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Multiplex PCR Detection of Aac(3)VIa and Aac(3)IIa Gentamicin Resistance Genes in Bacteria Isolated from Turtle Farms

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Multiplex PCR Detection of *Aac(3)VIa* and *Aac(3)IIa* Gentamicin Resistance Genes in Bacteria Isolated from Turtle Farms

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Abstract

The Louisiana turtle farming industry is a multimillion-dollar industry which is threatened by the emergence of gentamicin resistant strains of *Salmonella*. Prior to export of the turtles into commerce, hatchlings must be certified *Salmonella*-free. Recent turtle lots have failed certification despite the fact that the eggs had been treated with a gentamicin pressure-differential dip. This is due to the presence of two aminoglycoside resistance genes, *aac(3)VIa* and *aac(3)IIa*, detected in the turtle farm setting. A polymerase chain reaction (PCR) assay was developed to detect the presence of the *aac(3)VIa* gene to complement an existing PCR reaction for detection of the *aac(3)IIa* gene. The multiplex PCR assay can detect both aminoglycoside resistance genes responsible for gentamicin resistance in the bacterial population of the turtle farms. This assay was then tested on gentamicin resistant bacteria isolated from turtle farms in Pierre Part, Louisiana. One of the two resistance genes was present in every isolate tested and the both genes were found among every bacterial species tested.

Introduction

Aminoglycosides are potent, broad-spectrum antibiotics which are used principally in the treatment of gram-negative bacterial infections. This class of antibiotics, which includes kanamycin, streptomycin, tobramycin, and gentamicin, as well as many synthetic compounds, is defined by its structure of at least one aminated sugar joined in glycosidic linkages to a dibasic cyclitol. Aminoglycosides are bactericidal, but are also highly toxic to the host. They have limited ability to bind to the some eukaryotic cells, resulting in deafness, and kidney damage.

Aminoglycosides function by binding to prokaryotic ribosomes to impair bacterial protein synthesis. The drug passes through the prokaryotic outer membrane by a self-promoted uptake process. This process is mediated by the aminoglycoside-induced disruption of Mg^{2+} bridges between adjacent lipopolysaccharide molecules. Subsequent passage through the inner membrane, a step known as energy-dependent phase I, is mediated by an unknown carrier and is dependent upon electron transport. Energy dependent phase II occurs when the drug binds to the prokaryotic 30s ribosomal subunit. Binding impairs the proofreading process controlling translational accuracy, thus causing mutations and inhibiting elongation. These aberrant proteins are inserted into the cell membrane, causing increased permeability of the cell membrane to the aminoglycoside. Cell death occurs when the drug binds all bacterial ribosomes and no functional proteins can be produced.

There are three common methods of aminoglycoside resistance: ribosome alteration, decreased permeability of the cell to the aminoglycoside, and enzymatic

modification of the drug. Ribosome alteration, which confers significant resistance to streptomycin and spectomycin only, works because the altered ribosome has less binding affinity for the aminoglycoside. Decreased permeability, seen mostly in *Pseudomonas* and other non-fermenters, confers only a moderate level of aminoglycoside resistance. The reduced permeability can be caused by an alteration in the aminoglycoside transport system, inadequate membrane potential, or modification of the lipopolysaccharide.

The most significant method of aminoglycoside resistance is enzymatic inactivation of the antibiotic. Bacteria with this mode of resistance have genes encoding enzymes which catalyze the modification of amino or hydroxyl groups on the aminoglycoside. This modification reduces the ability of the antibiotic to bind to the 30s ribosomal subunit, thus reducing the antibiotic's activity. There are three major classes of aminoglycoside resistance enzymes: nucleotidyltransferases (abbreviated ANT), phosphotransferases (APH), and acetyltransferases (AAC). Nucleotidyltransferases and phosphotransferases both use ATP as a donor and act on hydroxyl groups. Acetyltransferases use acetyl-coenzyme A as a donor and acetylate the amino groups of aminoglycosides. Aminoglycoside modifying enzymes are typically encoded on plasmids, but can also be associated with transposons. Some of these genes are thought to be derived from naturally occurring genes in bacteria which are overexpressed in the presence of aminoglycosides, thus conferring resistance.

The acetyltransferase class contains the two genes with which this paper is concerned: *aac(3)IIa* and *aac(3)VIa*. The 876 base pair a*ac(3)IIa* confers resistance to gentamicin, tobramycin, debekacin, netilmicin, 2'-*N*-ethylnetilmicin, 6'-*N*-ethylnetilmicin, and sisomicin (Vliegenthart, *et al,* 1989). *Aac(3)VIa,* identified by

Rather, *et al,* is 900 base pairs and confers resistance to gentamicin and 6'-*N*-ethylnetilmicin.

