product(s) is reported in Table 1.

Table 1. Antibiotic resistance patterns and integrons present in bacteria recovered from swine.

Sample	Fecal	Rectal	Nasal	Amp	Tet	Km	Cam	PCR products (kb)
61		√		+	+	+		0.5*
62			√		+			0.5
63		√		+	+	+		0.5
64			√			+		0.5
65			√	+	+	+		2.0
67			√			+		2.0
68		√		+	+	+		0.5, 2.0
69		√		+	+	+		0.5, 2.0

Sample	Fecal	Rectal	Nasal	Amp	Tet	Km	Cam	PCR products (kb)
70			√	+	+	+		0.5, 2.0
71			√		+			0.5, 1.3
72	√			+	+		+	1.0, 1.3
74			√	+	+			0.5, 1.0
75	√			+	+		+	0.5
76	√			+	+			0.5
77			√	+	+			0.5
80	√			+	+			0.5
85	√						+	1.0
86			√				+	2.1
96			√		+			1.2
99		√		+				2.1
102			√	+				2.1
103			√		+			2.1
103			√	+				1.0
105		√					+	2.0
105		√			+			2.0
106		√					+	2.1
107		√					+	1.6
107			√		+			1.0
107			√	+				1.0
109		√		+				2.0
113					+			1.0

**-the 0.5 kb fragment was found to be a gene coding for aconitase which by coincidence has the sequence used for the forward primer duplicated in a convergent orientation*

Antibiotic resistant organisms were recovered from all sources with some having microorganisms resistant to more than antibiotic. This does not imply that a given organism is multiply resistant as no cross screening of a given isolate on a panel of antibiotics was carried out. A few rectal samples had microorganisms that were resistant to three antibiotics (ampicillin, tetracycline and kanamycin). In contrast, nasal samples tended to harbor microorganisms that were resistant to only one antibiotic. Isolates from this initial screen were then subjected to PCR analysis using the integron primers. At least four different size classes of PCR products were identified, 0.5, 1.0, 1.3, and 2.0 kb. In some cases more than one PCR product was identified with 0.5 kb product being the most prevalent.

Environmental screening for integron sequences

To complement the above efforts we have also been 'panning' for integron sequences out of environmental samples using direct PCR amplification. The integron primers have been used to identify potential antibiotic resistance genes. In parallel, primers that amplify the 16S rRNA have been used to identify the microbial flora in the same samples. A total of six environmental samples were analyzed by direct sequencing. The predominant PCR products identified in environmental samples were 0.8 and 1.0 kb (Table 2). In all but one case the sequence of the PCR product revealed it to encode resistance to aminoglycoside, with individual isolates homologous to either *Enterococcus*, *E. coli*, *C. glutamicum* or *Salmonella*. One sample, 44a gave rise to a 1.0 kb PCR product which was homologous to a trimethoprim resistance gene that is found in *E. coli*. The 16S rRNA analysis of these environmental samples failed to show any clear indication of the microbial flora perhaps due to the complex nature of the population. One sample did result in 16S rRNA sequences that would indicate the that *Streptococcus bovis* was present. The corresponding integron sequence from the same sample was for an aminoglycoside resistance found in *Salmonella*.

Integron sequences and 16S rRNA identification

A survey of isolates recovered by direct plating on agar medium containing either ampicillin, tetracycline or chloramphenicol was carried out. The isolates were recovered as described above using swab samples from animals. The resulting isolates were characterized by their integron sequences and 16S rRNA analysis (Table 2). The integron sequences from a total of 22 isolates was examined and sequences recovered from 12 isolates. In most but not all cases, positive identification of the isolate was obtained by 16S rRNA sequence analysis. The predominant size integron PCR products were 1.0 and 2.0 kb in length. The sequences of these integron PCR products reveal them to mainly encode beta-lactamase and/or aminoglycoside resistance. In many cases the larger, 2.0-2.1 kb fragment encoded both resistances and these sequences were homologous to those found in *Salmonella* and in the case of 107 amp and 86 cam homologous to *Salmonella* DT104. Interestingly the 107 amp and 86 cam derived genes carried a 1.0 kb PCR product with homology to the aminoglycoside resistance gene found in *Salmonella* DT104. All beta-lac genes sequenced displayed homology to those found in *Salmonella.* The 107 amp isolate was identified as a *Klebsiella pneumoniae*, while 86 cam was identified as *E. coli*. The predominant antibiotic resistant flora recovered was identified as *E. coli* and none were identified as *Salmonella* (Table 2).

Table 2. Integron and 16s sequence alignments integron and 16s rRNA sequence alignments

Sample	Int PCR (kb)	16S rRNA identification	Gene identification by homology	Organismic origin by BLAST (>99%)
Environmental isolates				
44a	1.0		trimethoprim	*E.coli*
9	1.0		aminoglycoside	*Enterococcus*
11	1.0	*Streptococcus bovis*	aminoglycoside	*Salmonella typh.*
45a	.8		aminoglycoside	*E.coli*
45b	1.0		aminoglycoside	*Corynebacterium glutamicum*
43b	1.0		aminoglycoside	*Corynebacterium glutamicum*
Cultured isolates				
72 tet	1.0	*E.coli*	aminoglycoside (pro)	*Corynebacterium glutamicum*
107 amp	1.0	*K. pneumoniae*	aminoglycoside	*Salmonella DT104*
86 cam	2.1	*E.coli*	beta-lac, aminoglycoside	*Salmonella DT104*

