# ANTIBIOTIC RESISTANCE IN THE FOOD CHAIN- A CASE STUDY OF *CAMPYLOBACTER* SPP. IN POULTRY

by

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# DECLARATION

I hereby declare that the contents of this thesis entitled "ANTIBIOTIC RESISTANCE BY THE FOOD CHAIN - A CASE STUDY OF *CAMPYLOBACTER* SPP. IN POULTRY" represent my own work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, all the sources used or quoted, are indicated and acknowledged by means of a complete reference either in the published articles or *Reference* section.

L.A. Bester

26/3/2013

Date

## DEDICATION

This thesis is dedicated to my mother

**Babs Livanos**

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# ABSTRACT

The sub-therapeutic use of antibiotics for growth promotion in food animal production, has engendered substantial debate on the dissemination of antibiotic resistance via the food chain, specifically, the probability of antibiotic use in food production creating a reservoir of resistant bacteria and/or resistance genes that may spread to humans thereby limiting the therapeutic value of antimicrobial drugs. In the absence of any surveillance programme on food-borne bacteria in South Africa, this study focussed on *Campylobacter* spp. in poultry and encompassed a literature review on the prevailing debate on the dissemination of antibiotic resistance via the food chain, a phenotypic observational study on the prevalence and antibiotic resistance profiles of *Campylobacter* spp. isolated within and across different poultry farming systems and a genotypic component that covered identification methods, plasmid profile determination and strain typing.

Identification methods for *Campylobacter* spp., viz, biochemical tests and matrix assisted laser desorption ionization- time of flight (MALDI-TOF) mass spectrometry was compared to the PCR which is considered the gold standard as a molecular method of identification. The MALDI-TOF was shown to be superior to the biochemical tests for the identification of *C. coli* but equivalent to the biochemical tests for *C. jejuni*. Of the 363 samples collected in total, the frequency of thermophilic *Campylobacter* was 68 % in rural farms (or informally reared poultry), 47 % in both commercial free-range and industrial broilers and the highest in industrial layers at 94 %. Antibiotic resistance analysis showed that isolates from the rural farming systems were significantly ($P < 0.01$) more susceptible to ciprofloxacin, tetracycline and erythromycin when compared

to the other farming systems. Significant ($P < 0.001$) antibiotic resistance differences were detected between broilers (5 - 8 week lifespan), and layers (36 - 52 week lifespan) for gentamicin, ciprofloxacin and tetracycline.

Plasmids were found be harboured by isolates in all the farming systems; in 84 % of isolates from free-range broilers, 77 % of isolates from industrial broilers, 83 % of isolates from industrial layer hens and 75 % of isolates from the rural farming system. The PFGE genotyping of 42 *Campylobacter* isolates generated 39 *Sma*I types. Substantial and substantive genetic diversity was observed between and within farming systems. The lack of correlations amongst the parameters within and between farming systems attested to the diversity and complexity of phenotypes and genotypes and indicated *de novo* evolution in response to antibiotic selection pressure and animal husbandry practices.

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# CHAPTER ONE

## 1.1 Background

Antibiotic drugs can be used in food animals both therapeutically (to treat a disease) and sub-therapeutically. The latter usually occurs over long periods in smaller quantities to improve the animal's growth rate and feed conversion efficiency, this practice being referred to as growth promotion. The possible mechanisms for growth promotion appear to include enhancement of vitamin production by gastrointestinal microorganisms, elimination of subclinical populations of pathogenic organisms, and increased intestinal absorption of nutrients (Bilgili, 2005; Chopra and Roberts, 2001). Theoretically, it is possible for the environment and animals to act as antibiotic resistant reservoirs.

Molecular biologists differentiate the pan-genomes, which are a collection of the total gene make-up of the bacteria, into the ancestral genome (also called core genomes), and the dispensable genome. The latter is any additional genetic material that the bacteria acquired and can differ from strain to strain within species (Medini *et al.*, 2005). According to reviews by Kelly *et al.* (2009), the transfer of genetic entities, including those carrying antibiotic resistance qualities, is highly possible in environments with high bacterial loads (Kelly *et al.*, 2009a; Kelly *et al.*, 2009b). This could occur through well-known pathways used during horizontal gene transfers such as conjugation, transduction and transformation. Additional role players using these pathways to assist the bacterial strains to obtain antimicrobial resistance genes are plasmids, pathogenicity islands, transposons and integrons (Kelly *et al.*, 2009a; Singh *et al.*, 2005).

Plasmids in particular are known to play an active role in allowing the movement of genetic material, including antimicrobial resistance genes, between bacterial species and genera. They mediate resistance to multiple antimicrobials, the consequence being a pathogen acquiring resistance to all or most clinically relevant antimicrobials (Sherley *et al.*, 2004). Conjugation allows transmissible plasmids and chromosomal DNA of even very large sizes to be transferred from cell to cell either within or outside the species, mediating antibiotic resistance through specified enzyme activities. Conjugative plasmids that have the capability to replicate independently in the newly acquired or recipient host have a greater chance of spreading through a bacterial flora community than those without conjugative abilities (Maurelli, 2006). The resulting increased potential of treatment failures of first-line antimicrobial drugs, hospitalisation and even death may necessitate administering additional second-choice drugs and prolonged treatments.

Food animals exposed to additives, such as antimicrobials used for growth promotion, may serve as a reservoir of resistant bacteria and/or resistance genes that may spread to humans, thereby limiting the medical value of antimicrobial drugs in the latter (Aarestrup *et al.*, 2001), although the ability of bacteria from animal origin to colonize the human gut and transfer resistance genes remains contentious (Phillips *et al.*, 2004). Figure 1 provides a theoretical demonstration of how the use of antibiotics in food animal production may pose a human health risk. It is known that in the natural environment, such as soil and water, bacteria exchange genetic information (for example resistant genes) with great facility and lack of species specificity (Dessen *et al.*, 2001). Mass therapy practice is a method used to treat large groups of animals, especially poultry, by supplementing their food or water supply. In the process, healthy animals are also treated, but the doses

received by each animal differ, as it depends on the amount of food and water that they each consume (Shea, 2004).

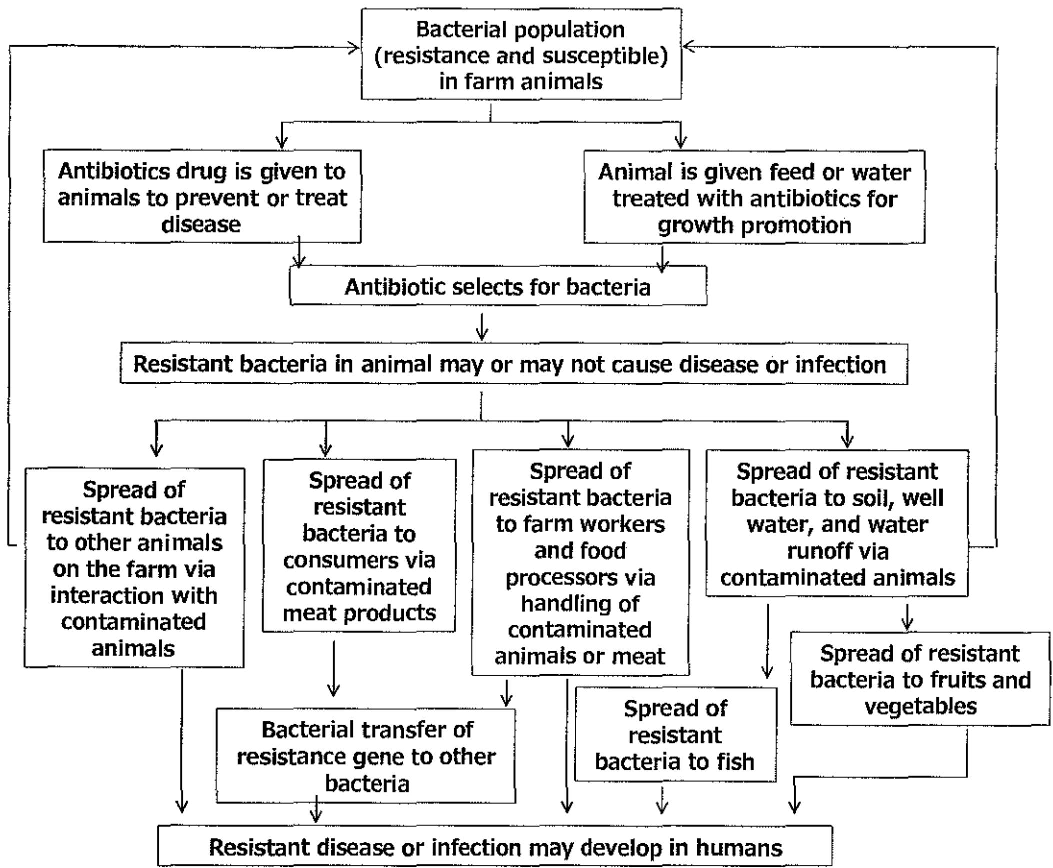


**Figure 1**: Demonstration of the theoretical transfer of antibiotic resistant genes potentially causing a public health risk (From GAO Report/04-490) (GAO, 2004).

The largest obstacle in support of the termination of the use of antibiotics in food animals is the limited data available in the *in-vivo* and *in-situ* food chain environment. While gene transfer was reported in diverse environments, including animal and human intestinal tracts, animal faeces, human cell cultures, bovine rumen fluid, and sewage and surface waters (Walsh and Fanning, 2008), the evidence of resistance via the food chain is mostly

circumstantial. A typical example of how an animal reservoir for resistant bacteria was concluded was the detection of nourseotricin resistant faecal *E. coli* in farm personnel in the former German Democratic Republic. Resistance to nourseotricin, a streptotricin antibiotic, was detected soon after its implementation in pig farming production. Nourseotricin was not prescribed in human health care and it did not cause any cross-resistance of bacterial strains to other antibiotics used in human health care. The resistance to nourseotricin was also later detected in human *Shigella* spp. not associated with animal origins. In addition, nourseotricin resistance was not detected in any neighbouring countries as it was never utilised for the local animal production and therefore in the absence of any reported human consumption, an animal resistant reservoir was therefore considered a likely source (Van den Bogaard and Stobberingh, 1999).

Another example is the presence of *tet*Q genes, which confer tetracycline resistance and are classified as 'ribosomal protection genes' (Chopra and Roberts, 2001) that were described in *Bacteriodes* spp. and *Prevotella intermedius*. Although these are host-specific commensal intestinal bacteria of humans, an identical *tet*Q gene was also described in *Prevotella ruminicola,* which is a host-specific member of the commensal flora in cattle (Van den Bogaard and Stobberingh, 1999). *Tet*Q genes are frequently linked to conjugative transposons and are expressed by both Gram-positive and Gram-negative bacteria (Chopra and Roberts, 2001). The question arises whether the *tet*Q gene in human commensal bacteria originated from an animal source or from an alternative source, including human tetracycline therapy.

In 2000, a ban on the use of all antibiotics as growth promoters was originally implemented on the basis of a "Precautionary Principle", as the scientific evidence that its

contribution to the prevalence of resistant strains experienced in human health care was found to be inconclusive. The 'precautionary principle' acknowledged that although science was not yet able to provide plausible proof, the human risk was a major uncertainty factor (Van Asselt and Vos, 2006). In 2002, the "European Court of First Instance" (Luxemborg), following a ruling on the "Commission's Communication on the Precautionary Principle of 2000", ruled that the ban would continue as a "Precautionary Principle", as reports after the initial ban were contradictory and concrete scientific evidence was still lacking (Phillips, 2007; Van Asselt and Vos, 2006). As a result of these studies, the European Union banned the use of all antibiotics as growth promoters in animal food from 1 January 2006. In the UK, antibiotics used to improve growth efficiency can be purchased as prescription-only medicines (Sarmah et al., 2006).

The termination of antibiotic use and the implementation of more efficient regulations regarding the use of veterinary antibiotics were a direct result of multiple studies around the world reporting antibiotic resistance of clinical isolates, particularly bacteria strains associated with zoonotic diseases (Aarestrup *et al.*, 2001; Sarmah *et al.*, 2006). However, the effectiveness of the ban is difficult to evaluate, as it is evident that it depends on how and what data is interpreted. Persons travelling across district and country borders, as well as the import food markets (from countries where other and/or more lenient food production regulations concerning antimicrobial usage are followed), have complicated scientists' efforts to arrive at strong conclusions about the effect of the ban on human health (Hammerum et al., 2007). A typical example of these difficulties is the low quinolone resistance of *Campylobacter jejuni* (8 %) from poultry reported in the 2005 Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP), in contrast to the higher 29 % clinical resistance reported in humans

(DANMAP, 2005). DANMAP has provided intermediate surveillance reports since 1997, and is one of the few reports that have provided some continuity in a specific region or country since before the ban.

Given the present knowledge of horizontal resistance transfer, co-resistance and co-transfer across bacterial species, the potential risk to human health is serious if food animals become reservoirs of resistance. However, models created to calculate the risks are yet to conclusively explain how much antibiotic usage in food animal production would result in resistance of subsequent human use of related drugs (Bailar and Travers, 2002). Increased therapeutic use of antimicrobials to maintain animal welfare of food animals was further argued to be contributing to the discrepancies found in the antimicrobial resistance surveillance reports after the ban (Bengtsson and Wierup, 2006). This increased therapeutic antimicrobial usage could also have emerged following other opportunistic sources of infection that materialised after the ban, and may not necessarily have been as a direct result of the ban on growth promoters (Phillips, 2007). Data on human clinical isolates provided after the EU 2006 ban is further skewed by the increased therapeutic prescriptions in the EU human health care system, such as higher prescribed doses as a result of increased pharmacological understanding of certain antibiotics (Hammerum *et al.*, 2007). It is thus difficult to conclude what the effect of the EU ban was even after being in effect for more than 15 years, notwithstanding simultaneous influential changes that took place in other areas of the health care (human and animal) systems.

Despite international concern, South Africa has not followed similar banning restrictions set by the EU, and the debate on the use of antibiotics in food production has been less prominent from a public health perspective. However, some public concern does exist, and

South Africa may encounter international pressure to ban the use of antibiotics in food animal production, particularly in those reared for export (Didiza, 2006). The Animal Feed Manufacturers Association (AFMA) acknowledges the responsibility that producers have when using antimicrobials for food production, specifically those related to human health care (AFMA, 2006). AFMA further states that the prudent use of antimicrobials according to "Global Basic Principles" (WHO, 2000) should be followed but adapted for the local legislation and regulations of a country. The adaptation in South Africa involves the registration of therapeutic antimicrobials under the 'Medicines and Related Substances Control' Act 101 of 1965, which allows for their prescription by a veterinarian, while growth promoters are registered under Act 36 of 1947 as 'Stock Remedies', and require no veterinary supervision (Table 1) (AFMA, 2006).