These particular acetyltransferase genes are of interest because of their importance in the Louisiana pet turtle farming industry. The Louisiana turtle farming industry is an economically important industry which is threatened by the human pathogen *Salmonella enterica*. *Salmonella s*pecies, of the family Enterobacteriaceae, are part of the normal flora of turtles, but cause illness in humans upon ingestion. Although infection with *Salmonella* can cause significant distress, mortality rates are low. Despite this, *Salmonella* is dangerous to the very young and old, as well as the immunocompromised and in 1975 the sale of pet turtles was banned. However, the export of these turtles is still a thriving business. Turtles hatchlings must be certified *Salmonella* free by FDA approved labs before export. Since the early 1980's, a gentamicin pressure differential dip has been used to treat turtle eggs in an effort to eliminate *Salmonella* from newly hatched turtles (Siebeling *et al.* 1984). Prior to introduction of this method, untreated turtles had an infection rate of between forty and fifty percent. This rate dropped to nearly zero upon introduction of the gentamicin treatment.

However, in July of 1999, 11 out of 16 farms in Pierre Part, Louisiana had shipment lots fail certification. These lots tested positive for *Salmonella,* despite the fact that the eggs were treated with gentamicin. This gentamicin resistance is likely due to aminoglycoside resistance genes. For non-fermenters such as Pseudomonas, the genes responsible for the gentamicin resistance found in bacteria isolated from turtles have been primarily the two above-mentioned genes.

The polymerase chain reaction (PCR) is an accepted method for the detection of *Salmonella* in environmental samples and in detecting the *aac(3)IIa* gene in bacteria isolated from these turtles. However a workable procedure for the detection of the *aac(3)VIa* gene by PCR has never been developed. To monitor the dissemination of these genes, as well as to determine if there are any other gentamicin resistance genes in the bacterial population, it would be helpful to develop a method employing PCR to detect the *aac3VIa* gene in environmental isolates. This method can be used to create a single multiplex PCR protocol to detect the presence of both acetyltransferase genes and determine their role in the gentamicin resistance problem in the south Louisiana turtle farming industry.

Materials and Methods

Bacteria Isolates and Identification

Bacteria isolated from failed turtle shipment lots in south Louisiana (Pierre Part area) were used in this study. Turtles in each lot were determined to harbor *Salmonella,* despite the fact that the eggs had been treated with 1000 μg/ml gentamicin using a pressure differential method. Bacteria examined in this study were found to possess high levels of gentamicin resistance by either the Kirby-Bauer disk diffusion method or the through the use of the Minimum Inhibitory Concentration (MIC) method. (Diaz, personal communication). The bacteria examined in this study had been obtained after they were screened for *Salmonella* species. Because of this, they had been isolated on agar selective for *Salmonella* and thus were not representative of all bacteria isolated from the turtles.

Each isolate was identified by genus and species using the API system (bioMérieux Vitek, Inc). Lactose fermenters were identified using API 20-E and non-fermenters were identified using the API 20-NF. The API strips were inoculated according to the manufacturer's directions, incubated overnight, and then read the next day. Numeric values obtained from the results were matched with a computer database to identify the organism.

PCR Detection of Aminoglycoside Resistance Genes

Gentamicin resistant bacteria were examined by PCR to detect the presence of the *aac(3)VIa* and *aac(3)IIa* resistance genes. Three sets of PCR primers were used in this study. The first set of primers (RRN1A and RRN2B) amplify an approximately 330 base

pair segment of DNA within the 16s rRNA (Vliegenthart, *et al.* 1990). This gene is common to all bacterial species and can be used as a positive control to confirm the presence of amplifiable bacterial DNA within the sample. The second set of primers, aac3IIFE (5'GGGAATTCAGAGGAGA TATCGCGATGCATACG) and aac3IIRS (5'TTATCATTGTCGACGGCCTCTAACC), amplifies the 876 base pair *aac(3)IIa* gene and was developed previously in the laboratory (Achberger, personal communication).

The third set of primers was designed to detect the presence of the *aac(3)VIa* gene. Forward primer aac36a3 (5'-CGCTCAGGCGATATGGTGAT -3') and reverse primer aac36a4 (5'-CATAATGGAGCGCGGTGACT-3') amplify a 466 base pair fragment of the *aac(3)VIa* aminoglycoside resistance gene. Primer design was aided by the software Primer Designer 2 (Scientific & Educational Software, 1991). Figure 1 shows the *aac(3)VIa* sequence with the primers in bold-faced type.

Figure 1: Sequence of the *aac(3)VIa* gene (Rather, *et al.* 1990)

