The relationship between use of apramycin in the poultry industry and the detection of gentamicin resistant \textit{E. coli} in processed chickens

Jane Rosemary Millar, International Baccalaureate Diploma
St. Margaret’s College, Christchurch

Abstract
Apramycin is an aminoglycoside antibiotic used sparingly by the New Zealand poultry industry. Apramycin resistance is generally caused by an aminoglycoside modifying enzyme (AME), with the most common example being apramycin-acetyltransferase [AAC(3)-IV]. This enzyme can also inhibit the action of two common and medically important aminoglycosides, tobramycin and gentamicin. Development of apramycin resistance requires previous exposure to the compound, so consequently resistance to all three aminoglycosides can be triggered by exposure to apramycin. Research has found that antibiotic resistant bacteria can be introduced to the human gastrointestinal tract through food, where they can spread their resistance factors to other bacteria via horizontal gene transmission. Acquired antibiotic resistance in pathogenic bacteria prolongs infections and decreases treatment options.

Gram-negative bacterial strains were cultured from six different raw chickens from a variety of commercial sources, including organic farms. The susceptibility of these strains to a range of medically important aminoglycosides and apramycin was established using the Kirby-Bauer disc diffusion method. After incubation, the resulting zone sizes were measured to establish the susceptibility range.

Escherichia species from two chickens were found with apramycin, tobramycin and gentamicin resistances. This observation suggests that the resistance resulted from prior exposure to apramycin, causing resistance due to the acquiring of the [AAC(3)-IV] enzyme. This pathway is significant, as it suggests humans are at risk from apramycin use in the poultry industry.

Key words: apramycin, gentamicin resistant \textit{E. coli}, poultry


Introduction

Chicken represents 35 percent of all meat consumed in New Zealand (1). In 2006, the average New Zealander consumed 36.5 kg of chicken (2). To supply this heavy demand, New Zealand poultry farmers have adopted methods of intense chicken husbandry, minimizing the use of land space. However, this can lead to the chickens being raised in cramped conditions enabling rapid transmission of pathogenic bacteria within a flock. One way to control this spread is the sub-therapeutic use of antibiotics. Antibiotics are administered to flocks of chicken through the feed or water, acting not only as general disease-prevention insurance, but also as a growth promoter. The mechanism for the latter is not well-understood, but may result from the reduction of normal intestinal flora which compete with the host for nutrients. Harmful gut bacteria can spread their resistance factors to other bacteria via horizontal gene transmission. Acquired antibiotic resistance in pathogenic bacteria prolongs infections and decreases treatment options.

Apramycin, an aminoglycoside is important in animal husbandry around the world and is used by the Poultry Industry Association of New Zealand (PIANZ) members, who collectively account for 99% of New Zealand’s broiler chicken production. Apramycin is predominantly used in poultry breeder flocks for short periods, to treat clinical cases of colibacillosis and infections caused by \textit{E. coli} or \textit{Salmonella} species. During the last audited year (2004-2005) only 7.35 kilograms of apramycin was administered to chickens belonging to PIANZ; a mere 0.014% of all antibiotics used in New Zealand animals (3).

Gentamicin has been used in horticulture and agriculture in New Zealand (4), PIANZ states that their members have never used gentamicin (3). Tobramycin is an aminoglycoside with an extended spectrum capable of treating infections with gentamicin resistant bacteria. It is not used in agriculture, but is reserved for life-threatening infections in humans.

Although there are different mechanisms of aminoglycoside resistance, plasmid encoded enzymatic modification is the most common (5). Many aminoglycoside modifying enzymes (AME) exist and by testing the susceptibility of isolates against a range of aminoglycoside antibiotics, a pattern of resistance emerges that is unique to a specific enzyme (6). A commonly known apramycin inhibiting enzyme is apramycin-acetyltransferase [AAC(3)-IV] which also inactivates gentamicin and tobramycin (7). The resistance for apramycin due to this enzyme is unique in that it only occurs after exposure to apramycin. Interestingly, exposure to apramycin can cause the resistance to apramycin, gentamicin and tobramycin, but gentamicin exposure cannot cause apramycin resistance to form (J Aitken, personal communication).