**Table 1:** South African antimicrobial drugs of relevance to the study intended for human health that are registered antimicrobial feed additives (growth promoters) and/or ethical (scheduled) antimicrobial drugs (Swan *et al.*, 2007).

| **Class** | **Antibiotic** | **Growth promoters (Act 36 of 1947)** | **Scheduled drugs (Act 101 of 1965) Main indications** | |
|---|---|---|---|---|
| Beta-lactams | Amoxicillin | NO | YES | Infections caused by a variety of susceptible organisms |
| Tetracyclines | Oxytetracyclines<br>Chlortetracycline | YES<br>YES | YES<br>YES | Tick-borne haemo-parasitic diseases<br>Respiratory tract infections |
| Quinolones | Enrofloxacin<br>Norfloxacin | NO<br>NO | YES<br>YES | Respiratory tract infections<br>Bacterial and mycoplasmal infections in poultry only |
| Aminoglycosides | Neomycin | NO | YES | Bacterial enteritis |
| Macrolides | Tylosin<br><br>Kitasamycin | YES<br><br>YES | YES<br><br>NO | Mycoplasmal infections<br>Swine dysentery<br>Respiratory tract infections |

The first report of the South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs (SANVAD) showed that indicator organisms in healthy animals had reached concerning levels of resistance, this being attributed to the antimicrobial usage in the food production industry. *Escherichia coli* in poultry showed a prevalence of resistance to oxytetracycline (> 90 %), enrofloxacin (65 %), ampicillin (~28 %), trimethoprim (~32 %), neomycin (20 %), gentamicin (~7 %), sulfamethoxazole (> 85 %), nalidixic acid (~63 %) and ceftiofur (~3 %). It also showed resistance to chloramphenicol (~24 %), which has been banned since 1980, this rather being attributed to the use of florfenicol, a chloramphenicol analogue, registered for use in other food animals (cattle and pigs) (SANVAD, 2007). Cross-resistance may explain this resistance, while co-transfer of resistance may explain the phenomenon of chloramphenicol resistance in poultry *E. coli*, in the absence of chloramphenicol and florfenicol, but following phenicol exposure (Bywater *et al.*, 2004).

In a European Commission (EC) report of 1999, 14 key human pathogenic bacteria were listed and of those, only four bacterial strains were recognized to have animal origins: *Campylobacter*, *Salmonella*, *E. coli* and Vancomycin-resistant Enterococci (Follet, 2000). A variety of animals are thought to be asymptomatic reservoirs of *Campylobacter* spp. including food animals such as pigs, poultry (including turkeys) and cattle (WHO, 2011). It is only since the late 1970s with the development of more specialized techniques to isolate *Campylobacter* enhanced an awareness about its potential risk to public health. *Campylobacter* is a small Gram-negative spiral shaped rod that grows in micro-aerophilic atmosphere on medium that is supplemented with blood or blood substitute (Lastovica, 2006a). Presently, the genus comprise of 17 species of which *C. jejuni* spp. *jejuni* (further referred to as *C. jejuni*) and *C. coli* are the most common human pathogens (WHO, 2011),

although other species, namely *C. lari* and *C. upsaliensis,* have also been identified, but are prevalent to a lesser degree (Lastovica, 2006b; WHO, 2011).

Humans are normally infected by *Campylobacter* bacteria through an oral pathway by handling and consuming uncooked meat, especially poultry products, contaminated water and even raw vegetables (Chai *et al.*, 2007; Wilson *et al.*, 2009). *Campylobacter* cause gastroenteritis in humans, also known as campylobacterioses, and is normally considered self-limiting diarrhoea (Van Vuuren *et al.*, 2006). However, cases of bacteraemia, pancreatitis and hepatitis have been reported as complications. Post-infection links campylobacterioses to arthritis and the neurological Guillain-Barré syndrome, although this has been observed less frequently. Children, the elderly and immuno-compromised patients are particularly vulnerable, and infection may result in death (WHO, 2011). *Campylobacter* spp. have also shown to be important pathogenic opportunists in under-nourished children in South Africa (Mackenjee *et al.*, 1984).

Campylobacterioses is the most prevalent of all the foodborne diseases in the EU, with the cost to the health sector and employers regarding its impact on productivity being estimated to be EUR 2.4 billion per annum (EFSA, 2011). It is not known what the impact of *Campylobacter*-related diseases is in South Africa as no continuous surveillance programme in human and animal health care has been implemented to date, although sporadic research on its prevalence and resistance in both human and animal health has been reported (Jonker, 2009; Lastovica, 2006b; Samie *et al.*, 2007).

## 1.2 Problem Statement

In South Africa, antibiotics used as growth promoters in animals are regulated under the Department of Agriculture Act 101 of 1965 (Swan *et al.*, 2007), although these regulations are not as stringent as they are in the EU countries. There is however, a corporate awareness of the responsible usage of antibiotics in animal food production and the need to prevent the emergence of antibiotic resistance reservoirs in food animals (AFMA, 2006; South African Poultry Association, 2008). Despite this there is limited research and little public awareness of the probability of antibiotic resistance dissemination via the food chain in South Africa, necessitating a study of this nature.

## 1.3 Aim

To investigate the prevalence of antibiotic resistance in the food chain using *Campylobacter* spp. in poultry as a case study.

## 1.4 Objectives

- To isolate *Campylobacter spp* from caeca of poultry reared in rural, free-range and industrial production systems.
- To phenotypically and genotypically identify the *Campylobacter* spp. ascertaining the sensitivity, specificity, positive-predictive value and negative-predictive value of biochemical methods and the new matrix assisted laser desorption ionization- time of flight (MALDI-TOF) mass spectrometry with polymerase chain reaction as the gold standard.
- To ascertain antibiotic resistance profiles of *Campylobacter spp.* using the agar dilution method of the Clinical and Laboratory Standards Institute.
- To ascertain plasmid profiles of *Campylobacter spp.* using alkaline lysis method.

- To ascertain the number of genotypic strains using pulse-field gel electrophoresis.
- To analyse the phenotypic and genotypic similarities and/or differences within the different farming systems.

# CHAPTER TWO
# SCIENTIFIC PAPERS

## 2.1 Introduction

This is a PhD by publication and consists of three published papers (as is the requirement of the College of Health Sciences at the University of KwaZulu-Natal) and a fourth paper that is in preparation. The first published paper debated the contested terrain regarding the dissemination of antibiotic resistance via the food chain, the second paper confirmed that antibiotic resistance is a function of time and use by comparing the antibiotic resistance profiles of broiler and layer chickens in a single industrial poultry production system. The third paper presented the impact of selection pressure by describing antibiotic resistance profiles in three different poultry farming systems where birds were subjected to different antibiotic use and/or exposure. The fourth paper (submitted for publication) compared identification methods, strains and plasmid profiles on a representative sample of isolates described in the third paper.

# ANTIBIOTIC RESISTANCE VIA THE FOOD CHAIN: FACT OR FICTION?

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## ABSTRACT

The mechanisms that bacteria use to acquire additional genetic material, including genes coding for antibiotic resistance, are principally the secondary pathways that have been described as transformation and conjugation pathways. The farming industry often is reported as a hotspot for antibiotic-resistance reservoirs. In this review, we consider the exposure of food animals during the course of their lifespans to preventative, therapeutic or prophylactic treatment with antibiotic agents. In this context, zoonotic bacteria are commonly recognised as a potential threat to human health, with therapeutic treatment of pathogenic organisms on farms increasing the likelihood of selective antibiotic pressure influencing the commensal flora of the intestines. Existing literature indicates, however, that the effective impact on human health of such interventions in the food production process is still subject to debate.

## INTRODUCTION

Medini et al.[1] refer to the total genetic makeup of an individual bacterium species as the pan genome. They further differentiate the pan genome into the ancestral or core genome and the dispensable genome, which comprises additional or acquired genetic material and can differ between strains within a species. In situations in which bacteria carrying acquired genetic material are hosted by food animals, the question arises as to whether exposure of these food animals to therapeutic and/or prophylactic antibiotic treatments creates a potential threat to human health.[2,3] We review whether a specific gene encoding a particular antibiotic-resistant gene element, originally present in a bacterium derived from a food animal source, can be the same gene encoding antibiotic resistance subsequently residing in a human clinical isolate of a totally different bacterial strain, species or genus.

## TRANSFER OF ANTIBIOTIC RESISTANCE

Acquired antibiotic resistance is attributed to both direct and indirect pathways. Direct or primary pathways are mutations in the gene encoding resistance against the mechanisms of particular antibiotics. The most commonly known example is resistance of *Mycobacterium tuberculosis* to streptomycin that is associated with an adaptation of the ribosomal sites.[4,5,6] Indirect or secondary pathways are the gaining of small fragments of DNA coding for resistance. These indirect pathways for resistance acquisition can be further sorted into three categories: transformation, conjugation and transduction.[4,7]

### Transformation

Theoretically, even a dying bacterium cell may release its plasmids, or short fragments of its DNA into the environment, which allows for the possibility for a healthy bacterium cell, called a recipient, to acquire this material (and use it for its own benefit) directly through the cell wall. This kind of transfer via the cell wall is known as the transformation pathway. However, our present knowledge of this form of genetic transportation indicates that it has limitations; for bacteria, such an action can only be executed if the bacterium has the requisite genetic capacity to absorb 'loose' compatible DNA – usually in a plasmid form – and, to present knowledge, can only occur in a limited number of bacteria. However, some scientists believe that this pathway usage by bacteria is underestimated.[4,7,8,9]

### Conjugation

Conjugation allows transmissible plasmids and chromosomal DNA of very large sizes to be transferred from cell to cell, either within or between species, mediating the transmitted genetic material through variously specified enzyme activities. The conjugational pathway does not select DNA material simply from the environment, as in the case of transformation, but instead has a direct cell-to-cell exchange of genetic material, where one cell is the donor of the genetic material and the other the new host or recipient. The conjugation pathway uses a very specific, hair-like attachment on the surface of the bacterial cell, constructed mainly of oligomeric pilin protein, that acts as a bridge pulling the two cells together.[4] Enzymes further initiate the transportation of a single DNA strand to the new host, where both cells synthesise duplicates. Both the cells can now act as donor cells. Additional role players that use such pathways to assist the bacterial strain to obtain advantageous characteristics (such as antimicrobial resistance and pathogenicity) are plasmids, pathogenicity islands, transposons, integrons[7,10] and insertion sequences.[11] Conjugative plasmids that have the ability to replicate independently in the newly acquired (recipient) host have greater opportunity to spread through a bacterial flora community than those without conjugative abilities.[9] Gentamycin-resistant *Staphylococcus aureus*, for example, reflects the presence of aminoglycoside acetyltransferases and aminoglycoside phosphotransferases – enzymes responsible for drug inactivation – and is encoded on a gene [*aac(6')-Ie+aph(2")*] located on the transposon Tn4001. This transposon is common in *S. aureus* isolates and is also found on conjugational plasmids. The *aac* gene is not specific to Gram-positive bacteria as it has been found to cause multi-drug resistance in *Enterobacteriaceae* isolates.[12] The tetracycline-resistant *tet*(Q) gene is often found simultaneously with the erythromycin-resistant *erm(F)* gene on a conjugative transposon, resulting in multi-drug resistance.[13] But it is unclear whether the transfer of genetic elements through conjugation occurs voluntarily or equally between both cells (that is, whether both cells actively seek to exploit the possibility of transference) or whether a scenario of cell 'hijacking' takes place for the sake of survival of a more aggressive genetic element.[14]

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### Transduction

Transduction is the virus-like injection of genetic material into a host cell after attachment. To the best of current knowledge, transduction is a feature mainly of bacteriophages commonly acting as bacterial viruses,[9] and is not directly relevant to the present discussion.

## ANIMAL RESERVOIRS OF ANTIBIOTIC RESISTANCE

The farming industry is often alluded to as a hotspot for reservoirs of antibiotic resistance. During their lifespan, production animals are often subjected to preventative, therapeutic or prophylactic treatment with antimicrobial agents. Although subtherapeutic use of antibiotics in animal production is commonly considered to be a major contributor to the proliferation of antibiotic resistance amongst environmental bacteria, Ghosh and LaPara[6] concluded that the clearance of the animal pens of manure, followed by its subsequent disposal, had a greater impact on the lateral transfer (also known as horizontal transfer) of *tet* genes coding for antibiotic resistance amongst soil bacteria. In their study, manure that was allowed to accumulate on soil produced greater antibiotic resistance in natural soil bacteria. In addition, bacterial isolates from soil samples collected closer to the animal pens produced higher antibiotic resistance than those from a further distance. Generally, animals also have a high level of exposure to their environment, especially the soil environment, making it easier for them to be infected with bacteria that carry problematic genetic elements. D'Costa et al.[15] undertook a study on the antibiotic-resistance profiles of strains of soil bacteria that were chiefly from the familiar antibiotic-producing *Streptomyces* genus. They highlighted the high antibiotic resistance and multi-drug resistance found in the natural soil environment and concluded that it was possible that these soil sources could act as a reservoir for resistant elements.[15] Natural antibiotic-producing organisms mutate for the sake of survival in competition with other organisms sharing the same ecological environment. It was shown that erythromycin-resistance in *S. aureus*, caused by a plasmid, and corresponding resistance in soil bacteria, *Streptomyces erythraeus*, have a similar genetic mutation of the 23SrRNA. Methylation of this region is a natural process for the latter erythromycin-producing organism, suggesting that it could have originated from this source.[16]

As with humans, animals also are susceptible to diseases that necessitate therapeutic treatment. However, treatments of pathogenic organisms in a farming context heighten selective antibiotic pressure that influences the overall commensal flora of the intestines. While treating for mastitis caused by *S. aureus* in cattle (in Meylan, France), researchers located an R-plasmid (resistant plasmid), pTMS1 in *Escherichia coli*, hosted in the cattle and in the local veterinarian and some of the other animal handlers.[17] These findings were very similar to those reported in a groundbreaking study by Levy et al. in 1976,[18] in which the intestinal bacterial flora of farm personnel were studied after introduction of an antibiotic supplement to the animals' feed. The antibiotic was a low-dose, broad-spectrum tetracycline; within six months of its introduction, 31.3% of weekly faecal samples taken from farming personnel contained more than 80% tetracycline-resistant bacteria, compared with 6.8% of samples taken from neighbouring farm personnel who were not exposed to the farm animals.[18] Since then, it has been shown that tetracycline resistance may be caused by efflux proteins, 'ribosomal protection genes', or antibiotic inactivation enzymes, expressed by any one of a wide range of *tet* genes, which have been sequenced for both Gram-positive and Gram-negative bacteria.[12,13]

## ZOONOSES

Bacteria, normally associated benignly with animals, can also have natural qualities that allow them to replicate in humans with deleterious disease consequences. These bacteria have a greater chance of being vectors of antibiotic-resistant genes. South African veterinarians working in constant close proximity with farm animals have a three-fold greater likelihood of being infected with a zoonotic disease than their counterparts in the small-animal or other research fields.[19] This figure is highly significant when we consider how many people in South Africa, and in Africa generally, live in secluded or rural areas in close proximity to farm animals, with possible exposure to zoonotic bacteria. Zoonotic bacteria with pathogenic capabilities are generally a threat to the human health system, and more so if they become resistant to the antibiotics normally dictated for treatment. Geonaras et al.[20] undertook a susceptibility study of *E. coli* strains collected in poultry, measured against five antimicrobial agents used in the South African poultry industry. All strains showed susceptibility to danofloxacin and colistin, while 96% were resistant to two tetracyclines.[20] Moreover, in figures for antibiotic susceptibility of potentially human pathogenic bacteria isolates clinically, as well as from healthy farm animals, published in the first report of the South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs (SANVAD), both *E. coli* and *Enterococcus* species showed increased antibiotic resistance, but equivalent to that of comparative profiles from European counterparts. The highest resistance was reported for tetracycline and sulphonamides and the lowest resistance for ceftiofur (a third-generation cephalosporin). Highlighted in the report was evidence of *E. coli* isolates obtained from abattoir chickens, which showed higher resistance for tetracyclines, fluoroquinolones and sulphonamides, in particular, than isolates obtained from clinically ill chickens.[21]

In addition to zoonotic bacteria acquiring antibiotic resistance, pathogenicity can also be increased by the addition of virulent genetic elements. For example, in Ireland, a potential epidemic clone of *Salmonella entirica* serotype Typhimurium DT104 was detected in human gastroenteritis isolates, veterinary isolates, and food samples, with 78% of the isolates showing simultaneous resistance against ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline.[22] Similar resistances were found in the United States, France, Sweden, Russia and Canada for *Salmonella* serotypes as well as for *E. coli* and *Campylobacter* species.[23] The step-wise partial or complete banning in various countries of antibiotics used in food animal production was primarily in response to results reported on clinical zoonotic bacteria. However, the reported bacterial resistance profiles varied from country to country. Aquino et al.[24] relate the inconsistencies between antibiotic resistance profiles to the way therapeutic and prophylactic treatment of humans and animals was exercised in different demographical and geographical areas. The clinical resistances found were correlated with each country's regulations on the use of specific drugs for certain groups of food animals. Australia, for example, did not allow fluoroquinolones to be used in commercial food production and only cooked meat products were allowed to be imported, a fact which might explain why clinical fluoroquinolone-resistant Campylobacters were rarely detected in Australia, in comparison to what was experienced by other countries at the time.[25] The European Union consequently banned the use of all antibiotics as growth promoters as from 01 January 2006. In the United Kingdom, antibiotics used to improve growth efficiency have been restricted as prescription-only medicines.[26]

## FOOD ANIMALS AS A SOURCE OF ANTIBIOTIC RESISTANCE

It is difficult to prove that a specific gene (e.g. coding for antibiotic resistance) has been transferred from a food animal source and is now residing in a pathogenic human clinical isolate in a different bacterial strain, species or genus. However, two general opinions exist as to whether food animals can act as a source of a human pathogenic antibiotic-resistant bacteria.