ATGACTGATCCCCGCAAAAACGGCGATTTGCACGAACCCGCGACGGCACCCGCGACGCCCTGGTCCAAAAGC
GAGCTGGTCCGGCAATTGCGCGACCTCGGCGTG**CGCTCAGGCGATATGGTGAT**GCCGCATGTGTCGTTGCGC
GCCGTCGGGCCGCTGGCGGACGGACCGCAGACACTTGTCGATGCGCTGATCGAGGCCGTCGGCCCCACCGGG
AATATTCTCGCCTTCGTCTCGTGGCGCGATTCGCCCTATGAACAGACGCTGGGTCATGATGCGCCGCCCGCC
GCCATCGCCCAAAGCTGGCCTGCGTTCGACCCCGACCATGCGCCCGCCTACCCCGGCTTTGGCGCGATCAAC
GAATTTATCCGAACCTATCCGGGGTGTCGGCGCACGGCCCATCCCGACGCATCGATGGCGGCGATCGGGCCC
GATGCGGCGTGGCTGGTGGCGCCGCACGAGATGGGCGCCGCTTATGGCCCCCGCTCGCCGATCGCGCGTTTT
CTCGCCCACGCAGGAAAAATCCTGTCGATCGGCGCCGGGCCCGATGC**AGTCACCGCGCTCCATTATG**CCGAA
GCGGTGGCGCGGATCGAGGGCAAGCGCCGCGTCACTTATTCGATGCCCTTACTGCGCGAAGGCAAGCGCGTC
TGGGTCACCACGTCCGACTGGGATTCGAACGGCATCCTCGACGAATATGCCGCGCCCGACGGCCCCGACGCG
GTCGAACGGATCGCCCGCGACTATCTCGCCCGCACCAGGGTTGCGCAAGGCCCGGTCGGCGGCGCGCAATCC
CGGCTGATCGACGCGGCCGATATCGTTTCCTTCGGCATCGAATGGCTCGAGGCGCGCCACGCCGCGCCAGCG
GCGGCAGCGCTGAAGCCGAAACAACGCCGCGACTGA

Multiplex PCR reactions were run in a final volume of 25 µl. The DNA template, 2.5 µl of this reaction, was prepared by suspending a colony in a 1.5 ml snap cap centrifuge tube containing 0.5 ml of sterile distilled water or 5% Chelex 100 (Bio-Rad Laboratories). The tube contents were vortexed and then placed in a 95°C dry block

heater for five minutes, removed, and immediately placed on ice for five minutes. This heat treatment cycle was repeated one more time.

The remaining 22.5 µl of the reaction was a mixture which contained the following:

2.5 µl 10x Reaction Buffer
2 µl dNTP mix (2.5 mM each dGTP, dTTP, dATP, dCTP)
1.5 mM $MgCl_2$
0.2 µl AmpliTaq Gold DNA Polymerase (New England Biolabs)
1.25 µl aac36a3
1.25 µl aac36a4
1.25 µl aac3IIFE
1.25 µl aac3IIRS
0.2 µl RRN1A
0.2 µl RRN2B
9.7 µl sterile dH_2O

Because the 16s rRNA product was more readily amplified than either of the two aminoglycoside acetyltransferase products, the quantity of 16s rRNA gene primers was reduced to ensure that sufficient nucleotides would be available to detect the target acetyltransferase genes.

PCR reactions were run in a Perkin-Elmer thermocycler (model 480). The program was begun with nine minutes at 95°C to activate the AmpliTaq Gold DNA polymerase. Each cycle consisted of one minute at 95°C to denature the DNA template, 2 minutes at 55°C to allow the primers to anneal to the denatured DNA template, and two minutes at 72°C to allow for elongation. The last step was extended by twenty seconds in each of the 30 cycles.

The PCR products were separated on 6% polyacrylamide gels in a Tris-borate-EDTA buffer system (89 mM boric acid, 89 mM Tris, and 2.5 mM EDTA, pH 8.3). A 100 base pair ladder was included to estimate product size. The gels were

electrophoresed at 100 Volts for approximately 45 minutes and then stained with ethidium bromide for ten minutes and viewed under ultraviolet light with the Stratagene Eagle-Eye system.

Confirmation of Amplicon Identity: Restriction Enzyme Digestion

Presumptive identification of the *aac(3)VIa* amplicon was based on expected molecular size. Restriction enzyme digestion was performed to confirm the identity of the *aac(3)VIa* amplicon. The restriction enzymes *Cla*I and *Ssp*I were selected because both were predicted to cut the amplicon at one site, producing two fragments. Each endonuclease recognizes a six base pair sequence that would have a low probability of occurrence in a random 466 base pair DNA segment. The restriction enzyme *Cla*I was predicted to cut the *aac(3)VIa* amplicon to generate 159 and 307 base pair fragments and does not cut the 16s rRNA amplicon. The restriction enzyme *Ssp*I cuts the *aac3VIa* amplicon to create fragment sizes of 114 and 352 base pairs. In addition, *Ssp*I cleaves a 15 base pair fragment from the 16s rRNA gene amplicon.

Amplicons positive for the *aac(3)VIa* gene were randomly chosen for digestion. The PCR product was separated from mineral oil by chloroform extraction. Five µl of PCR product was digested by each restriction enzyme for approximately three hours in a 37°C water bath. Following digestion, the fragments were run on a 6% polyacrylamide gel for 45 minutes at 100 V. The gels were then stained with ethidium bromide for 10 minutes and viewed with the Stratagene Eagle Eye system under ultraviolet light.