Sample	Int PCR (kb)	16S rRNA identification	Gene identification by homology	Organismic origin by BLAST (>99%)
88 cam	2.1	*E.coli*	beta-lac, aminoglycoside	*Salmonella typh.*
85 cam	2.1	*E. coli*	beta-lactamase (pro), aminoglycoside	*Salmonella typh.*
96 tet	1.0			
99 amp	2.1			
99 tet	2.1			
105 cam	2.0		beta-lac, aminoglycoside	*Salmonella typh.*
106cam	2.1			
108 tet	2.2			
107 cam	10			
103 tet	2.1		beta-lac, aminoglycoside	*Salmonella typh.*
103 amp	1.0	*Pantoea sp.*	beta-lac, aminoglycoside	*Salmonella typh.*
105 tet	2.0	*E.coli*	beta-lac, ND	*Salmonella typh.*
102 amp	2.1	*E.coli*	beta-lac, ND	*Salmonella typh.*
107 tet	1.0	*K. pneumoniae*	aminoglycoside	*Pseudomonas*
109 amp	2.0	*E.coli*	beta-lac, aminoglycoside	*Salmonella typh.*
113 tet	1.0	*Staphylococcus*		

The antibiotic resistance profile of recombinant *E. coli* carrying the integrons from a number of the isolates was further examined by Sensititer (Table 3). Resistance to all antibiotics with the exception of spectinomycin was the same for all of the recombinant strains including the control pGEM. However 103 amp, 88 cam and 86 B cam all showed resistance to 32-64 µg/ml of spectinomycin. This is as expected given that these strains carry the aminoglycoside resistance gene. The failure of any of the other strains to be resistant to Spectinomycin which also carried similar resistance genes is currently under investigation.

Table 3. Antibiotic resistance profiles of recombinant *E. coli* carrying various integrons.

	103amp	88 cam	86 B cam	103 tet	113 tet	107 tet	pGEM
Amikacin	<4	<4	<4	<4	<4	<4	<4
Amoxicillin	16	16	16	16	16	16	16
Ampicillin	>16	>16	>16	>16	>16	>16	>16
Cefaxolin	8	8	8	8	8	8	8
Cefoxitin	<2	<2	<2	<2	<2	<2	<2
Ceftiofur	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Cephalothin	16	16	16	16	16	16	16
Clindamycin	>2	>2	>2	>2	>2	>2	>2
Enrofloxacin	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Erythromycin	>4	>4	>4	>4	>4	>4	>4
Gentamicin	<1	<1	<1	<1	<1	<1	<1
Imipenem	<1	<1	<1	<1	<1	<1	<1
Orbifloxacin	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Penicillin	>8	>8	>8	>8	>8	>8	>8
Spectinomycin	64	32	64	<8	<8	<8	<8
Sulphadimethoxime	<32	<32	<32	<32	<32	<32	<32
Tetracycline	<1	<1	<1	<1	<1	<1	<1
Ticarcillin	>64	>64	>64	>64	>64	>64	>64
Ticarcillin/clavulanic acid	>64	>64	>64	>64	>64	>64	>64
Trimethoprim/suphamethoxazole	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5

Riboprint™ analysis of E. coli isolates

The Riboprint™ pattern of five *E. coli* isolates was determined (Table 4). Only three were identical, 88 cam, 103 cam and 105 tet, but these were very similar to 109 amp. All four were

identified as *E. coli* in the LMT database. Two others, 72 tet and 86 cam were unique patterns and not found in the database. Riboprint patterns are presented in Figure 2.

Table 4. Riboprint™ pattern for various antibiotic resistant *E. coli* isolates

Sample	Riboprint™	Resistance	Genetic homology
72 tet	116-351-S-5	Aminoglycoside	*C. glutamicum*
88 cam	116-90-S-5	Beta-lactamase, Aminoglycoside	*Salmonella*
105 tet	116-90-S-5	Beta-lactamase, ND	*Salmonella*
109 amp	116-351-S-7	Beta-lactamase, Aminoglycoside	*Salmonella*
86 cam	116-342-S-1	Beta-lactamase, Aminoglycoside	*Salmonella* DT104
103 cam	116-90-S-5	ND	

Conclusions

Antibiotic resistances similar to those found in *Salmonella* DT104 were identified in environmental isolates in organisms other than *Salmonella* DT104. Organisms including *E. coli* and *K. pneumoniae* were found to harbor resistances similar to those found in *Salmonella* DT104. Since these were cross sectional studies, carried out over a very short time and without any overt experimental variables (*i.e.* effect of antibiotic usage) it is not possible to determine if these isolates were the origins of resistance in *Salmonella* DT104. It is also not possible to determine if these integrons could be transfers among genera giving rise to the development of antibiotic resistant strains. Further the effect of animal rearing practices on the rate of transfer cannot be assessed from this data.

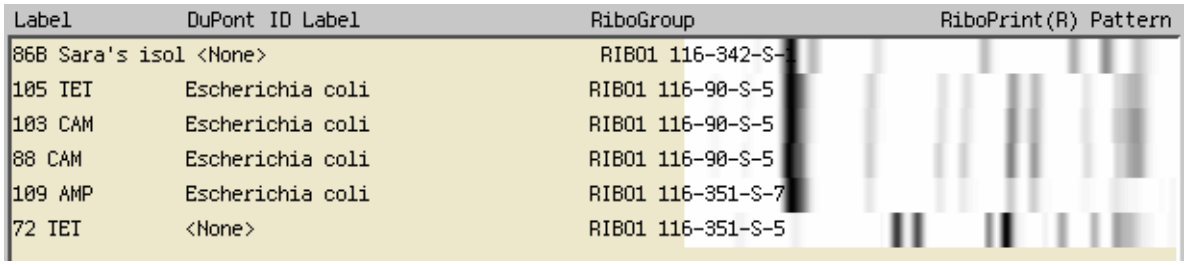

Figure 2. Riboprint patterns for *E. coli* isolates isolated from swine and the associated environment.

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