Methods

6 fresh chickens were purchased from Christchurch Merivale Fresh Choice supermarket on the 16th February 2006 where they had been displayed in an open 4ºC refrigeration unit. Each chicken was assigned a number to ensure anonymity of the producer. These chickens were:

1. Corn-fed free-range fresh chicken.
2. Fresh chicken, barn raised, no added hormones.
3. Fresh chicken, no added hormones, barn raised, size small.
4. Chicken, free range organic “bio-gro” certified, no growth promotants, no antibiotics.
5. Fresh chicken, no added hormones, barn-raised.
6. Fresh chicken, no hormones added, barn raised, size large.

Colonies of Gram-negative bacteria were isolated from the different brands of raw chickens using selective media and the aminoglycoside susceptibility profiles were determined using the Kirby-Bauer method as follows:

Using a sterile syringe, the plastic wrapping was punctured and approximately 100 mL of the fluid was aspirated from each chicken and transferred into a corresponding numbered test tube. 100 µL of the fluid from each chicken was transferred into five sterile test tubes containing MUG broth (a qualitative fluorescent detection media used to detect \textit{E. coli} in water samples). The tubes were then incubated for 24 hours at 37ºC with lids on.
After removal from the incubator, in a dark area a UV light was shone over each test tube. Any fluorescence was recorded and a blood MacConkey agar plate was inoculated and streaked for each test tube (i.e. 5 plates for each chicken). The plates were incubated for 24 hours at 37°C.

One sample colony of growth on each plate and was emulsified in a nutrient broth and this was used to prepare KB antibiotic sensitivity plates stamped with apramycin, gentamicin, tobramycin, kanamycin, streptomycin, neomycin, and amikacin discs, then incubated for 24 hours at 37°C. The antibiotic susceptibility plates were examined after incubation and susceptibility profiles were measured and recorded. Isolates exhibiting apramycin resistance were observed and a representative isolate was taken for further study. The antibiotic disc zone sizes on the plates were measured and CLSI based methodology was used to classify the bacteria as being resistant, intermediate or sensitive to the antibiotic. API20 bacterial identification kits were used to identify the genus and species of any growths showing significant aminoglycoside resistances.

Additionally, 5ml of saline was used to wash the chicken carcass and then centrifuged at 3000rpm for 15 minutes. The centrifugate was inoculated onto Campylobacter isolation media (Fort Richard) and incubated at 42°C for 48 hours in CO2. After incubation the plates were examined for growth of Campylobacter species.

Results

Campylobacter species were isolated from all six chickens. All samples fluoresced under the UV light, indicating the presence of but not the quantity. The different chickens’ isolate resistance patterns were as shown in Figures 1 to 7.

![Figure 1. Apramycin zone sizes.](image)

Figure 1 shows that the bacteria from chickens 5 and 6 were resistant to apramycin while the rest were sensitive. No intermediate resistances were found, indicating that apramycin resistance factors cause complete resistance.

![Figure 2. Amikacin zone sizes.](image)

Figure 2 shows that all the bacteria were sensitive to amikacin. This is to be expected, as amikacin is not used in agriculture. The lack of resistance also indicates that the AME causing amikacin resistances is not formed in response to apramycin exposure.

![Figure 3. Gentamycin zone sizes.](image)

Figure 3 shows that the susceptibility for gentamicin varied considerably between the bacteria from the different chickens. The bacteria from chicken 5 and 6 were considered to be resistant because of the limited zone size. The rest were classified as sensitive. Since gentamicin is not used in agriculture, resistance cannot have been caused by exposure. Gentamicin resistance only occurred where apramycin resistance occurred (Comparing Figures 1 and 3). Apramycin and gentamicin resistances are known to be caused by the same AME, [AAC(3)-IV], therefore these gentamicin resistances probably exist due to apramycin exposure.
Figure 4. Kanamicin zone sizes.