The first opinion is that food animals exposed to additives such as the antibiotics used for growth promotion may serve as a reservoir of resistant bacteria and/or resistance genes that may

spread to the human population, thereby limiting the medical value of antimicrobial drugs.[3]

The initial efficacy of antibiotics in food animal production and, specifically, as growth promoters, was achieved through (1) stabilising the gut microflora by suppressing subclinical pathogenic populations, (2) enhancing protein metabolism by improving digestibility and nitrogen upkeep, (3) enhancing vitamin production by gastrointestinal microorganisms, and (4) increasing intestinal absorption of nutrients by the host.[13,27] The practice of mass treatment of animals such as poultry by supplementing their food or water supply is of particular concern when considering the development of antibiotic resistance because, as the animals compete for food sources, the doses received differ between individuals. The consequence of some individuals receiving higher doses than others introduces another differential in the selective pressure on commensal bacteria[28] and opens the possibility for the transfer of genes encoding for antibiotic resistance and multi-drug resistance. For example, *E. coli* strains from commensal environments act as reservoirs of genes, such as class 1 integrons, with both antibiotic-resistant and transferable qualities; these genes can be horizontally transferred throughout a bacterial population and carry several antimicrobial resistance cassettes.[10]

The existence of transformational and conjugational pathways was demonstrated when *qnr* genes, coding for quinolone resistance, were transferred in the laboratory from human clinical non-Typhi *S. enterica* serotypes to an *E. coli* J53 strain. These variants of the *qnr* were found to be widely distributed across different states of the United States and found in a variety of animal hosts.[29] Kruse et al.[30] demonstrated the transfer of R-plasmids by conjugation after preparing an *Aeromonas salmonicida* subsp. *salmonicida* NVH4133 (recipient)-infected fish (salmon) on a plastic cutting board infected with *E. coli* DH5 cells (donor). They were also able to demonstrate the transfer between pathogenic bacteria in various other environments, including a hand towel and pig faeces.[30]

Meat and meat products seem to be particular loci of antibiotic-resistant bacteria. Contamination of meat can occur during carcass processing if the rumen is accidentally cut. In the case of poultry, the intestine may rupture when the carcass is gutted during processing, releasing its contents into the thoracic and abdominal cavities.[31] The gut contents of chickens, especially of the caeca, are considered to be peak environments for bacterial colonisation, providing a ready platform for the transfer of genetic entities[7,8] with subsequent spreading to other meat products that emanate from commercial abattoirs. The Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP) reported laboratory studies of the exchange of *vanA* genes in the intestines of mice and humans that code for vancomycin resistance. Special concern was raised for meat contaminated with enterococci transferring their *vanA* genes to human pathogenic *E. faecium* isolates.[32] Retail 'ready-to-eat' foods could present particular risk because they are consumed as purchased, rather than undergoing subsequent cooking.

Products containing poultry, beef and pork in Minneapolis, United States, were found to be contaminated with resistant *E. coli* and extraintestinal pathogenic *E. coli* carrying additional virulent factors. These findings corresponded closely with the prevalence of contamination found for animal carcasses exposed to immediate contamination from the intestines, as well as for processed meat further along the production line.[33]

This form of meat contamination was also found in Addis Ababa, Ethiopia, where 8% of the beef samples collected were contaminated with *E. coli* O157:H7, but less than 2.5% of sheep and goat meat samples were contaminated. This lower contamination rate was attributed to the lower faecal prevalence of *E. coli* O157:H7 in sheep and goats. The meat contamination rates thus corresponded closely with the prevalence rates in the respective hosts. The samples collected from butcheries also yielded higher contamination rates than those from abattoirs, including an export abattoir.[34] *E. coli* O157:H7 is a well-recognised pathogenic invasive strain of *E. coli*; Mizan et al.[35] have demonstrated the strain's susceptibility to the acquisition of resistant plasmids in laboratory conditions using bovine rumen fluid.

Following ingestion, either the complete bacterium starts to colonise the human digestive system or the genetic resistant elements are transferred to commensal or pathogenic *E. coli* strains in the human intestines, causing food-borne disease. The antibiotic-free labelling of production animal food sources apparently fails to take into account potential subsequent contamination during the processing of the food products.[33,36] Generally, bacteria with or without resistant genes present in the meat are killed in the course of cooking the meat. But the potential for infection still persists in contamination of the preparation environment, if, for example, hands are not washed properly or the same utensils are used to prepare both meat and salad dishes. In Malaysia, Chai et al.[37] frequently isolated *Campylobacter jejuni* and *Campylobacter coli* from raw vegetables used for popular salad dishes, probably attributable to animal-contaminated irrigation water.[37] The latter instance signals how easily bacteria normally associated with animals can be sustained in non-animal environments.

The second general opinion is that concerns of food animals being the major source of antibiotic-resistant genes are unfounded. Phillips et al.[38] believe that the likelihood that bacteria from food origin have the ability to colonise the human gut and transfer resistance genes is low, and that, if the possibility did exist, the clinical consequences would be insignificant.[38]

Food animal production usually incorporates some form of antibiotic usage, either therapeutic or prophylactic. But horizontal transfer of genes coding for antibiotic resistance happens with ease, even without selective pressure imposed by the presence of antibiotics.[6] According to Kruse et al.[30], the transfer of plasmids in faeces indicates that conjugational transfer of R-plasmids takes place in the digestive tract, irrespective of antibiotic usage. It appears that transfer can occur between bacteria of diverse origin – humans, food animals, fish and even sea water.[30] According to reviews by Kelly et al.,[7,8] the transfer of genetic entities, including those carrying antibiotic-resistance qualities, becomes all the more probable in environments with high bacterial loads.

In rural areas in developing countries, there is a high risk of exposure to food animals, and thus exposure to zoonotic bacteria,[19] even though the use of antibiotic growth promoters may not be a normal farming practice. The most common poultry production systems in these localities consist of indigenous-species animals in small numbers (i.e. less than 50) that rely on scavenging as a feed source.[39] Dhlamini[40] reported that 87% of family poultry systems studied in KwaZulu-Natal used traditional remedies originating predominantly from plant material, although commercial products, in particular Terramycin and, to a lesser degree, potassium permanganate, also were used for treating the animals. Thus the resistance found in clinical samples from developing countries may have different origins to that found in developed countries. The 70th Report of the Joint FAO/WHO Expert Committee on Food Additives (JECFA)[41] presents evaluations of certain veterinary drugs for subsequent drug residues in food products destined for human consumption. The report states that within a few hours of oral administration, more than 30% of ingested tylosin (a macrolide antibiotic) was bound to the faeces inside the intestinal tract of the animal, reducing the antimicrobial activity of the antibiotic. The antibiotic itself undergoes chemical breakdown as it passes through the digestive system, further reducing its antimicrobial activity.[41] So although tylosin is in the same antibiotic class as

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erythromycin, which is used in human treatment, it does not remain active in the digestive system to initiate bacterial stress.

## THE HUMAN CONTRIBUTION

In recent decades, veterinary and agricultural practices have come under frequent censure for their role in establishing and spreading antibiotic-resistant bacteria. At the same time, as noted in the Danish DANMAP report[32], there has also been increased clinical use of antimicrobials in human health care, probably because of an improved understanding of the pharmaceutics of antibiotic drugs, which has often resulted in higher dosages, increased hospitalisations, but shorter hospital stays.[32] In addition to this, the quantity of antimicrobials being used in human health systems is further augmented by drugs prescribed in the treatment of syndromes (e.g. AIDS), where the antimicrobials are not necessarily pathogen-specific.[42] European human epidemiological studies have shown a wide presence of integrons, including integron 1, with additional gene cassettes coding for various forms of resistance.[12] The origin of the host, namely humans, is suspected to contribute to the way gene transfer occurs. For both humans and other animals, the bacterial flora in the gut is significantly determined from birth by the host's diet and environment (prior to birth, the digestive tract is bacteria-free). Not only is the eventual composition of the gut flora in adults dictated by this early colonisation of the gut, but it is also uniquely shaped for each individual by the present and future welfare, and environmental hygiene to which a newborn is exposed.[43,44,45,46] Adlerberth et al.[45] reported that breastfeeding and environmental exposure resulted in increases in pathogenic Gram-negative bacteria in the gut flora of newborns in Sweden (a developed country) and Pakistan (a developing country).[47] This initial establishment of the eventual composition of the gut flora dictates the host's future immune response and prevents colonisation of foreign bacteria.[43] Mechanisms, not yet well defined, seem to assist in the establishment of the microbiota and it is speculated that there is a very specific relationship relating to the host-microbe interaction.[48] There is a clear likelihood that this relationship, founded in infancy and specific to each individual, could also be disturbed by the early administration of antibiotics and even probiotics. It has been suggested that clinical treatment should be based on the unique microbiota of each individual[48] – a possibility with future technology development.

## CONCLUSION

A large amount of information on the transfer of antibiotic-resistance genes has been derived from developed countries, including the prevalence of antibiotic resistance in food animals, the possible correlation to human health, subsequent antibiotic management strategies and ongoing surveillance programmes that have often led to new legislation and regulations. Molecular tools have highlighted genes linked to known, unknown and undefined bacteria.[27] The influence of these unknown organisms on the transfer of antibiotic resistance is speculative. Early-infancy colonisation of the gut determines the future bacteriological welfare of an individual. Taking into account the divergences in healthcare systems (including the prevalence of acute and chronic diseases with accompanied short-term and long-term antibiotic therapy), economic welfare and cultural habits, the average gut bacteria composition for African people is likely to be distinct from their Western counterparts. In all probability, bacterial evolution is a continual process determined by a survival strategy. Our focus on the continually changing micro-environment of bacteria has undoubtedly become sharper as we view it through 'the lens of antibiotic resistance'.

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# Prevalence of antibiotic resistance in *Campylobacter* isolates from commercial poultry suppliers in KwaZulu-Natal, South Africa

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***Objectives*: *Campylobacter jejuni* isolated from broiler and layer chickens from registered abattoirs in KwaZulu-Natal, South Africa, were tested for their susceptibility to eight antibiotics.**

***Methods*: Using agar dilution, susceptibility to eight antibiotics was determined for *C. jejuni* recovered from the caeca.**

***Results*: A total of 155 isolates were collected of which 77 were identified as *C. jejuni* (broilers $n = 56$ and layers $n = 21$). Resistance was highest to tetracycline (broilers 98.2% and layers 100%) and ceftriaxone (broilers 96.4% and layers 100%). High susceptibility was found to ciprofloxacin (broilers 91% and layers 76%) and gentamicin (broilers 98% and layers 81%). Susceptibilities to each of the antibiotics for the broilers and layers, respectively, were: 50% and 57% for erythromycin, 45% and 24% for clarithromycin, 68% and 43% for ampicillin and 64% and 48% for nalidixic acid. Statistically significant differences were detected for the $MIC_{50}$ of gentamicin, ciprofloxacin and tetracycline between broilers and layers ($P < 0.001$) with the $MIC_{90}$ of gentamicin also of significant difference ($P = 0.01$). Multiresistance was detected in 23% and 43% of the isolates from broiler and layer chickens, respectively.**

***Conclusions*: Mass therapy procedures used in animal husbandry have a potential impact on antibiotic resistance development in *C. jejuni*.**

Keywords: *Campylobacter jejuni*, antimicrobial resistance surveillance, food animals, growth promoters, animal reservoirs

## Introduction

Although banned in the European Union, many developing countries use antibiotics sub-therapeutically as growth promoters and as prophylaxis, usually as food/water supplements. *Campylobacter* species, especially *Campylobacter jejuni* and *Campylobacter coli*, are known zoonotic bacteria causing symptomatic diarrhoea in humans.[1] Humans are normally exposed to *Campylobacter* spp. through an oral pathway by handling and consuming uncooked meat, especially poultry products, faecal contaminated water and even raw vegetables.[2] Campylobacteriosis is generally described as a self-limiting disease that rarely requires antimicrobial intervention.

The aim of this study was to determine the susceptibility profiles of *C. jejuni* collected at slaughter from commercially produced broilers and layers in KwaZulu-Natal. Antibiotic susceptibility was determined for antibiotics commonly used in human therapy for Enterobacteriaceae infections in humans, namely fluoroquinolones, quinolones, tetracyclines, macrolides, aminoglycosides and β-lactam antibiotics (cephalosporins and penicillins).

## Materials and methods

### *Bacteria isolation*

Ethical approval was granted by the University of KwaZulu-Natal (AE/Bester/06). Caecal samples were collected from commercial broiler and layer chickens soon after slaughter from four registered chicken abattoirs in KwaZulu-Natal. Samples were filtered on Butzler plates and incubated at 41.5°C in a micro-aerophilic atmosphere (CampyGen, Oxoid) for 48 h.

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*Identification*

*Campylobacter* spp. were screened using conventional methods for campylobacters, which included oxidase and catalase tests, characteristic morphology after Gram stain, hippurate hydrolysis and susceptibility to nalidixic acid and cefalotin.

*Antibiotic susceptibility testing*

MICs were determined by agar dilution using a modification of Columbian agar supplemented with 7% lysed horse blood and *Campylobacter* growth supplements (Oxoid). Except for erythromycin, breakpoints for ciprofloxacin, tetracycline, ceftriaxone, clarithromycin, ampicillin, nalidixic acid and gentamicin were adapted from those used for Enterobacteriaceae: ciprofloxacin, MIC $\leq$ 1 mg/L; tetracycline, MIC $\leq$ 4 mg/L; ceftriaxone, MIC $\leq$ 8 mg/L; clarithromycin, MIC $\leq$ 2 mg/L; ampicillin, MIC $\leq$ 8 mg/L; nalidixic acid, MIC $\leq$ 8 mg/L; and gentamicin, MIC $\leq$ 4 mg/L.[3] A proposed guideline was followed for erythromycin: MIC $\leq$ 8 mg/L.[4] *Escherichia coli* (ATCC 25 922) and *Pseudomonas aeruginosa* (ATCC 27 853) were used as controls. STATA was used to analyse percentile values.

## Results

Of the 155 samples, 77 were identified as *C. jejuni*, of which 56 were collected from broilers and 21 were collected from layers. Table 1 shows the percentage resistance and $MIC_{50}$ and $MIC_{90}$ results with significant differences between broilers and layers evident for ciprofloxacin, tetracycline and gentamicin. Multiresistance, described as resistance to four or more antibiotics, was detected in 23% and 43% of the isolates from broiler and layer chickens, respectively.