Results

Identification of Bacterial Isolates

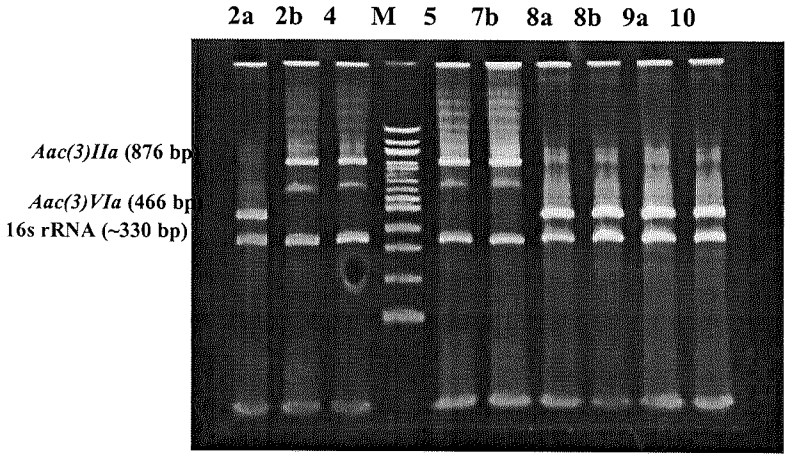
The API system was used to identify the species of each unknown bacterial isolate recovered from turtles or turtle eggs. The bacteria were isolated from a selective medium that had been inoculated from broth cultures designed to enrich for *Salmonella*. Of the 36 bacterial species resistant to gentamicin, 21 were identified as *Salmonella arizonae,* five as *Citrobacter frendii*, four as *Pseudomonas aerogenes*, three as *Providentia stuartii*, and one as *Alcaligenes faecalis*. Two isolates could not be identified by the API system.

PCR Detection of Aminoglycoside Resistance Genes

PCR was used to identify the gene which imposed gentamicin resistance for each isolate. Each gentamicin resistant isolate was examined on the multiplex PCR to test for the presence of each aminoglycoside acetyltransferase gene as well as a 16s rRNA gene segment common among all bacterial species. Figure 2a-2e shows the polyacrylamide gel electrophoresis results of all the bacterial isolates tested.

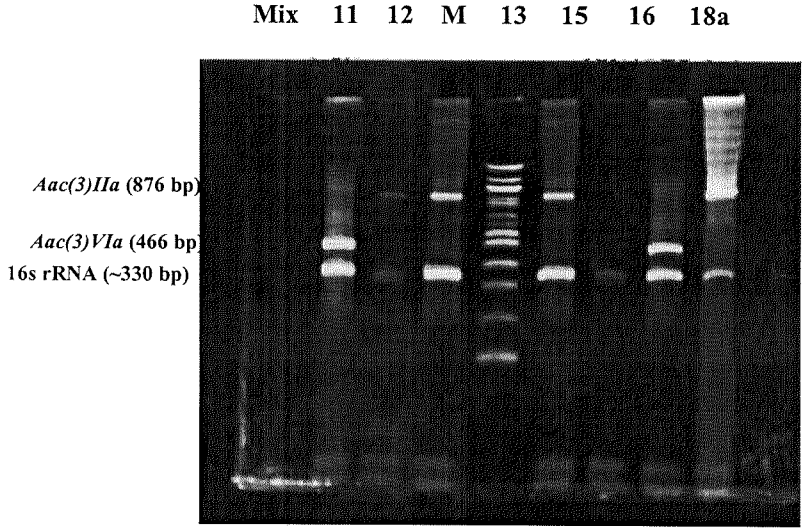
A mixture of DNA templates prepared from two bacterial isolates representing both acetyltransferase genes was also subjected to PCR to determine if there was any interference between the two sets of primers for the acetyltransferase genes. This was done to ensure that, if present, both genes could be detected in one sample. The lane titled "Mix" in Figure 2b shows that while the *aac(3)VIa* gene amplified more efficiently than the *aac(3)IIa*, both genes could clearly be detected. This difference in amplification efficiency could be due to differing template concentrations in the two samples used.

Figure 2a



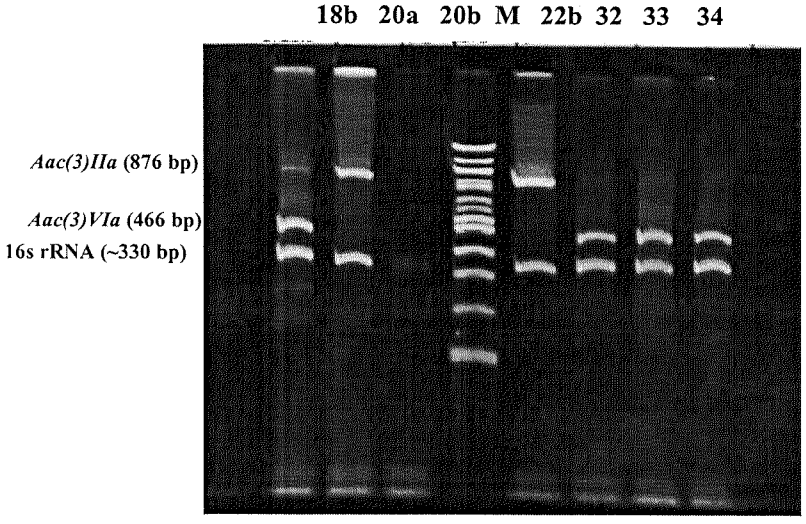
Isolate #	Genus and Species
2a	*Citrobacter freundii*
2b	*Salmonella arizonae*
4	*Citrobacter freundii*
5	*Citrobacter freundii*
7b	*Pseudomonas aeroginosa*
8a	*Salmonella arizonae*
8b	*Salmonella arizonae*
9a	*Salmonella arizonae*
10	*Salmonella arizonae*