Figure 4 shows that the bacteria cultured from all of the chickens were sensitive to kanamicin. This is to be expected, as kanamicin is allegedly not used in agriculture. The lack of resistance also indicates that the AME causing kanamicin resistance is not formed in response to apramycin exposure.

Figure 5. Neomycin zone sizes.

Figure 5 shows that the majority of the bacteria were intermediate resistant to neomycin. Since neomycin is registered for use by PIANZ, this intermediate resistance probably resulted from neomycin exposure.

Figure 6. Streptomycin zone sizes.

Figure 6 shows that there was little variation in resistance to streptomycin and that the majority contained bacteria intermediate resistant. Streptomycin is not used by PIANZ and it is not believed to be a substrate of the AME caused by exposure to apramycin. Streptomycin, however, is used in large amounts in the pip fruit industry and this activity could result in a significant reduction in streptomycin sensitivity in the environment that the chickens may have acquired.

Figure 7. Tobramycin zone sizes.

Figure 7 shows the large variation in resistance to tobramycin. The bacteria from chicken 6 was completely resistant to tobramycin, the bacteria from chicken 5 had a very restricted zone size, classifying it also as resistant, while the rest of the bacteria was sensitive. Since tobramycin use is restricted to human medical use only, resistance cannot have been caused by exposure. Tobramycin resistance only occurred where apramycin and gentamicin resistance occurred (comparing Figures 1, 3 and 7). Therefore these tobramycin resistances probably were caused by apramycin exposure causing a resistance due to the [ACC(3)-IV] enzyme, resulting in apramycin, gentamicin and tobramycin resistances.

Campylobacter species was grown from all six samples, including the organically raised chickens. Results from the Api20 test kits identified the bacterial isolate from chicken 5 as *Escherichia coli* and chicken 6 as *Escherichia fergusonii*.

Discussion

In both apramycin resistant isolates, resistance was found to gentamicin and tobramycin, medically important antibiotics. Since apramycin, tobramycin and gentamicin resistance can all be caused by the production of the [AAC(3)-IV] enzyme in the bacterium due to apramycin exposure, these results suggest that the use of apramycin by PIANZ has triggered medically important aminoglycoside resistant bacteria in chickens.

Campylobacter is one of the most common causes for human gastroenteritis, and its occurrence in all 6 sampled chickens is unsurprising, given the established link between Campylobacter infection and raw chickens.

In 2003 Cook et al (8) examined vancomycin-resistant *Enterococcus faecalis* isolates in humans, which appeared to be indistinguishable or genetically closely related to the dominant poultry vancomycin-resistant *E. faecalis* clone. It was suggested that there was either a transfer of antibiotic resistant bacteria from poultry to humans by the food chain, or gene transfer of resistance in the human gastrointestinal tract. Similarly it is possible for the aminoglycoside resistant bacteria found in this investigation to be introduced into the human gastrointestinal tract where they can survive for up to six weeks (9), enabling gene transfer to take place. This can lead to the transference of resistance to other pathogens, thus preventing the possibility of therapeutic treatment for that pathogen with the aminoglycoside antibiotics. If infections caused by resistant bacteria fail to respond to treatment it can result in prolonged illness and a greater risk of death. Failed treatment can also
mean longer periods of infectivity, increasing the number of infected people in the community. And finally when infections become resistant to first-line antibiotics, second or third-line drugs are used, which are nearly always much more expensive and sometimes more toxic as well.

The acquisition of resistant plasmids may have been a consequence of selective pressure exerted by the use of apramycin in poultry. The two isolates which are assumed to contain the [AAC(3)-IV] AME belong to the *Escherichia* genus, which are ubiquitous inhabitants of the intestine of warm-blooded animals, including avian species and are usually harmless. The resistant strains were identified as *E. coli*, and *E. fergusonii*. These bacteria are potential pathogens as new strains arise all the time from natural mutations including particularly virulent strains such as *E. coli* 0157:H7. Effective use of antibiotic therapy will result usually in the eradication of the infecting organism, therefore the presence of resistant bacteria suggests that bacteria survived apramycin exposure through a natural selection process caused by sub-therapeutic use.