## Discussion

Fluoroquinolones and macrolides are the drugs of choice for early treatment of campylobacteriosis.[5] The prevalence of ciprofloxacin resistance is a cause for concern, especially for the layer chickens (24%) that do not have as intensive exposure to antibiotics as do the broilers. Approximately half the isolates from the broilers (50%) and layers (47%) were resistant to erythromycin.

Even though layer chickens are not continuously exposed to growth promoter antibiotics in their feed, in the long run the therapeutic use of some drugs does have the potential to affect the susceptibility of *C. jejuni* when one considers the nature of poultry production. Normal commercial production methods make it difficult to treat individual animals mainly because of the large numbers of animals grouped together; thus, mass medication is the only option. However, addition of antimicrobial agents to water or feed may result in individual animals receiving inadequate curative doses or excessive prophylactic doses.[6] Such production practices may result in reservoirs of resistant *C. jejuni* and possibly other zoonotic pathogenic bacteria.

Cui *et al.*[7] examined *Campylobacter* originating from conventionally and organically reared chickens in the USA and showed similar levels of resistance to tetracycline (78%), erythromycin (46%) and ciprofloxacin (8%). In contrast, a Belgian study showed lower levels of resistance in broiler and layer chickens, respectively, for: tetracycline, 34.4% and 20%; and erythromycin, 6.3% and 8.6%. The study also showed a higher level of ciprofloxacin resistance in their broiler and layer chickens: 44.2% and 27.6%, respectively.[8] These fluctuations in resistance to common antibiotics differ from country to country and are reflective of local legislations that regulate the use of antimicrobials for animal production. The termination or more efficient regulation of veterinary antibiotics resulting in decreased resistance in zoonotic bacteria has been reported by several studies globally.[9] Unfortunately, South Africa has not taken any stance regarding the use of antibiotics in animal feed. The use of antibiotics, either sub-therapeutically or as growth promoters, in animal feeds is legislated under Act 36 of 1947 as *Stock Remedies* and is regulated by the Department of Agriculture.[10]

Children are particularly vulnerable to campylobacterioses through zoonotic acquisition. Clinical *Campylobacter* isolates collected at the Red Cross Children's Hospital in Cape Town, South Africa, from the period 1998 to 2005 have shown a steady increase in resistance to antibiotics: resistance to ciprofloxacin increased from 1.4% to 29%; to erythromycin from 3.4% to 7.2%; to nalidixic acid from 5.7% to 41%; and to ceftriaxone from 3.6% to 24.6%. For the first time, multiresistant

**Table 1.** Percentage resistance and $MIC_{50}$ and $MIC_{90}$ results for *C. jejuni* isolates collected from broiler and layer chickens in KwaZulu-Natal, South Africa

| | % resistance | | $MIC_{50}$ (mg/L) | | | $MIC_{90}$ (mg/L) | | |
|---|---|---|---|---|---|---|---|---|
| Antibiotic | broilers | layers | broilers | layers | *P* | broilers | layers | *P* |
| Ciprofloxacin | 8.9 | 23.8 | 0.031 | 0.25 | <0.001 | 0.5 | 64 | 0.06 |
| Tetracycline | 98.2 | 100 | 64 | 128 | <0.001 | 128 | 128 | NA |
| Erythromycin | 50 | 42.9 | 12 | 4 | 0.6 | 128 | 128 | NA |
| Ceftriaxone | 96.4 | 100 | 32 | 32 | 0.9 | 128 | 64 | NA |
| Clarithromycin | 55.4 | 76.2 | 16 | 64 | 0.3 | 128 | 128 | NA |
| Ampicillin | 32.1 | 57.1 | 8 | 16 | 0.07 | 16 | 32 | 0.9 |
| Nalidixic acid | 35.7 | 52.4 | 4 | 16 | 0.8 | 128 | 128 | NA |
| Gentamicin | 1.8 | 19 | 1 | 2 | <0.001 | 2 | 8 | 0.01 |

NA, not applicable.

*Campylobacter* isolates were also observed in South Africa.[11] Steady increases in fluoroquinolone and macrolide resistance and increased penicillin resistance were also observed in the Venda district, South Africa, from 2002 to 2007; resistance to ciprofloxacin has increased from 8% to 13%, to erythromycin from 25% to 53%, and to gentamicin from 8% to 17.3%. However, fewer clinical isolates were resistant to tetracycline (27%) and ceftriaxone (7%), which showed very high resistance among both broiler and layer chickens in this study.[12] The Venda district study did not specify the *Campylobacter* species, but it is accepted that the *Campylobacter* species mostly responsible for acute diarrhoea in humans is *C. jejuni*.

Unlike European surveillance programmes, such as DANMAP, the prevalence and antibiotic resistance of *Campylobacter* infections in South Africa are not monitored and no surveillance programme exists.[1] The importance of such bacteria is overshadowed by the existence of more severe diseases, for example, HIV infections, malaria, tuberculosis and salmonellosis.

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## Transparency declarations

None to declare.

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**Research Note**

# Observational Study of the Prevalence and Antibiotic Resistance of *Campylobacter* spp. from Different Poultry Production Systems in KwaZulu-Natal, South Africa

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## ABSTRACT

Campylobacter bacteria are important foodborne pathogens that cause acute diarrheal illness, and infection is often associated with contaminated poultry. In a blind observational study, the prevalence and resistance profiles of thermophilic *Campylobacter* strains collected from different poultry production systems were tested against the clinically used antibiotics ciprofloxacin, tetracycline, erythromycin, gentamicin, and streptomycin. *Campylobacter* strains were isolated from chickens in rural production systems, a free-range commercial facility, and industrially raised broiler and egg-laying chickens all situated in KwaZulu-Natal, South Africa. Isolates were collected from the chicken cecae and were identified with conventional methods and tested for antibiotic resistance with the Clinical and Laboratory Standards Institute agar dilution method. The prevalence of *Campylobacter* spp. isolates in chickens was 68% (56 samples) in rural production, 47% (140 samples) in commercial free-range broilers, 47% (133 samples) in industrial broilers, and 94% (34 samples) in industrial layer hens. Isolates from the rurally raised chickens showed significantly ($P < 0.01$) less resistance against ciprofloxacin (7.9%), erythromycin (0%), and tetracycline (21.6%) than those from commercially produced chickens. Isolates from the commercially raised chickens (free range and industrial) were highly resistant to tetracycline (98.9 to 100%). The incidence of gentamicin and streptomycin resistance was 1.6 and 11.5%, respectively, in commercial free-range broilers, 1.7 and 16.4%, respectively, in industrially raised broilers, and 12.9 and 40%, respectively, in industrially raised layers. It is possible that variations among the poultry production systems, including antimicrobial usage, result in differences in antibiotic resistance profiles in *Campylobacter*.

Bacterial stress in the gastrointestinal tract is caused by the therapeutic or prophylactic use of antibiotics during meat production, prompting the occurrence of antibiotic-resistant strains *(19)*. *Campylobacter jejuni* subsp. *jejuni* and *Campylobacter coli* have both been identified as pathogens that frequently cause acute diarrhea in humans, especially in children and the elderly *(15, 30)*. *Campylobacter* infection acquired through the oral route has been shown to have a possible connection with the development of Guillain-Barre syndrome *(18)*, and *Campylobacter* is the most frequently isolated bacterium that causes diarrhea in AIDS patients *(17, 25)*. The sources of these *Campylobacter* infections are often linked to meat products and, in particular, to poultry products *(21)*. Although the diarrhea caused by campylobacters is normally self-limiting, failure of the immune response results in the need for therapeutic intercession. The preferred first-line antibiotics are macrolides and fluoroquinolones, and thus, concern has been expressed about the health risk to humans of antibiotic-resistant *Campylobacter* strains associated with the use of antibiotics, often belonging to classes used for human therapeutic treatment, during meat and poultry production *(10, 31)*.

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In South Africa, the poultry industry accounts for 43% of the national total of animal-derived products *(27)*. Commercial poultry production is undertaken by industrialized and free-range farming systems, and antibiotics are utilized both therapeutically and prophylactically. However, there is also a third farming system widespread in suburban and rural KwaZulu-Natal and, indeed, throughout sub-Saharan Africa, where antibiotic usage is either limited or absent: namely, informal small-scale family farming (henceforth referred to as rural production), in which indigenous poultry roam freely and scavenge for food themselves *(8)*.

South Africa has no public health or food production surveillance program for *Campylobacter* spp. The objective of the present study was to evaluate the frequency of isolation and the antibiotic resistance profiles in *Campylobacter* isolates collected from each of the poultry production systems, namely, rural, commercial free-range broilers, industrialized broilers, and industrialized layers, in relation to the differing antibiotic sources and usage in each system.

## MATERIALS AND METHODS

Ethical clearance was obtained from the University of KwaZulu-Natal Animal Ethics Sub-committee (015/07/Animal). Except for the rural chickens, all samples were collected randomly from abattoirs during 2008 and 2009. No data on the specific

TABLE 1. *Prevalence of* Campylobacter *isolates collected from the different farming systems*

| | | % (no.) with isolates of: | | |
|---|---|---|---|---|
| Farming system | No. of samples | *C. jejuni* | *C. coli* | *Campylobacter* spp.[a] |
| Rural chickens | 56 | 29 (11) | 71 (27) | |
| Commercial free-range broilers | 140 | 48 (32) | 52 (34) | |
| Industrial broilers | 133 | 70 (44) | 25 (16) | 5 (3) |
| Industrial layers | 34 | 69 (22) | 9 (3) | 22 (7) |

[a] *Campylobacter* spp. excluding *C. jejuni* and *C. coli*.

antimicrobials used on the farms that supplied the abattoirs are available except what is known from commercial feed suppliers and legal usage. Antibiotics used in South African commercial poultry production as growth promoters and therapeutic treatment include tetracyclines (oxytetracycline and chlortetracycline) and macrolides (tylosin and kitamycin). Antibiotics indicated for therapeutic treatment only are beta-lactams (amoxycillin), quinolones (enrofloxacin and norfloxacin), and aminoglycosides (neomycin and spectinomycin) *(12, 28)*. A single large commercial free-range farm in KwaZulu-Natal province assisted with samples from an in-house abattoir for broiler chickens (5 to 8 weeks old). Samples from industrialized chickens, both broilers (5 to 8 weeks old) and layers (36 to 54 weeks old), were collected at four abattoirs situated in KwaZulu-Natal. Adult indigenous chickens were collected from rural communities in Port Shepstone, Mvoti, Maphumulo, and Shongweni, all in KwaZulu-Natal. Two adult chickens were collected from every third household in each locality. The birds were slaughtered at the Biomedical Resource Unit (a laboratory animal science unit in the University of KwaZulu-Natal), and samples collected from the cecae.

**Bacterial isolation and identification.** The Cape Town protocol *(14)* was used for isolating *Campylobacter* spp., but instead of isolating the organisms on antibiotic-free tryptose blood agar, a saline suspension of fecal matter collected from the ceca of each animal was passed through a 47-mm cellulose nitrate filter of 0.65-μm pore size (Sartorius Stedim Biotech, GmbH, Goettingen, Germany) onto a Butzler plate (*Campylobacter*-selective medium SR0085E, Oxoid, Ltd., Basingstoke, UK) and *Campylobacter* growth supplement SR0232E (Oxoid) containing 5% lysed horse or sheep blood. Suspected *Campylobacter* colonies were screened according to Gram staining and characteristic spiral morphology and further identified using biochemical testing based on indoxyl acetate hydrolysis, hippurate hydrolysis, growth at 42 and 24°C, and sensitivity to nalidixic acid (30 μg; Oxoid) and cephalothin (30 μg; Oxoid).

**Antimicrobial susceptibility testing.** *Campylobacter* strains were stored in tryptose soy broth (Oxoid) supplemented with 10% glycerol (ACE Pty., Johannesburg, South Africa) at −60°C until tested for antimicrobial susceptibility. The MIC (micrograms per milliliter) of an antibiotic required for total growth inhibition was determined by the agar dilution method of the Clinical and Laboratory Standards Institute (CLSI) *(4)*. The susceptibility breakpoints of *Enterobacteriaceae* for ciprofloxacin (Fluka AG, Buchs, Switzerland), tetracycline (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), and gentamicin (Sigma) *(3)* were used, together with the proposed CLSI guideline for susceptibility of *Campylobacter* spp. to erythromycin (Sigma) *(2)*. Epidemiological cut-off values in surveillance monitoring were used for streptomycin (Sigma) *(9)*. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 served as controls as described in the CLSI standard parameters for MIC susceptibility testing. *C. jejuni* ATCC 29428 served as a growth control.

**Statistical analysis.** Statistical validity was predetermined at 95%. Pairwise comparisons were made with Wilcoxon's rank sum test in cases where overall differences in the percentages of resistant thermophilic *Campylobacter* spp. were significant. The Stata version 10 statistical package (Stata, Inc., College Station, TX) was used for data analysis.

## RESULTS

**Prevalence of *Campylobacter* in the different poultry production systems.** There were 38 *Campylobacter* isolates collected from 56 samples from poultry at rural production facilities, 66 isolates from 140 samples from commercial free-range chickens, 63 isolates from 133 samples from industrial broilers, and 32 isolates from 34 samples from industrial layer chickens. The frequency of thermophilic *Campylobacter* spp. isolates in KwaZulu-Natal was 68% in poultry raised in rural farming systems, 47% in commercial free-range broilers, 47% in industrial broilers, and 94% in industrial layers. The ratio of *C. jejuni* subsp. *jejuni* prevalence to *C. coli* prevalence varied between the production systems, with *C. coli* more prominent in the rural (71%) and commercial free-range broiler (52%) groups, while *C. jejuni* subsp. *jejuni* was more dominant in the industrialized broiler (70%) and layer groups (69%) (Table 1).

**Antibiotic susceptibility.** The results show a relationship between restricted antibiotic usage and the prevalence of *Campylobacter* antibiotic-resistant strains in poultry production. Compared to the other groups, the rural group showed the lowest occurrence of resistance (Table 2). Isolates from the commercial free-range broiler and industrial broiler and layer groups showed higher resistance to tetracycline (100, 98.9, and 100%, respectively). Except in the case of erythromycin, the industrial layer group (normally older birds, aged 36 to 52 weeks) showed more resistance than the industrial broilers (normally younger birds, aged 5 to 8 weeks), although the significance was not determined.