Figure 2b



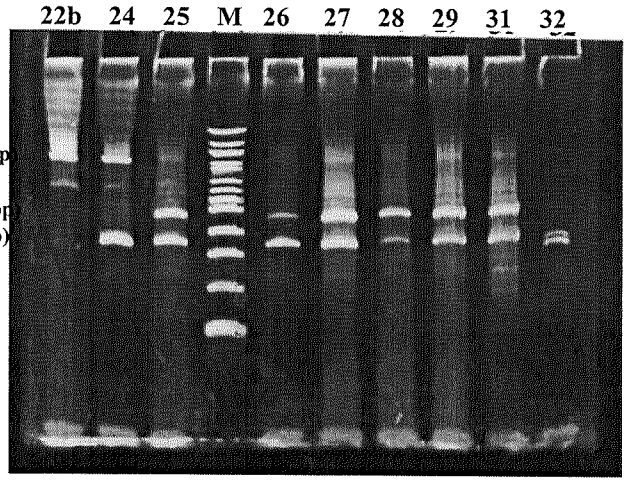
Isolate #	Genus and Species
Mix	Mix of isolates containing both resistance genes
11	*Pseudomonas aeroginosa*
12	*Alcaligenes faecalis*
13	*Pseudomonas aeroginosa*
15	*Salmonella arizonae*
16	*Salmonella arizonae*
18a	*Salmonella arizonae*

Figure 2c



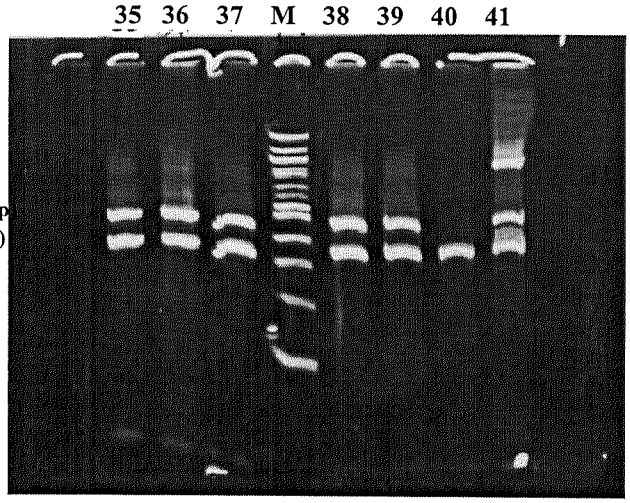
Isolate #	Genus and Species
18b	*Salmonella arizonae*
20a	*Salmonella arizonae*
20b	*Providentia stuartii*
22b	*Citrobacter freundii*
32	Unidentifiable
33	*Salmonella arizonae*
34	Unidentifiable

Figure 2d



Lane or Isolate #	Genus and Species
22b	*Citrobacter freundii*
24	*Pseudomonas aeuroginsosa*
25	*Salmonella arizonae*
26	*Salmonella arizonae*
27	*Salmonella arizonae*
28	*Citrobacter freundii*
29	*Salmonella arizonae*
31	*Salmonella arizonae*
32	unidentifiable

Figure 2e



Lane or Isolate #	Genus and Species
35	*Salmonella arizonae*
36	*Salmonella arizonae*
37	*Salmonella arizonae*
38	*Salmonella arizonae*
39	*Salmonella arizonae*
41	*Salmonella arizonae*
42	*Providentia stuartii*

Figures 2a-2e: Polyacrylamide gels of multiplex PCR reactions to identify the presence of two Gentamicin resistance genes: These figures show photographs of polyacrylamide gels, stained with ethidium bromide, which were used to separate the products of the multiplex PCR reaction. The bottom band, representing approximately 330 base pairs, is present in all lanes and is the 16s rRNA amplicon. The band representing approximately 466 base pairs is the *aac(3)VIa* amplicon, and the top band, at 876 base pairs, is the *aac(3)IIa* amplicon. The tables to the side of each photograph identify the genus and species, by isolate code, from which the DNA template for each lane was prepared.