Finally, apramycin is used mostly for infection control of breeder flocks of chickens, which are not processed for consumption. Finding the resistant bacteria in breeder chicken implies spread of bacteria between flocks, suggesting that the consequences of antibiotic use are more widespread than anticipated.

Apramycin resistant bacteria may have been developed by intensive use of apramycin previously, so may not have been caused by the current therapeutic use. This is a possibility because antibiotic resistant bacteria can persist long after the removal of the selection pressure (10). Aminoglycoside resistances that have arisen may therefore be difficult, if not impossible to reverse. A comprehensive field evaluation of apramycin use by PIANZ would be needed to further investigate this likelihood. If these resistances were caused by the historic use of apramycin, it would be a strong indicator that the use of apramycin causes other resistances to develop.

PIANZ justify the use of antibiotics by saying the chickens must be "protected from and, the rapidly diagnosed of any significant injury of disease." (11). According to PIANZ, when used for health maintenance, antibiotics allow better nutrient utilisation and reduce the amount of feed needed and allow the chickens to grow to full potential. Antibiotics also lower the incidence of sickness and death in the chicken flocks, consequently reducing the pain and suffering of the chickens. PIANZ have stated "To date, there is only indirect scientific evidence linking the use of antibiotics in food animals with the potential to compromise the efficacy of related antibiotics in humans." and present results from overseas studies (conducted by the Heidelberg Appeal Netherland Foundation 1999), which found that there was no evidence that antibiotics used in animal production compromise the efficacy of related antibiotics in human medicine. In my opinion, the small sample of this investigation contradicts their claim, raising the possibility that industry may select scientific information displaying results benefiting their business.

Intermediate resistances to neomycin and streptomycin were also found but suggested a different resistance mechanism than the AME caused by apramycin exposure. The neomycin resistance may have been caused by its use in the poultry industry and the streptomycin resistance may have been caused by use in horticulture. Not enough is known about the resistance mechanisms of neomycin and streptomycin to make any definitive links to use in agriculture.

Streptomycin is not used by PIANZ, but is extensively used in other areas of agriculture and horticulture. The discovery of streptomycin resistances in the chicken has indicated the extent of the possible widespread distribution of antibiotic resistant bacteria. The transmission of antibiotic resistant bacteria is not limited to food-borne transmission; other methods include surface water via eating crops and meat pet food via pets. These methods have not been investigated in-depth, so are poorly understood, but still pose a significant risk to human health.

The Expert Panel on Antibiotic Resistance convened by the New Zealand Food Safety Association (NZFSA) in 2005 did not express concern about aminoglycoside use in poultry because it was used minimally and in highly supervised conditions (3). However, an external review of the Expert Panel’s review said, “There is no discussion of the strong cross-resistance relationship between apramycin and gentamicin.” (12). It is possible that the NZFSA were unaware of the environmental impact of apramycin, or that the pressure to supply the growing population’s food demand is too overwhelming to consider other options. Either way, there has not been enough monitoring or investigation of apramycin usage for satisfactory answers to the questions raised by this research, which suggests that restricted use of apramycin, even for therapeutic purposes cannot guarantee that resistance to aminoglycosides important in the human medicine will not be selected.

In order to preserve and protect the effectiveness of medically important antibiotics, the spread of antibiotic resistant bacteria needs to be reduced. This should start with a ban of apramycin use in agriculture.

References


8 Cook GM, Manson JM, Smith JMB. Time to ban all non-therapeutic use of antimicrobials in New Zealand animals N Z Biosciences 2003.


Address for correspondence: Jane Millar, 10 Sedgewick Way, Westmoreland, Christchurch. Email: jane@millar.org.nz