**$MIC_{50}$s and $MIC_{90}$s.** The $MIC_{50}$s (MICs required to inhibit growth by 50%) and $MIC_{90}$s for the different production systems are described in Table 3. The rural production group's $MIC_{50}$s were low for ciprofloxacin (0.13 μg/ml), tetracycline (0.125 μg/ml), erythromycin (0.125 μg/ml), gentamicin (0.25 μg/ml), and streptomycin (1 μg/ml). The $MIC_{50}$s for commercial free-range chickens for ciprofloxacin (4 μg/ml), tetracycline (64 μg/ml), and

TABLE 2. *Frequencies of antibiotic resistance of* Campylobacter *isolates collected from the different farming systems*

| Antibiotic Class MIC breakpoint | No. and % of resistant isolates | | | | | | | | P values |
|---|---|---|---|---|---|---|---|---|---|
| | Rural | | Commercial free-range broilers | | Industrial broilers | | Industrial layers | | |
| | No. | % | No. | % | No. | % | No. | % | |
| Ciprofloxacin | | | | | | | | | |
| Quinolones | | | | | | | | | |
| MIC ≤ 1 | 38 | 7.9 | 66 | 95.4 | 63 | 15.9 | 32 | 17.7 | Rural vs commercial free-range broilers, $P < 0.01$; rural vs industrial broilers, $P = 0.4$; rural vs industrial layers, $P = 0.3$; commercial free-range broilers vs industrial broilers, $P < 0.01$ |
| Tetracycline | | | | | | | | | |
| Tetracyclines | | | | | | | | | |
| MIC ≤ 4 | 37 | 21.6 | 66 | 100 | 63 | 98.9 | 32 | 100 | Rural vs commercial free-range broilers, $P < 0.01$; rural vs industrial broilers, $P < 0.01$; rural vs industrial layers, $P < 0.01$; commercial free-range broilers vs industrial broilers, $P = 0.5$ |
| Erythromycin | | | | | | | | | |
| Macrolides | | | | | | | | | |
| MIC ≤ 8 | 38 | 0 | 66 | 87.9 | 63 | 47.6 | 32 | 43.7 | Rural vs commercial free-range broilers, $P < 0.01$; rural vs industrial broilers, $P < 0.01$; rural vs industrial layers, $P < 0.01$; commercial free-range broilers vs industrial broilers, $P = 0.04$ |
| Gentamicin | | | | | | | | | |
| Aminoglycosides | | | | | | | | | |
| MIC ≤ 4 | 37 | 0 | 61 | 1.6 | 60 | 1.7 | 31 | 12.9 | Rural vs commercial free-range broilers, $P = 0.9$; rural vs industrial broilers, $P = 0.9$; rural vs industrial layers, $P = 0.04$; commercial free-range broilers vs industrial broilers, $P = 0.9$ |
| Streptomycin[a] | | | | | | | | | |
| Aminoglycosides | | | | | | | | | |
| MIC ≤ 2[b] | 37 | 5.4 | 61 | 11.5 | 55 | 16.4 | 20 | 40 | Rural vs commercial free-range broilers, $P = 0.5$; rural vs industrial broilers, $P = 0.2$; rural vs industrial layers, $P < 0.01$; commercial free-range broilers vs industrial broilers, $P = 0.6$ |
| MIC ≤ 4[c] | | 0 | | 8.2 | | 12.7 | | 25 | Rural vs commercial free-range broilers, $P = 0.2$; rural vs industrial broilers, $P = 0.04$; rural vs industrial layers, $P < 0.01$; commercial free-range broilers vs industrial broilers, $P = 0.5$ |

[a] Due to the lack of a CLSI breakpoint for streptomycin, the European Food Safety Authority cut-offs for *C. jejuni* subsp. *jejuni* and *C. coli* were used *(9)*.
[b] MIC for *C. jejuni* subsp. *jejuni*.
[c] MIC for *C. coli*.

erythromycin (64 μg/ml) were significantly higher ($P < 0.01$) than those for poultry from the rural system. The $MIC_{50}$ for industrialized broilers for tetracycline (64 μg/ml) was significantly higher ($P < 0.01$) than that in the rural poultry system (0.125 μg/ml). The $MIC_{50}$ for industrial layers for tetracycline (128 μg/ml) was significantly higher than that for poultry in the rural system ($P < 0.01$).

The $MIC_{90}$s for ciprofloxacin in *Campylobacter* isolates from commercial free-range broilers (8 μg/ml), industrial broilers (16 μg/ml), and industrial layers (16 μg/ml) were significantly higher ($P < 0.01$) than those in *Campylobacter* isolates from poultry in the rural system (1 μg/ml). The $MIC_{90}$s for tetracycline were consistently high (128 μg/ml) for all the poultry systems investigated. The $MIC_{90}$s for erythromycin were significantly higher ($P < 0.01$) for commercial free-range broilers (128 μg/ml), industrial broilers (128 μg/ml), and industrial layers (128 μg/ml) than for poultry in the rural system (1 μg/ml). The $MIC_{90}$s for gentamicin were significantly higher in *Campylobacter* isolates from industrial layers (8 μg/ml) than from poultry in the rural system (0.25 μg/ml). The $MIC_{90}$s for streptomycin were significantly higher in *Campylobacter* isolates from industrial layers (20 μg/ml) than from poultry in the rural system (2 μg/ml).

TABLE 3. *MICs required to inhibit 50 and 90% of the growth of the* Campylobacter *isolates collected from the different farming systems*

| Antibiotic and % inhibition | MIC (µg/ml) Rural poultry | Commercial free-range broilers | Industrial broilers | Industrial layers | *P* values |
|---|---|---|---|---|---|
| Ciprofloxacin | | | | | |
| $MIC_{50}$ | 0.13 | 4 | 0.06 | 0.375 | Rural vs commercial free-range broilers, $P < 0.01$; rural vs industrial broilers, $P < 0.01$; rural vs industrial layers, $P < 0.01$; commercial free-range broilers vs industrial broilers, $P < 0.01$ |
| $MIC_{90}$ | 1 | 8 | 16 | 16 | Rural significantly lower than free-range broilers and industrial broilers and layers, $P < 0.001$ |
| Tetracycline | | | | | |
| $MIC_{50}$ | 0.125 | 64 | 64 | 128 | Rural vs commercial free-range broilers, $P < 0.01$; rural vs industrial broilers, $P < 0.01$; rural vs industrial layers, $P < 0.01$; commercial free-range broilers vs industrial broilers, $P = 0.5$ |
| $MIC_{90}$ | 128 | 128 | 128 | 128 | No difference in $MIC_{90}$s, $P = 0.06$ |
| Erythromycin | | | | | |
| $MIC_{50}$ | 0.125 | 64 | 4 | 4 | Rural vs commercial free-range broilers, $P < 0.01$; rural vs industrial broilers, $P < 0.01$; rural vs industrial layers, $P < 0.01$; commercial free-range broilers vs industrial broilers, $P < 0.01$ |
| $MIC_{90}$ | 1 | 128 | 128 | 128 | Rural significantly lower than free-range broilers and industrial broilers and layers, $P < 0.001$ |
| Gentamicin | | | | | |
| $MIC_{50}$ | 0.25 | 0.25 | 0.5 | 2 | Rural vs commercial free-range broilers, $P = 0.2$; rural vs industrial broilers, $P < 0.01$; rural vs industrial layers, $P < 0.01$; commercial free-range broilers vs industrial broilers, $P < 0.01$ |
| $MIC_{90}$ | 0.25 | 0.25 | 2 | 8 | Rural significantly lower than industrial broilers and layers, $P < 0.001$; free-range broilers significantly lower than industrial broilers, $P < 0.001$ |
| Streptomycin | | | | | |
| $MIC_{50}$ | 1 | 1 | 2 | 1.5 | Rural vs commercial free-range broilers, $P = 0.2$; rural vs industrial broilers, $P < 0.01$; rural vs industrial layers, $P = 0.3$; commercial free-range broilers vs industrial broilers, $P < 0.01$ |
| $MIC_{90}$ | 2 | 4 | 8 | 20 | All $MIC_{90}$s significantly different, $P < 0.008$ |

## DISCUSSION

**Limitations of the study.** Among the limitations of our study was its blind observational nature, since only one commercial free-range farm participated in the study. In addition, the prevalence of hippurate-negative *C. jejuni* subsp. *jejuni* isolates was not confirmed with molecular identification. It is probable that a small number of such isolates would have represented organisms mistakenly identified as *C. coli* or other hippurate-negative thermophilic *Campylobacter* spp. *(24)*.

**Prevalence.** Woodward et al. *(32)* found that humans and a variety of animals, including wild birds, rodents, pigs, and cattle, can spread contamination of *Campylobacter* spp. In the present study, poultry in rural farming systems are often only confined to a holding area in the evenings and have interaction with other animals, for example, cattle, sheep and rodents, in the areas they scavenge freely for food sources *(8)*. In a Tanzanian study, thermophilic *Campylobacter* spp. were isolated from rural chickens significantly more often than from commercial broiler chickens (76 versus 60%, $P < 0.01$) *(20)*, in a percentage comparable to the 68% prevalence of thermophilic *Campylobacter* spp. isolated from rurally raised poultry in the present study.

The ratio of the prevalence of *C. jejuni* subsp. *jejuni* isolates to that of *C. coli* isolates in the different farming systems suggested that *C. coli* dominated in the rural and commercial free-range broilers. However, this study is only observational and, thus, further investigations will be needed to clarify this dominance. Most other studies *(11, 13)* have found *C. jejuni* subsp. *jejuni* to be typically the predominant species in poultry, as was found in the industrialized broiler and layer groups in this study. Corry and Atabay *(5)*, in a study of reports from The Netherlands

and Northern Ireland, found that the prevalence of *C. coli* can often equal that of *C. jejuni* subsp. *jejuni* in live chickens and carcasses.

**Resistance.** In South Africa, the Department of Agriculture regulates the usage of antibiotics, prophylactically or as growth promoters, under the Stock Remedies Act No. 36 of 1947 *(7)*. Antibiotics for agricultural usage in South Africa are more freely available than in, for example, the United Kingdom, where growth-promoting antibiotics can only be obtained by prescription *(26)*. In rural settings, 87% of the family poultry systems studied in KwaZulu-Natal used traditional remedies originating mostly from plant material. In addition, the use of commercial products, and in particular, "Terramycin" and potassium permanganate, has also been reported *(8)*. The ciprofloxacin resistance found in the commercial production systems can be attributed to the use in these farming systems of enrofloxacin and norfloxacin. It has been reported that the use of enrofloxacin in poultry production (broilers) dramatically increased the resistance of *C. jejuni* subsp. *jejuni* isolates to ciprofloxacin *(22)*, nalidixic acid, and ofloxacin *(29)*.

Large amounts of tetracycline are used in the South African animal production system. A recent study in the Gauteng and Western Cape provinces of South Africa also reported high resistance of *Campylobacter* isolates from broiler poultry against tetracycline (95%), doxycycline (60%), and chlortetracycline (70%) *(12)*. The present study found that all the highly (>64 μg/ml) tetracycline-resistant isolates among the rural group were identified as *C. jejuni* subsp. *jejuni*. It is uncertain whether this was by coincidence and whether it has something to do with the expression of the *tet*(O) gene in *C. jejuni* subsp. *jejuni* and *C. coli*: tetracycline-resistant *C. coli* expresses the *tet*(O) gene at a chromosomal site, while the tetracycline-resistant *C. jejuni* subsp. *jejuni* expresses the gene on a conjugative plasmid *(6)*.

In a study (in this case a human study) by Putnam et al. *(23)*, *Campylobacter* isolates were obtained from stool samples collected from young children in a rural farming district of Egypt from 1995 to 2000. The study found low antibacterial resistance linked to an absence of antibiotic exposure; the isolates showed no resistance to the macrolides erythromycin and azithromycin and low resistance to ciprofloxacin, although this was observed to have increased from 17% in 1995 to 58% in 2000 *(23)*. At the time of the Putnam et al. study, fluoroquinolones were not indicated for pediatric treatment in the locality of the study but they were used in other community health contexts, and norfloxacin was used in food animal production. The investigators suggested that the increased antibiotic resistance of *Campylobacter* isolates from 1995 to 2000 may have come from exposure of the children to an antibiotic-resistant gene pool *(23)*.

The use of the macrolides tylosin and kitamycin in commercially produced free-range and industrialized poultry was reflected in the erythromycin resistance of *C. jejuni* subsp. *jejuni* and *C. coli* isolates encountered in our study. Lin et al. *(16)* noted that a single dose of tylosin given to a poultry flock had little effect on selection for erythromycin resistance in *Campylobacter* and that multiple exposures, typically the case when tylosin is used as a growth-promoting agent, were needed to establish erythromycin-resistant strains. Conversely, one would not expect to find resistance in rurally reared chickens.

This study is an expansion on the limitations of a previous study by Bester and Essack in 2008 entitled "Prevalence of antibiotic resistance in *Campylobacter* isolates from commercial poultry suppliers in KwaZulu-Natal, South Africa" *(1)*. The results of the present study highlight the prevalence of aminoglycoside resistance in commercially bred poultry.

In conclusion, the prevalence of *Campylobacter* isolates was higher in the rural and industrial layer chickens than in the industrial broilers and commercial free-range broilers, although study limitations do not permit extrapolation of the results as representative of the different poultry production systems. The study showed that when comparison is made between chickens from commercial production systems and rurally reared chickens, there is an association between lower antibiotic usage (in the rurally reared chickens) and reduced incidence of antibiotic-resistant *Campylobacter* isolates. The rurally reared chickens showed the lowest incidence of resistant *Campylobacter* isolates; the commercial free-range broiler and industrial broiler and layer groups showed high levels of tetracycline-resistant *Campylobacter* isolates, most likely attributable to on-going high levels of tetracycline use in commercial animal production systems, specifically, poultry production systems, in South Africa. The fact that antibiotic-resistant *Campylobacter* strains were also recovered from the rurally reared chickens is an indication that sources of antibiotic-resistant *Campylobacter* exist other than the development of resistance following therapeutic or prophylactic antibiotic treatments in animal production. A factor to consider in this regard is that the levels of hygiene are often lower in rural communities, heightening the incidence of common illnesses, and with the added prevalence of HIV/AIDS and tuberculosis, there is a likelihood of long- and short-term antibiotic use and abuse in the public health sector. The study also indicated that differing poultry production systems and antimicrobial programs can generate unique antibiotic resistance profiles.

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# Campylobacter species from different poultry farming systems – Identification, Antibiograms, Strain Typing and Plasmid Profiles.



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1 Title page

2 **_Campylobacter_ species from different poultry farming systems – Identification,**

3 **Antibiograms, Strain Typing and Plasmid Profiles.**

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24 strain typing

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26 **Synopsis**

27 **Objectives:** The objective of the study was to delineate the similarities/difference (if any) in
28 the antibiograms, plasmid profiles and strain types of *Campylobacter* spp. from different
29 poultry farming systems in KwaZulu-Natal, South Africa. The study additionally compared
30 phenotypic and genotypic identification methods of Campylobacter against the gold standard
31 of polymerase chain reaction (PCR) in terms of sensitivity, specificity, positive-predictive
32 value and negative-predictive value. **Methods:** Thermophilic *Campylobacter* isolates was
33 identified using conventional biochemical tests, specifically hippurate hydrolysis, MALDI-
34 TOF mass spectrometry and PCR with primers unique to *C. jejuni* and *C. coli*. Plasmid
35 analysis was undertaken using an alkaline lysis method and PFGE was executed according to
36 the Pulse Net protocol described for *Campylobacter* bacteria. **Results:** The MALDI-TOF was
37 shown to be superior to the biochemical tests for the identification of *C. coli* but equivalent to
38 the biochemical tests for *C. jejuni*. Plasmids were harboured in 84 % (16/19) of the isolates
39 from the free-range broilers, 83 % (10/12) of isolates from industrial layers, 72 % (18/25) of
40 the isolates from industrial broilers, and 77 % (10/13) of isolates collected from rural or
41 informally reared poultry. The PFGE genotyping of 42 *Campylobacter* isolates generated 39
42 *Sma*I types (47.6 % in *C. coli*, 33.3 % in *C. jejuni* and 2.4 % *C. lari*) of which 28 % were
43 amongst industrial broilers, 26 % amongst the free-range broilers, 26 % amongst industrial
44 layers, and 21 % amongst rural or informally reared poultry. There were no correlations of
45 any of the parameters within and between farming systems attesting to the diversity and
46 complexity of phenotypes and genotypes and indicating *de novo* evolution to antibiotic
47 selection pressure.