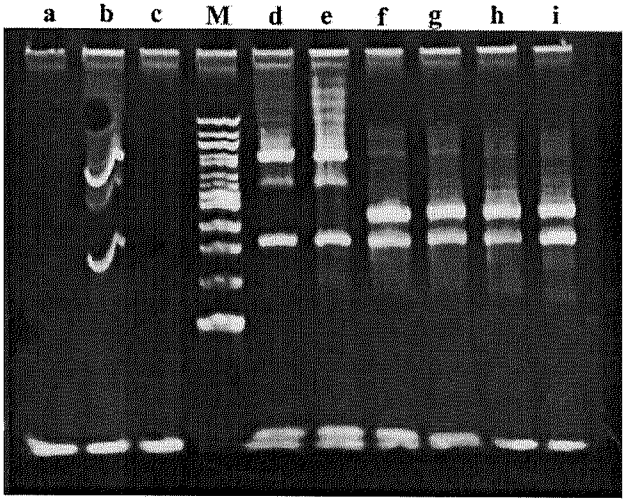
One of the two aminoglycoside acetyltransferase genes was detected in each gentamicin resistant bacterial isolate determined to be gentamicin resistant. However, isolate 12 (*Alcaligenes faecalis)* had only a very faint visible 16s rRNA band, and no acetyltransferase genes were apparent. PCR reactions separating the two resistance gene primer pairs were run after treating the DNA template with 5% Chelex 100 (Bio-Rad Laboratories) which revealed that isolate 12 possessed the *aac(3)VIa* gene. Isolate 32 (genus and species unknown) also failed to show an acetyltransferase gene after two runs (Figure 2c and 2d). The strain was lost and further analysis was not possible.

Isolate 41 (Figure 2e) did not seem to exhibit either acetyltransferase gene. This isolate, *Salmonella arizonae,* was streaked to agar containing gentamicin, and new DNA templates were prepared from isolated colonies in 5% Chelex 100, and when retested, each template showed the presence of the *aac(3)VIa* gene (Figure 7, lanes f-i).

In Figure 2e, isolate 42 (*Providentia stuartii)* showed both acetyltransferase genes were present in the reaction mixture. This isolate had been identifed as either *Providentia stuartii* or *Proteus vulgaris.* Subsequent analysis of this isolate showed the *aac3IIa* gene only (Figure 3). Therefore, it is likely that the original preparation was a mixed culture of *Providentia stuartii* and *Proteus vulgaris*, with each containing one acetyltransferase gene. By the time the second analysis was done, the species with the *aac(3)VIa* gene had been lost leaving only the bacteria with the *aac(3)IIa* gene.

Lanes a and c (Figure 3) failed to show discernible bands, which was not due to a flaw in procedure. The absence of the 16s rRNA band suggests that there was no amplifiable bacterial DNA present in the sample, which may reflect error in colony transfer to the snap cap tube containing 5% Chelex 100.

Figure 3: Further Analysis of Samples 41 and 42



Lane	Identification
a	colony from 42
b	colony from 42
c	colony from 42
d	colony from 42
e	sweep of plate 42
f	colony from 41
g	colony from 41
h	colony from 41
i	colony from 41

Table 1 shows the distribution of the *aac(3)IIa* and *aac(3)VIa* resistance genes among the 36 bacterial isolates recovered from a failed turtle lot in Pierre Part, Louisiana.

Table 1: Breakdown of Presence of Gentamicin Resistance Genes by Isolate

Isolate code	Genus and Species[1]	*Aac(3)IIa*	*Aac(3)VIa*
2a	*Citrobacter freundii*		+
2b	*Salmonella arizonae*	+	
4	*Citrobacter freundii*	+	
5	*Citrobacter freundii*	+	
7b	*Pseudomonas aeroginosa*	+	
8a	*Salmonella arizonae*		+
8b	*Salmonella arizonae*		+
9a	*Salmonella arizonae*		+
10	*Salmonella arizonae*		+
11	*Pseudomonas aeroginosa*	+	
12	*Alcaligenes faecalis*		+
13	*Pseudomonas aeroginosa*	+	
15	*Salmonella arizonae*		+
16	*Salmonella arizonae*		+
18a	*Salmonella arizonae*		+
18b	*Salmonella arizonae*		+
20a	*Providentia stuartii*	+	
20b	*Providentia stuartii*	+	
22b	*Citrobacter freundii*	+	
24	*Pseudomonas aeroginosa*	+	
25	*Salmonella arizonae*		+
26	*Salmonella arizonae*		+
27	*Salmonella arizonae*		+
28	*Citrobacter freundii*		+
29	*Salmonella arizonae*		+
31	*Salmonella arizonae*		+
32	unknown[2]		
33	*Salmonella arizonae*		+
34	unknown[2]	+	
35	*Salmonella arizonae*		+
36	*Salmonella arizonae*		+
37	*Salmonella arizonae*		+
38	*Salmonella arizonae*		+
39	*Salmonella arizonae*		+
41	*Salmonella arizonae*		+
42	*Providentia stuartii*	+	

[1] Identified by API 20E or NE system (bioMerieux Vitek, Inc.)
[2] unidentifiable by the API system

Of the 36 gentamicin-resistant isolates tested, 12 (34%) had the *aac(3)IIa* gene, and 23 (66%) had the *aac(3)VIa* gene. Table 2 summarizes the distribution of the resistance genes among each species. Of the non-fermenters (*Pseudomonas aeuroginosa* and *Alcaligenes faecalis*), most had the *aac3IIa* gene. Nearly all *Salmonella arizonae* possessed the *aac(3)VIa* gene, but sixty percent of *Citrobacter freundii* isolates possessed the *aac(3)IIa* gene.