48

49

50

51 **Introduction**

52 Several studies have reported on the host specificity of *Campylobacter* spp. with strains of
53 animals and humans found to be less adapted for colonization and infection outside their
54 original hosts. [1, 2] Although animal-derived strains often show very little similarity to
55 clinical strains, researchers are still finding it difficult to confirm campylobacter species as
56 those of human origin because of the high genetic variations. [3] Thakur *et al.* (2009)
57 suggested that the manifold methods of food animal production selects for "ecologically fit
58 strains" when studying *C. coli* using pulse-field-gel-electrophoreses (PFGE) and multi-locus
59 sequence typing (MST) analysis.[4] Fitzgerald *et al.* (2001) indicated that the survival or
60 dominancy of a *Campylobacter* strain lies is in its ability to genomically adapt by rearranging
61 its genome, [3] while Taboada *et al.* (2004) reported in a study using microarray genomic
62 hybridization analysis that the genomic rearrangement of *C. jejuni* spp. *jejuni* was not
63 random, but restricted to certain areas of the genome leaving a large portion stable.[5]
64 However, genetic diversity may be higher in production systems where there is exposure to a
65 variety of environmental sources contaminated with *Campylobacter*. [6]

66

67 The study compared *Campylobacter* spp. isolated from different farming systems in terms of
68 antibiograms, strain typing and plasmid analysis. Secondarily it compared the phenotypic and
69 genotypic identification methods, viz., biochemical testing and matrix assisted laser
70 desorption ionization- time of flight (MALDI-TOF) mass spectrometry to the gold standard
71 of polymerase chain reaction (PCR) method in terms of sensitivity, specificity, positive-
72 predictive value and negative-predictive value.

73

74

75

76 **Materials and methods**

77 *Campylobacter* strains

78 A total of 69 isolates obtained from three different farming systems were used, viz., 13
79 isolates from rural or informal farms, 19 from a free-range system and 37 from an industrial
80 farming system, the latter further stratified into industrial broilers (25 isolates) and industrial
81 layers (12 isolates). These isolates were screened as thermophilic *Campylobacter* using the
82 conventional methods including Gram staining for unique spiral morphology, growth at
83 41.5°C, naladixic acid and cephalothin disc susceptibility and hippurate hydrolysis. *C. jejuni*
84 spp. *jejuni* is positive and *C. coli* negative for the hippurate hydrolysis tests.

85

86 Antibiotic Susceptibility MIC's

87 The minimum inhibitory concentration (MIC) were done using the CLSI agar dilution
88 method [7] as described previously [8] on an antibiotic panel consisting of tetracycline,
89 ciprofloxacin, erythromycin, gentamicin and streptomycin. *Escherichia coli* ATCC 25922
90 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls.

91

92 PCR

93 PCR identification of *Campylobacter* was conducted according to Linton *et al.* (1997) [9].
94 DNA extraction was accomplished using the heat-lysis method.. The DNA samples were
95 quantified spectrophotometrically using the NanoDrop 2000 (ThermoScientific, South
96 Africa) and standardised to 15 ng/µl with nuclease free water. For the identification of *C.*
97 *jejuni*, the region of the hippurate gene (HipO) consisting of 735 base pairs was used. The
98 primer pairs used were: forward 5′-GAA GAG GGT TTG GGT GGT-3′ and reverse 5′-AGC
99 TAG CTT CGC ATA ATA ACT TG-3′ [9]. For *C. coli* identification a region of the putative
100 aspartokinase gene was used with an amplicon of 500 base pairs. The primer pairs used were:

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forward 5′-GGT ATG ATT TCT ACA AAG CGA G-3′ and reverse 5′-ATA AAA GAC TAT CGT CGC GTG-3′ [9]. The primers were commercially synthesized by Inqaba Biotechnical Industries (Pty) Ltd. (Johannesburg, South Africa).

The PCR reaction volume for each sample was 40μl, comprising of: GoTaq® Flexi Buffer, 1.5mM $MgCl_2$, 200μM of each dNTP, 0.5 U GoTaq®DNA polymerase (Promega), and 45ng genomic DNA template. For the HipO (735) 30 pmol of forward and reverse primers was used and for the putative aspartokinase (500) 10 pmol of forward and reverse primers were added to the mastermix. Amplification conditions were as follows: initial denaturation (95 °C, 5 min), followed by 35 cycles of denaturation (95 °C, 30 sec), annealing (58 °C, 30 sec) and extension (72 °C, 30 sec). Final extension was achieved at 72°C for 5 minutes using the Chromo4 DNA Engine Thermocycler (Bio-Rad, South Africa).

MALDI-TOF analysis

The isolates were identified using MALDI-TOF mass spectrometry (Autoflex, Bruker Daltonics) by the direct application method. Using a wooden applicator a small quantity of colonies of 24 - 48 hr cultures from a micro-aerophilic atmosphere were smeared on a microtitre spot of a 384 polished steel plate (Bruker Daltonics, Bremen, Germany). This was overlayed with 1 μL matrix which is a saturated cyano-4-hydroxycinnamic acid solution (HCCA) (Bruker Daltonics, Bremen, Germay) and allowed to dry. Calibration was done with the Bruker Daltonics bacteria test standard (BTS) which is an *E. coli* control sample. Each isolate was executed in duplicate and set in linear mode with the parameters set at mass range of 2 – 20 kDa, ISI 20 kV, ISI 18.62 kV, lens 6.5 kV. Spectra were analysed using the MALDI Biotyper 3.0 software (Bruker Daltonics, Germany) and the Bruker Genotypic database was selected for the identification framework. The peaks generated were automatically compared

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126 to the data in the genotypic data base and its probability conveyed to a score. The range of
127 scores and descriptions were analysed as follows: 2.300 to 3.000 are highly probable species
128 identification; 2.000 to 2.299 are a secure genus identification and probable species
129 identification; 1.700 to 1.999 are probable genus identification; and 0.000 to 1.699 are not a
130 reliable identification (Bruker Daltonik MALDI Biotyper classification results).
131
132 Plasmid DNA screen
133 Plasmids were extracted using the alkaline lysis method (GeneJET Plasmid Miniprep kit,
134 Thermo Scientific) according to the manufacturer's plasmid purification protocol using the
135 buffers, solutions and columns supplied in the kit. The purified plasmid DNA was stored at
136 -20 °C until electrophoretic analysis. The agarose gel (1 %) was made with 0.5 x Tris-borate-
137 EDTA buffer (TBE buffer) (Fluka BioChemika, Sigma-Aldrich Chemie, Buchs). The
138 markers used were an *E. coli* NCTC 50192 strain with plasmid sizes 154 kb, 66.1 kb, 37.6 kb
139 and 7.7 kb and a 1 kb DNA ladder (Thermo Scientific O'GeneRuler, Lithuania). Following
140 ethidium bromide staining plasmids were visualised using UV light in a Bio Imaging System
141 (Chemi Genius2 Syngene). UVIgeltec.Ink (Jencons-PLS, UK) 'image acquisition software'
142 was used for the analysis.
143
144 Pulse-field gel electrophoreses (PFGE)
145 PFGE were performed using the guidelines of the Pulse Net protocol [10]. Plugs were prepared
146 from cultures that were incubated overnight on trypticase blood agar supplemented with 5%
147 lysed sheep blood. Plugs were transferred to 5 ml lysis solution which is TE buffer
148 containing 1 % sodium N-lauroylsarcosine (Fluka BioChemika, Sigma-Aldrich Chemie,
149 Buchs) and 25 μl of the proteinase K (20 mg/ml proteinase K, Thermo Scientific, Lithuania).
150 After 2 hrs of agitation plugs were rinsed with 3 washes of TE buffer and stored at 4 °C until

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151 analysis. Small slices of plug were treated with 2.5 μl *Sma*I (Thermo Scientific FastDigest,
152 Lithuania) restriction enzyme for 2 hrs. Plugs were loaded in a gel prepared with 1 % agarose
153 (for PFGE, Sigma-Aldrich, St Louis, USA) in 0.5 x TBE buffer together with a lambda
154 ladder (CHEF DNA size standard, Bio-Rad laboratories, Inc. USA) and *C. jejuni* ATCC
155 29428 as a positive control to evaluate the enzyme activity. Gels were electrophoresed in
156 0.5 x TBE buffer on a Bio-Rad CHEF MAPPER™ with the following auto algorithm setting:
157 low molecular weight set at 50 kb and high molecular weight set at 500 kb; calibration factor
158 1.00; gradient 6.0 V, running time 19 hrs, included angle 120°, initial switch time 6.75 s, final
159 switch time 38.35 s and ramping factor linear. Agarose gels were stained in ethidium bromide
160 solution (Fluka BioChemika, Sigma-Aldrich Chemie, Buchs) and visualised using UV light
161 in a Bio Imaging System (Chemi Genius$^2$ Syngene). Dendogram analysis was performed by
162 Bionumerics software version 6.6 (Applied Maths NV, Belgium). The similarities using the
163 band locations between the isolates were calculated using the Dice correlation coefficient set
164 at 1% tolerance.

165

166 **Results**

167 The results of the identification, minimum inhibitory concentrations, plasmid profiles and
168 strain types, are depicted for each of the farming systems in Table 1 - 4.

169

170 Identification and specificity

171 The sensitivity, specificity, positive predictive value and negative predictive value of the
172 hippurate and MALDI-TOF mass spectrometry were compared with PCR methods as the
173 gold standard for the identification of *C. coli* and *C. jejuni* bacteria as shown in Table 5. Of
174 the 68 isolates in the study 26 were identified as *C. jejuni* and 38 as *C. coli* with a single
175 isolate identified as *C. lari*. The MALDI-TOF mass spectrometry was shown to be superior to

176 the biochemical tests for the identification of *C. coli* but equivalent to the biochemical tests
177 for *C. jejuni*.

178 **Table 5**: Comparison of hippurate and MALDI tests to the gold standard (PCR) for
179 sensitivity, specificity, positive predictive and negative predictive values
180

| Test | Sensitivity | Specificity | Positive predictive | Negative predictive |
|---|---|---|---|---|
| Hippurate (*C. coli*) | 100 % | 86.7 % | 90.5 % | 100 % |
| Hippurate (*C. jejuni*) | 96.3 % | 100 % | 100 % | 97.6 % |
| MALDI-TOF (*C. coli*) | 100 % | 96.7 % | 97.4 % | 100 % |
| MALDI-TOF (*C. jejuni*) | 96.3 % | 100 % | 100 % | 97.6 % |

181
182
183 Plasmid profiles
184 The molecular weights of plasmids from the 19 isolates from free-range broilers ranged from
185 ~ 163 kb to ~ 57 kb and were harboured in 84.2 % (16 isolates) of the isolates. Of the 25
186 isolates from the industrial broilers 77.3 % (18 isolates) was found to harbour plasmids with
187 molecular weight ranging from ~ 170 kb to < 7.7 kb while industrial layer hens evidenced
188 plasmids in 12 isolates ranging in size from from ~ 293 kb to < 7.7 kb in 83.3 % (10 isolates)
189 of the isolates. A plasmid range of ~210 kb to ~39 kb was found in 75 % (9 isolates) of the
190 isolates from the rural farming system. Collectively plasmids were harboured in 76 % (29/38
191 isolates) of the *C. coli* isolates and in 80 % (20/25 isolates) of the *C. jejuni* strains. Images of
192 the plasmids from the different farming systems are depicted in Figure 1 (A-D).
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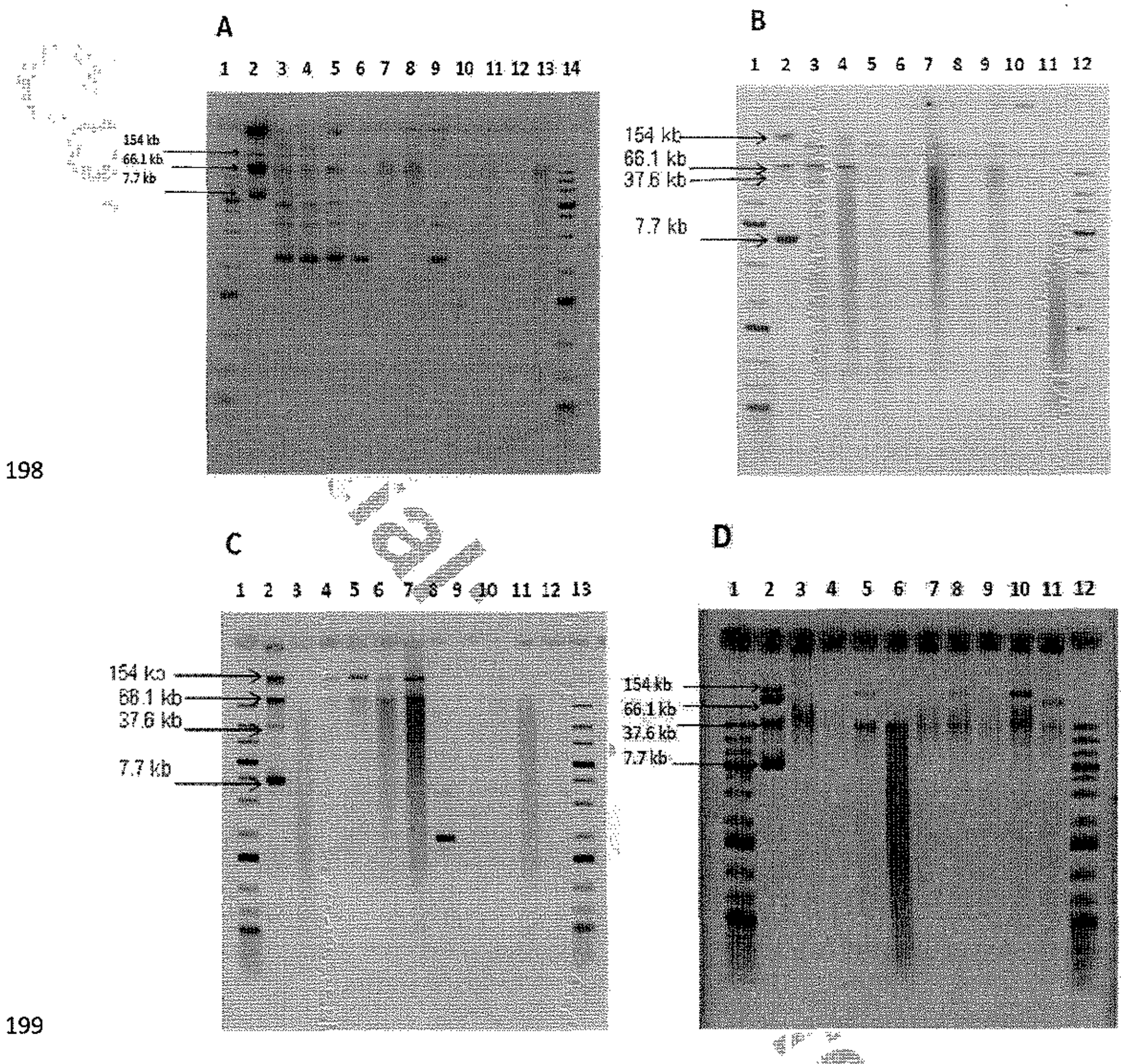


198

199

200

201 **Figure 1**: Images of the plasmid profiles of the isolates from the 3 farming systems (A) and
202 (C) were images from the industrial broilers and layer hens respectively; (B) free-range
203 broilers and (D) rural chickens. Lane 1 is the 1 kb ladder, and lane 2 the *E. coli* ATCC 50192
204 with plasmid sizes 154 kb, 66.1 kb, 37.6 kb and 7.7 kb.

205

206

207

208

209 Pulse-field gel electrophoreses

210 The PFGE genotyping of 42 *Campylobacter* isolates generated 39 *Sma*I types (Figure 2). Of
211 these 20 (47.6 %) were *C. coli*, 14 (33.3 %) *C. jejuni* and 1 (2.4 %) *C. lari* isolate. Substantial
212 and substantive genetic diversity was observed between and within farming systems although
213 various clusters of ~70 % similarity were seen within farming systems for both industrial and
214 free-range broilers. Just 3 identical isolates were identified amongst the 3 *C. jejuni* isolates
215 from the rural farming system and 2 amongst *C. coli* isolates from the free-range farming
216 system.

217

218

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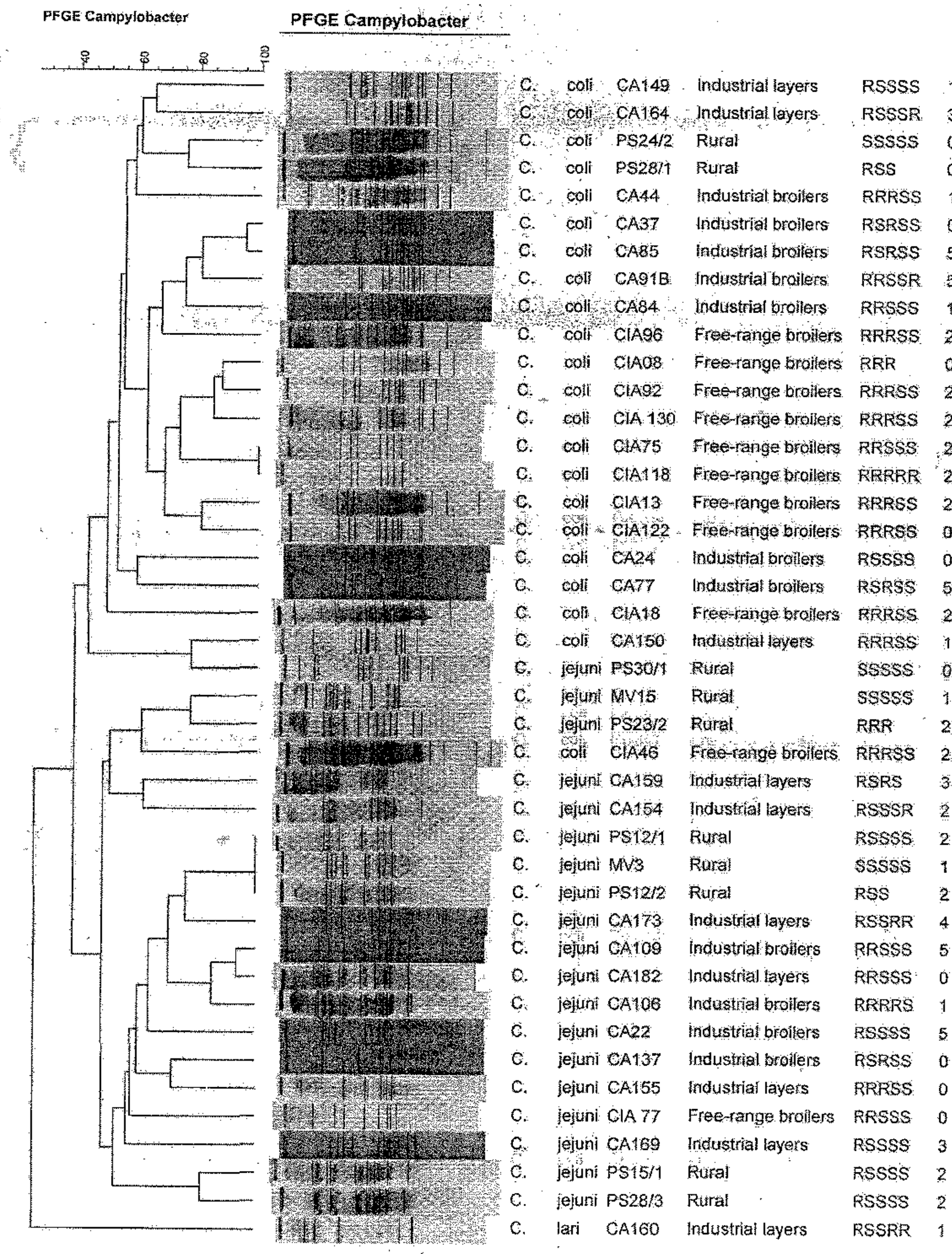


219

220 **Figure 2**: PFGE *Sma*I genotypic types generated from isolates from different farming systems. The R
221 and S indicate resistance or susceptibility respectively for tetracycline, ciprofloxacin, erythromycin,
222 gentamicin and streptomycin (in 4 cases the isolates were not tested for gentamicin and streptomycin).
223 The numeric values in the last column indicate the number of plasmids.

224 **Discussion**

225 Sensitivity of identification methods

226 Biochemical tests have been the initial form of bacterial identification and are based on the metabolic
227 characteristics of bacterial species. Together with a dichotomous key tree the results of a series of
228 biochemical tests can distinguish between bacteria even if they are closely related. These are similarly
229 used to identify *Campylobacter* [11]. However, within species a percentage of natural 'exception to the
230 rule' may exist [12] and together with technical errors in the laboratory the identification of a bacterial
231 species can be incorrectly concluded. The hippurate hydrolysis test is traditionally used to distinguish
232 between *C. jejuni* and *C. coli*, but is subject to inaccuracy if the laboratory execution was not optimal.
233 In addition, the existence of hippurate-negative *C. jejuni* species is well documented [13-15] and could
234 thus be incorrectly defined as a *C. coli* species. This was found with a single isolate from the
235 rural farming system that was identified as a hippurate negative *C. jejuni*, but without PCR
236 would have been incorrectly identified as a *C. coli* isolate. The polymerase-chain-reaction
237 (PCR) is a molecular based identification tool that focuses on a complete or partial gene that is unique
238 for the bacterial species. PCR identification for *Campylobacter* remains the gold standard
239 despite the methodology being time-consuming and labour intensive and despite
240 standardisation difficulties because of very different primers and PCR target genes have been
241 published to optimise PCR identification for *Campylobacter* [16]. As in the case of this study
242 the PCR primers chosen were very specific for either *C. jejuni* or *C. coli* excluding other
243 species. This was highlighted when testing isolate CA 160 with results showing that it was
244 neither *C. jejuni* nor *C. coli*. This isolate was found to be 99 % related to the *C. lari* ATCC
245 35221 strain when using primers that target the 16S ribosomal RNA gene.

246

247 Although the MALDI-TOF mass spectrometry apparatus is locally available albeit at a very
248 high price it has the ability to identify pure bacteria culture within a relatively short time (less
249 than an hour). This can be done directly without any biochemical testing. It is less labour

250 intensive especially when opting to use the direct method (indirect method also available) and
251 much less sample preparation is needed. In principle the MALDI-TOF mass spectrometry
252 uses a "soft" ionization technique by irradiating the crystalline structure of a prepared sample
253 resulting in the production of gas-phase ions. The ions accelerate through an electric field that
254 is detected and measured by calculating the "time-of-flight" (TOF), smaller molecules travel
255 faster and larger molecules take longer resulting in a spectrum compiled from the masses
256 measured. The spectrum created is compared to an existing data base of which the relatedness
257 is produced as a numeric score. [17, 18] Bessède *et al.* (2011) compared the identification
258 accuracy of MALDI-TOF with PCR methods where it was shown to be 100% comparable
259 with most thermophylic campylobacters showing only a 0.6 % error on *C. jejuni.* [19] Here the
260 indirect method has shown to be 100 % accurate and could be used as an alternative. The
261 indirect method is an extraction method where the cell suspension is pre-treated with ethanol
262 followed by formic acid and acetonitrile treatment to disrupt the cell walls. The supernatant is
263 then dispensed on the polished steel spot. Alispahic *et al.*, (2010) have been able to achieve a
264 100 % accuracy using the indirect method. [20] Dendogram peak analysis were also accurately
265 achieved when compared to PCR analysis using main spectra projection (MSP) and principle
266 component analysis (PCA) software [21], but the addition of the software can become labour
267 intensive and the MALDI-TOF is largely reserved as a research as opposed to a routine
268 diagnostic tool. If the MALDI-TOF mass spectrometry is going to replace traditional PCR
269 methods as an identification tool it might be worthwhile for the Bruker Daltonik agency to
270 continually update the Bruker Genotypic reference data base as new *Campylobacter* species
271 and strains are documented frequently.
272
273
274

275 Plasmids

276 Plasmids were present in isolates from all of the farming systems investigated in this study.
277 Although the sample size in this study was small it was noted that the isolates from the rural
278 chickens harboured the least plasmids (75 % of the isolates). The prevalence of plasmid in the
279 other farming systems, free-range broilers, industrial broilers and industrial layer hens were
280 84.2 %, 77.3 % and 83.3 % respectively. The frequency of *C. coli* (75 %) isolates harbouring
281 plasmids was found to be less than the *C. jejuni* (80 %) isolates. This is similar to a study by
282 Baserisalehi *et al.* (2008) who suggested that the incident of plasmids harboured by *C. coli* is
283 less than for example *C. jejuni* or *C. lari.* [22] In that particular study 50 % of the *C. coli* and 60
284 % of the *C. jejuni* harboured plasmids with the highest frequency, 80 %, found in *C. lari*
285 isolates. Of importance was the variation of the plasmid profiles noted between farming
286 systems. In industrial productions large numbers of birds are huddled together. These animals
287 are not exposed to the outside environment as free-range or rural farming systems. It is
288 suspected that in this environment genetic mutation, transformation and plasmid exchange
289 take place more rapidly as would be found in the other farming systems. Stress factors clearly
290 play a huge role in each of these faming systems and influence the health and ultimately how
291 both commensal and pathogenic organisms in the gastro-intestinal tract would react for the
292 sake of survival. Increased transformation was also found to take place with *Campylobacter*
293 spp. that supports the theory of a "specific recognition sequence" when DNA originates from
294 the same specie or related species. [23]

295

296 Pulse-field-gel-electrophoreses

297 The diversity of the *Campylobacter* genotypes is well-documented[30, 31] and supported in this
298 study. Genotypic diversity was evident for both *C. jejuni* and *C. coli* isolates within and
299 between farming systems although some degree of homogenousity is applicable to isolates



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300 from similar farming systems. It was also clear that *C. jejuni*, *C. coli* and *C. lari* are
301 genetically diverse although *C. coli* and *C. jejuni* are more closely related. It was not possible
302 to relate the genotypes to specific farming systems where growth promotor antibiotics were
303 either employed or not. Denis *et al.* (2008) studies much larger poultry population and also
304 failed to correlate clusters to specific production farms. [31] Anderson *et al.* (2011) found that
305 poultry rearing systems where the poultry have more environmental exposure increases the
306 number of *Campylobacter* genotypes, because of the multiple sources of contamination the
307 animals are exposed to. [6] Thus both free-range and rural farming systems have the ability to
308 generate diverse genotypes irrespective of the presence or absence of antibiotics.
309
310 **Conclusion**
311 The identification of *Campylobacter* species is challenging given that current PCR methods
312 are not standardised. However, MALDI-TOF spectrometry is a possible alternative diagnostic
313 tool. The variety of plasmid sizes found in the different farming systems indicate that
314 *Campylobacter* have the ability to carry and exchange genetic elements that would be more
315 prevalent during antibiotic stress. The lack of correlations amongst the parameters within and
316 between farming systems attested to the diversity and complexity of phenotypes and
317 genotypes and indicating *de novo* evolution in response to antibiotic selection pressure and
318 animal husbandry practices.
319
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326

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330

331 **Transparency declarations**

332 None to declare

333

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Table 1: Isolates from the free-range broilers showing the 3 identification methods, plasmid profiles and MIC's.

| Code | Identification | | | Plasmid analysis | | MIC mg/L | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | HIP | MALDI-TOF | PCR | Number of bands | MW of bands | TET | CIP | ERY | GEN | STR |
| CIA 13 | neg | *C. coli* 2.057 | *C. coli* | 2 | 151.739 68.375 | 128 | 4 | 64 | 0.25 | 2 |
| CIA 92 | neg | *C. coli* 2.106 | *C. coli* | 2 | 149.479 68.375 | 64 | 4 | 64 | 0.25 | 2 |
| CIA 75 | neg | *C. coli* 2.357 | *C .coli* | 2 | 154.000 64.044 | 256 | 64 | 2 | 0.25 | 1 |
| CIA 14 | neg | *C. coli* 2.123 | *C. coli* | 2 | 149.479 73.489 | 32 | 4 | 128 | 0.25 | 0.5 |
| CIA 20 | neg | *C. coli* 2.285 | not done | 2 | 147.221 69.584 | 64 | 4 | 64 | 0.5 | [a] |
| CIA 006 | neg | *C. coli* 2.292 | *C. coli* | 2 | 156.197 64.044 | 32 | 4 | 64 | 0.125 | 1 |
| CIA 18 | neg | *C. coli* 2.348 | *C. coli* | 2 | 149.479 69.584 | 32 | 4 | 64 | 0.125 | 1 |
| CIA 46 | neg | *C. coli* 2.232 | *C. coli* | 2 | 155.870 82.445 | 64 | 4 | 128 | 0.25 | 1 |
| CIA 130 | neg | *C. coli* 2.059 | *C. coli* | 2 | 144.941 59.971 | 128 | 4 | 128 | 0.25 | 0.5 |
| CIA 118 | neg | *C. coli* 2.464 | *C. coli* | 2 | 162.790 64.044 | 64 | 4 | 256 | 8 | 64 |
| CIA 96 | neg | *C. coli* 2.346 | *C. coli* | 2 | 154.00 66.100 | 32 | 4 | 64 | 0.125 | 1 |
| CIA 77 | pos | *C. jejuni* 2.281 | *C. jejuni* | 0 | --- | 256 | 32 | 2 | 0.25 | 2 |
| CIA 53 | neg | *C. coli* 2.317 | *C. coli* | 2 | 142.716 77.785 | 128 | 4 | 64 | 0.25 | 2 |
| CIA 56 | neg | *C. coli* 2.346 | *C. coli* | 2 | 151.735 57.958 | 64 | 8 | 128 | 0.25 | 0.5 |
| CIA 41 | neg | *C. coli* 2.192 | *C. coli* | 2 | 157.740 87.440 | 32 | 4 | 64 | 0.125 | 1 |
| CIA 30 | pos | *Enterococcus feacalis* 2.236 *C. coli* 2.126 | *C. jejuni* | 1 | 92.74 | 128 | 4 | 64 | 0.5 | 64 |
| CIA 48 | pos | *C. jejuni* 2.388 | *C. jejuni* | 1 | 72.215 | 256 | 64 | ≤1 | [a] | [a] |
| CIA 08 | neg | *C. coli* 2.347 | *C. coli* | 0 | --- | 64 | 4 | 64 | [a] | [a] |
| CIA 122 | neg | *C. coli* 2.420 | *C. coli* | 0 | --- | 32 | 4 | 32 | 0.25 | 1 |

HIP hippurate test; TET tetracycline; CIP ciprofloxacin; ERY erythromycin; GEN gentamicin; STR streptomycin