Table 2: Summary of Acetyltransferase Genes in Bacterial Species

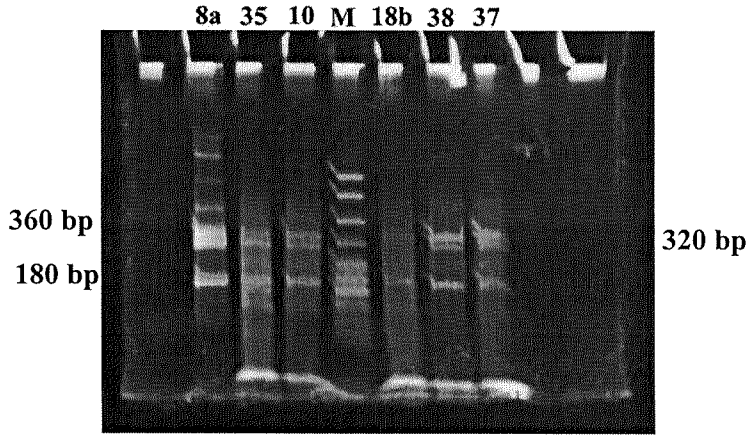
Genus	*Aac(3)IIa*	*Aac(3)VIa*
Salmonella arizonae	1 (4.8%)	20 (95.2%)
Citrobacter frendii	3 (60%)	2 (40%)
Pseudomonas aeuroginosa	4 (100%)	0
Providentia stuartii	3 (100%)	0
Alcaligenes faecalis	0	1 (100%)
Total	**12 (34%)**	**23 (66%)**

Confirmation of Amplicon Identity by Restriction Enzyme Digestion

A restriction enzyme digestion with the enzymes *Ssp*I and *Cla*I was performed to confirm the identity of the 465 base pair *aac(3)VIa* amplicon. The endonuclease *Cla*I was expected to generate 159 and 307 base pair fragments. *Ssp*I was predicted to create 114 and 352 base pair DNA fragments. The 16s rRNA gene amplicon present in the PCR reaction mix should not be digested by *Cla*I. The endonuclease *Ssp*I, however, should cleave a 15 base pair fragment from the 330 base pair amplicon, producing a 315 base pair DNA fragment.

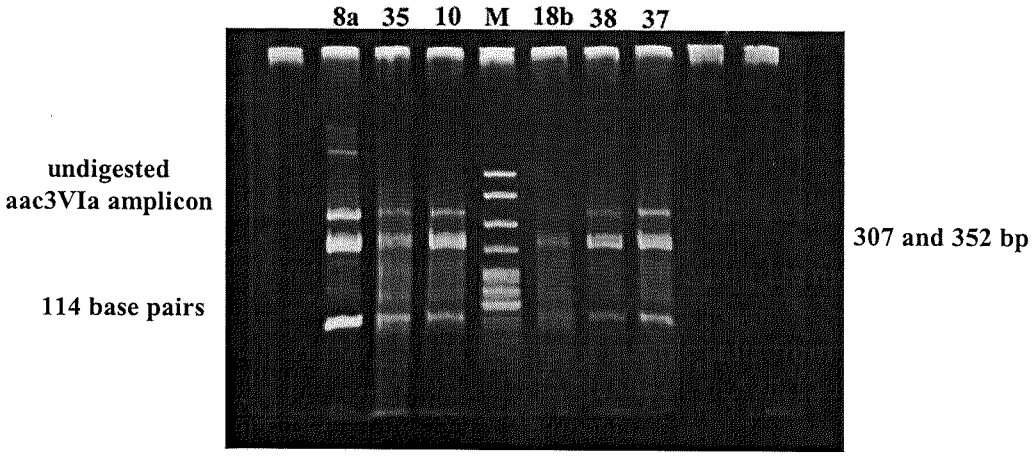
The *Cla*I digestion produced fragments with an observed size of 360, 320, and 180 base pairs. The band representing 360 base pairs is the 330 base pair 16s rRNA amplicon, while the band at 320 base pairs is the 307 base pair fragment expected from the restriction enzyme digestion. The 180 base pair DNA fragment represents the expected 159 base pair amplicon. Occassionally a band representing the undigested *aac(3)VIa* gene was seen, as in the lane labeled 8a in Figure 4. The DNA fragments migrated more slowly than expected relative to the pBR322 -*Msp*I digest marker, and thus appear larger than expected. This is likely because the digestions also contained "ionizable salts" such as $MgCl_2$, nucleotides and pyrophosphates from the PCR reaction and restriction enzyme digestion. An ethanol precipitation to remove these salts which inhibit the movement of the fragments in the polyacrylamide gel would have prevented this.

Figure 4: Restriction Enzyme Digestion with *Cla*I



In the *Ssp*I digestion, an observed DNA fragment size of 120 base pairs was seen. This band corresponds to the 114 base pair DNA fragment. In addition, a very wide band around 330 base pairs was observed. This band is thought to contain the 315 base pair 16s rRNA amplicon fragment as well as the 352 base pair DNA fragment expected from the digestion of the *aac(3)VIa* amplicon. The digestion was incomplete, and undigested amplicon is also visible on the gel in some lanes. The digestion fragments here also seemed to migrate more slowly relative to their size than the marker did. Again, this is because ionizable salts from the PCR and restriction reactions retard the migration of the DNA fragments.

Figure 5: Restriction Enzyme Digestion with *Ssp*I



Discussion

The goal of this investigation was to design a PCR assay to detect the presence of the *aac3VIa* gene in environmental isolates. This PCR assay was then combined with the existing PCR protocol for detecting the *aac3IIa* gene to create a single multiplex PCR assay capable of detecting the two genes commonly responsible for gentamicin resistance in Louisiana turtle farms. In addition, a primer pair specific for the bacterial 16s rRNA gene was also included in these multiplex PCR reactions. This 16s rRNA gene is common to all bacterial species and confirms the presence of amplifiable bacterial DNA in the sample.

Primers for the *aac3VIa* gene were chosen based on the reported sequence by Rather, *et al.* (1990). The primers amplified a 466 base pair sequence that was easily distinguishable from the 876 base pair *aac3IIa* amplicon and the approximately 330 base pair 16s rRNA amplicon. For DNA templates of organisms known to have the *aac3VIa* gene, detection of the *aac3VIa* gene using the aac36a3 and aac36a4 primers was near 100%. The 466 base pair amplicon was confirmed to be the *aac3VIa* amplicon through restriction enzyme digestion with *Cla*I and *Ssp*I.

The multiplex PCR assay was used to test gentamicin resistant isolates from turtle farms in Pierre Part, Louisiana for the presence of the acetyltransferase genes. Each of the 36 gentamicin resistant isolates subjected to the PCR assay possessed one of the acetyltransferase genes, however no isolate exhibited both. The results were consistent with what observed earlier using recombinant DNA techniques to clone and identify the resistance gene from a limited nuber of isolates (E. Achberger, personal communication).

The *aac3VIa* and *aac3IIa* genes seem to be responsible for the vast majority of the gentamicin resistance found in the Louisiana turtle farms.

The *aac3IIa* gene was responsible for gentamicin resistance in 34% of the isolates, while the *aac3VIa* gene was responsible for gentamicin resistance in the other 66% of the isolates. In a previous study, the *aac3IIa* gene was found in 69% of the isolates in Louisiana turtle farms (Thibodeaux, 1994). The difference between the results of this study and Thibodeaux's results may be attributed to the fact that Thibodeaux's isolates came from farms in two other areas in addition to Pierre Part, while the ones used in this study came only from Pierre Part. There may be a slight difference in the distribution of genes among the geographic locations of the turtle farms.

This PCR assay is important because it allows a quick and simple determination of the distribution of these two genes in the bacterial population. To date, every high-level gentamicin resistant isolate from south Louisiana turtle farms has had one of these two gentamicin resistant genes. Because of this, quick detection of these two genes allows new genes that may be entering the population to be discovered more quickly. If a PCR assay of an isolate possessing a high level of gentamicin resistance fails to detect the presence of either of these two genes, than it is possible that a new source of gentamicin resistance has entered the population.

The multiplex PCR assay for these two acetyltransferase genes seems to be reliable. Each acetyltransferase gene was only detected in the absence of the other one, which is consistent with previous results. Occasionally, a prominent band for the 16s rRNA amplicon was visible, however neither acetyltransferase gene was detected, as in isolate 41. In these cases, the isolate was streaked out on a plate containing gentamicin

and a new assay was then run again. One of the PCR genes was detected in every case where the PCR assay was repeated. This does not necessarily indicate a lack of reliability of the assay. If the isolate is not kept in an environment containing gentamicin, plasmids containing the gene for gentamicin resistance may be lost. In addition, mishandling of the DNA template may cause degradation of the DNA.

There did not seem to be a pattern for the distribution of the two gentamicin resistant genes among bacterial species. In the *Enterobactericaea*, all *Salmonella arizonae* isolates except one had the *aac(3)VIa* gene. The distribution for *Citrobacter frendii* was shifted the opposite way, with 60% having the *aac(3)IIa* gene. Among the non-fermenters, (e.g. *Pseudomonas* and *Alacaligenes*) all four of the *Pseudomonas aerogenes* isolates had the *aac(3)IIa* gene, while the sole *Alcaligenes faecalis* isolated had the *aac(3)VIa* gene. A larger sample would be necessary to tell if these distributions were significant.

The wide distribution of these genes among all the bacterial species indicates that both genes are passed very easily among bacteria. The genes must be associated with transposons or plasmids to be transmitted among the bacteria as easily as these findings indicate. The fact that both genes are present in the species where there is a significant number of isolates suggests that there is widespread conjugation among different bacterial species in the environment of the turtle farm. These factors point toward an increase in the severity of the antibiotic resistance problem in the future for the Louisiana turtle farming industry. Because these genes are so easily spread, it is possible that any new gentamicin resistance may be spread very easily as well.

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