469 Table 2: Isolates from the industrial broilers showing the 3 identification methods, plasmid profiles
470 and MIC's.
471

| Code | Identification | | | Plasmid analysis | | MIC mg/L | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Hippurate | MALDI-TOF | PCR | Number of bands | MW of bands | TET | CIP | ERY | GEN | STR |
| CA 91 B | neg | *C. coli*<br>2.266 | *C. coli* | 5 | 91.901<br>35.562<br><7.7<br><7.7<br><7.7 | 16 | 16 | 1 | 0.125 | 128 |
| CA 77 | neg | *C. coli*<br>2.404 | *C. coli* | 5 | 86.028<br>35.562<br><7.7<br><7.7<br><7.7 | 32 | 0.007 | 16 | 0.5 | 2 |
| CA 22 | pos | *C. jejuni*<br>2.351 | *C. jejuni* | 5 | 169.696<br>38.959<br><7.7<br><7.7<br><7.7 | 8 | 0.007 | 0.062 | 0.25 | 2 |
| CA 28 | pos | *C. jejuni*<br>2.358 | *C. jejuni* | 5 | 91.901<br>38.959<br><7.7<br><7.7<br><7.7 | 16 | 0.015 | 0.062 | 0.25 | 1 |
| CA 27 | neg | *C. coli*<br>2.188 | *C. coli* | 1 | 84.949 | 128 | 0.062 | 1 | 0.25 | 2 |
| CA 42 | neg | *C. coli*<br>2.279 | *C. coli* | 1 | 70.84 | 64 | 0.031 | 32 | 0.25 | 2 |
| CA 85 | neg | *C. coli*<br>2.165 | *C. coli* | 5 | 165.221<br>82.445<br><7.7<br><7.7<br><7.7 | 64 | 0.007 | 16 | 0.5 | 1 |
| CA 44 | neg | *C. coli*<br>2.173 | *C. coli* | 1 | 42.575 | 128 | 4 | 32 | 0.25 | 2 |
| CA 29 | neg | *C. coli*<br>2.335 | *C. coli* | 1 | 66.1 | 128 | 0.062 | 1 | 0.25 | 1 |
| CA 109 | pos | *C. jejuni*<br>2.354 | *C. jejuni* | 5 | 150.332<br>91.901<br><7.7<br><7.7<br><7.7 | 128 | 128 | 4 | 0.5 | 1 |
| CA 23 | neg | *C. coli*<br>2.145 | *C. coli* | 0 | — | 2 | 0.062 | 0.5 | 0.25 | 1 |
| CA 31 | neg | *C. coli*<br>2.315 | *C. coli* | 0 | — | 32 | 0.007 | 16 | 0.25 | 2 |
| CA 106 | pos | *C. jejuni*<br>2.358 | *C. jejuni* | 1 | 46.963 | 128 | 32 | 128 | 8 | 1 |
| CA 21 | neg | *Bacillus circulans*<br>1.717 | not *C. jejuni* /*C. coli* | 1 | 84.245 | 32 | 0.031 | 0.125 | 0.5 | 2 |

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| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| CA 34 | neg | *C. coli* 2.376 | *C. coli* | 1 | 76.925 | 64 | 0.031 | 16 | 0.25 | 2 |
| CA 45 | pos | *C. jejuni* 2.028 | *C. jejuni* | 2 | 160.279<br>33.509 | 128 | 0.125 | 0.125 | 0.5 | 2 |
| CA 82 | neg | *C. coli* 2.232 | *C. coli* | 3 | 63.480<br>38.154<br>35.935 | 64 | 0.007 | 16 | 0.25 | 8 |
| CA 133 | neg | *C. coli* 2.108 | *C. coli* | 0 | ---- | 128 | 0.25 | 128 | 2 | 1 |
| CA 41 | neg | *C. coli* 2.187 | *C. coli* | 0 | ---- | 32 | 0.015 | 16 | 0.25 | 1 |
| CA 25 | neg | *C. coli* 1.985 | *C. coli* | 1 | 66.1 | 64 | 0.062 | 0.125 | 0.5 | 2 |
| CA 37 | neg | *C. coli* 2.329 | *C. coli* | 0 | ---- | 64 | 0.031 | 32 | 0.25 | 1 |
| CA 24 | neg | *C. coli* 2.418 | *C. coli* | 0 | ---- | 64 | 0.062 | 0.125 | 0.25 | 2 |
| CA 137 | pos | *C. jejuni* 2.058 | *C. jejuni* | 0 | ---- | 128 | 0.5 | 128 | 2 | 1 |
| CA 84 | neg | *C. coli* 1.998 | *C. coli* | 1 | 73.321 | 16 | 16 | 4 | 0.5 | 2 |
| CA 40 | neg | *C. coli* 2.308 | Not *C. coli* or *C. jejuni* | 1 | 138.232 | 64 | 0.031 | 16 | 0.5 | 1 |

472 HIP hippurate test; TET tetracycline; CIP ciprofloxacin; ERY erythromycin; GEN gentamicin; STR
473 streptomycin
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500 Table 3: Isolates from the industrial layer hens showing the 3 identification methods, plasmid
501 profiles and MIC's.
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| Code | Identification | | | Plasmid analysis | | MIC mg/L | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | HIP | MALDI-TOF | PCR | Number of bands | MW of bands | TET | CIP | ERY | GEN | STR |
| CA 151 | pos | *C. jejuni* 2.396 | *C. jejuni* | 2 | 117.383 64.890 | 128 | 0.25 | 4 | 2 | 1 |
| CA 160 | neg | *C. lari* 2.151 | Not *C. jejuni* nor *C. coli* | 1 | 63.747 | 128 | 1 | 4 | 8 | 32 |
| CA 159 | pos | *C. jejuni* 2.12 | *C. jejuni* | 3 | 283.652 178.173 68.730 | 128 | 0.5 | 128 | 2 | [a] |
| CA 169 | pos | *C. jejuni* 2.358 | *C. jejuni* | 3 | 178.173 76.462 30.111 | 128 | 0.5 | 4 | 1 | 2 |
| CA 173 | pos | *C. jejuni* 2.368 | *C. jejuni* | 4 | 292.443 173.777 76.462 30.111 | 128 | 0.125 | 0.5 | 16 | 128 |
| CA 164 | neg | *C. coli* 2.220 | *C. coli* | 3 | <7.7 <7.7 <7.7 | 128 | 0.125 | 0.5 | 0.5 | 4 |
| CA 150 | neg | *C. coli* 2.305 | *C. coli* | 1 | 113.143 | 128 | 16 | 128 | 2 | 1 |
| CA 154 | pos | *C. jejuni* 2.385 | *C. jejuni* | 2 | 163.767 66.100 | 128 | 0.125 | 1 | 1 | 8 |
| CA 155 | pos | *C. jejuni* 2.383 | *C. jejuni* | 0 | ---- | 128 | 8 | 128 | 2 | 4 |
| CA 140 | pos | *C. jejuni* 2.305 | *C. jejuni* | 2 | 112.438 33.300 | 32 | 0.5 | 4 | 2 | 4 |
| CA 182 | pos | *C. jejuni* 2.297 | *C. jejuni* | 0 | ---- | 128 | 64 | 4 | 1 | 2 |
| CA 149 | neg | *C. coli* 2.338 | *C.coli* | 1 | 160.511 | 128 | 0.125 | 0.5 | 2 | 0.5 |

503
504 HIP hippurate test; TET tetracycline; CIP ciprofloxacin; ERY erythromycin; GEN gentamicin; STR streptomycin
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511 **Table 4:** Isolates from the **rural chickens** showing the 3 identification methods, plasmid
512 profiles and MIC's.

| Code | Identification | | | Plasmid analysis | | MIC mg/L | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Hippurate tests | MALDI-TOF | PCR | Number of bands | MW of bands | TET | CIP | ERY | GEN | STR |
| PS 23/1 | pos | *C. jejuni* 2.303 | *C. jejuni* | 1 | 63.108 | 32 | 4 | 1 | 0.25 | 1 |
| PS 15/1 | pos | *C. jejuni* 2.28 | *C. jejuni* | 2 | 209.940 42.753 | 128 | 1 | 0.5 | 0.25 | 1 |
| PS 19/2 | neg | *C. jejuni* 2.084 | *C. jejuni* | 2 | 196.455 41.442 | 16 | 0.06 | 0.125 | 0.25 | 1 |
| PS 28/3 | pos | *C. jejuni* 2.203 | *C. jejuni* | 2 | 175.096 20.495 | 128 | 0.5 | 0.5 | 0.25 | 1 |
| MV 15 | pos | *C. jejuni* 2.14 *C. coli* 2.385 | *C. jejuni* | 1 | 66.1 | 0.5 | 0.5 | 0.25 | 0.25 | 0.5 |
| PS 28/1 | neg | *C. coli* 2.273 | *C. coli* | 0 | ---- | 128 | 0.5 | 0.5 | [a] | [a] |
| PS 23/2 | pos | *C. jejuni* 2.414 | *C. jejuni* | 2 | 137.180 74.105 | 32 | 2 | 0.5 | [a] | [a] |
| PS 12/2 | pos | *C. jejuni* 2.200 | *C. jejuni* | 2 | 127.984 74.105 | 256 | 0.25 | 0.25 | [a] | [a] |
| MV 3 | pos | *C. jejuni* 2.102 | *C. jejuni* | 1 | 140.897 | 4 | 0.5 | 4 | 0.25 | 1 |
| MV 8/2 | neg | [a] | *C. coli* | 2 | 182.970 41.840 | 128 | 4 | 1 | 2 | 2 |
| PS 30/1 | pos | *C. jejuni* 2.238 | *C. jejuni* | 0 | ---- | 0.125 | 0.5 | 0.125 | 0.03 | 1 |
| PS 24/2 | neg | *C. coli* 2.368 | *C. coli* | 0 | ---- | 0.125 | 0.125 | 0.125 | 0.25 | 1 |
| PS 12/1 | pos | *C. jejuni* 2.147 | *C. jejuni* | 2 | 205.445 39.445 | 256 | 0.5 | 0.5 | 0.25 | 1 |

513 [a] Not done
514 TET tetracycline; CIP ciprofloxacin; ERY erythromycin; GEN gentamicin; STR streptomycin
515
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## 2.4 Conference proceedings

2.4.1 Bester L.A. and Essack. Antibiotic resistance in *Campylobacter* isolates from poultry in KwaZulu-Natal. FIDSSA 2nd joint congress 28-31 October 2007 Cape Town. Poster presentation (SENS/003).

### 7. Antibiotic resistance in *Campylobacter* isolates from poultry in KwaZulu-Natal

*LA Bester, SY Essack*

**Introduction**

Humans are exposed to *Campylobacter* spp through pathways that include animals, food and the environment. The use of antibiotics as growth promoters by commercial food animal producers has the ability to select for bacteria resistant to antibiotics. The aim of this study was to determine antibiotic sensitivity profiles of *Campylobacter* isolates from poultry against antibiotics commonly used in human therapy.

**Methods**

Thermophilic *Campylobacter* spp were isolated from the caecum of broiler (5-7 weeks) and cornish (38-42-week old) chickens. Isolates were subjected to MIC determinations using the CLSI agar dilution method.



South Afr J Epidemiol Infect 2007; Vol 22 (2,3)



**Results**

The percentage resistance detected in *C. jejuni* isolated in broilers (n=55) was 4% in ciprofloxacin, 93% in tetracycline, 49% in erythromycin, 31% in ceftriaxone, 55% in clarithromycin, 7% in ampicillin, 25% in nalidixic acid and 0% in gentamycin. A definite shift towards increased resistance was evident in cornish chickens (n=17) which showed 17% resistance to ciprofloxacin, 100% to tetracycline, 44% to erythromycin, 50% to ceftriaxone, 67% to clarithromycin, 33% to ampicillin, 39% to nalidixic acid and 17% to gentamycin. Multi-resistance was detected in 18% of the isolates from broiler chickens showing resistance to 4 or more antibiotics. In contrast, 41% of the isolates from cornish chickens showed resistance to 5 or more antibiotics.

**Conclusion**

Reservoirs of resistance genes in food animals have the potential to spread to the human population, thereby decreasing already dwindling antibiotic treatment options.

2.4.2 Bester LA, Basardien L. and A.J. Lastovica. Antibiotic-resistance patterns of *Campylobacter jejuni*, *C. concisus* and *C. upsaliensis* isolated from stools of paediatric patients in Cape Town, South Africa, 1998-2011. Poster and oral presentation presented at the 16th *Campylobacter*, *Helicobacter* and Related Organisms (CHRO) workshop, 26 Aug - 1 Sep 2011, Vancouver, Canada.

A150

**Antibiotic resistance patterns of *Campylobacter jejuni, C concisus* and *C upsaliensis* isolated from paediatric patients in Cape Town, South Africa, 1998-2011**

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**Introduction:** From 1998 to 2011, a 14-year period, 17,802 paediatric diarrhoetic stools were submitted to the diagnostic microbiology laboratories at Red Cross Children's Hospital and Groote Schuur Hospital in Cape Town, South Africa. 2,946 isolates of *Campylobacter* spp. were identified. Antibiotic susceptibility studies were done by disc diffusion at 37°C with erythromycin (15 µg), ceftriaxone (30 µg), ciprofloxacin (1 µg), and tetracycline (5 µg). During 2010 and 2011, *Campylobacter* isolates were collected from commercial poultry farming systems (free-range and industrialized) in KwaZulu-Natal, South Africa. MICs for tetracycline were determined using agar dilution.

**Results:** Since 1998, erythromycin resistance for *C jejuni* (n=1,232) increased from 3.4% to 7.2%. Ciprofloxacin resistance increased from 1.4% to 31.0%; ceftriaxone resistance increased from 3.6% to 24.6%; and tetracycline resistance increased from 14.2% to 78.0%. *C concisus* isolates (n=680) showed increasing resistance to ciprofloxacin 6.9% to 18.0%; to erythromycin 4.8% to 27.3%; and to tetracycline 4.3% to 100%. Ceftriaxone resistance remained stable at 2.0%. *C upsaliensis* isolates (n=702) showed increasing resistance to tetracycline 4.3% to 100%. Erythromycin resistance was constant at 9.0%, ciprofloxacin at ~1.0% and no ceftriaxone resistance was noted. An increase in antibiotic-resistant *Campylobacter* was also noted for paediatric blood cultures, adult stools and chicken isolates. In 2005, and subsequently, multiple antibiotic resistant isolates (i.e. ciprofloxacin + erythromycin + ceftriaxone) of *Campylobacter* were isolated from paediatric stools and blood. Collective *Campylobacter* isolates from chicken (n=161) showed 98.0% resistance to tetracycline.

**Conclusion:** Observed increases in antibiotic resistance in clinical and chicken isolates of *Campylobacter* are relevant for an understanding of transmission, and other parameters. Tetracycline usage in the poultry industry is possibly contributing to the increasing resistance observed in the clinical isolates.

# CHAPTER THREE

# CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS

## 3.1 Conclusion

The following are the main conclusions of the study:

- *Campylobacter* spp. were prevalent in all the farming systems, ranging from 47- 94 %, with the lowest rates of isolation in the broilers and the highest in layer hens.

- *Campylobacter* spp. were present in industrial poultry production systems in South Africa with concerning levels of resistance against first-line antibiotics, such as tetracycline, ciprofloxacin and erythromycin, which are used to treat campylobacterioses in human health care.

- Resistance was highest to tetracycline (industrial broilers 98.2% and layers 100%), with the highest susceptibility noticed for ciprofloxacin (broilers 96.4% and layers 100%). In addition, multi-resistance was detected in isolates from the industrial broiler (23%) and layer hens (43%).

- There is a clear relationship between restricted antibiotic usage and the prevalence of *Campylobacter* antibiotic-resistant strains in poultry production. Compared to the other groups, isolates from rural farming systems were significantly less resistant than isolates from commercial free-range and industrial broiler and layer systems.

- Plasmids were harboured in 75% and above of the isolates tested, and originated from all the different farming systems. However, no correlation between the presence or absence of plasmids and the antibiogram profile could be concluded.

- The PFGE genotyping of 42 *Campylobacter* isolates generated 39 *Sma*I types, and it was evident that substantial and substantive genetic diversity was observed between and within farming systems.

- There were no correlations among parameters within and between farming systems attesting to the diversity and complexity of phenotypes and genotypes and indicating *de novo* evolution to antibiotic selection pressure.

- MALDI-TOF mass spectrometry was shown to be superior to the biochemical tests for the identification of *C. coli* but equivalent to the biochemical tests for *C. jejuni*. The identification of *Campylobacter* species is challenging, given that current PCR methods are not standardised. However, although MALDI-TOF spectrometry technology has shown to have important advantages, in South Africa the apparatus is expensive to purchase and it is uncertain if it could be considered as a diagnostic tool in the near future. Thus, biochemical screening is still cheaper and the use of alternative advanced molecular techniques to confirm *Campylobacter* identifications at specie level considered a preferred method of diagnosis.

In addition, the study has shown a number of important aspects concerning antibiotic resistance via the food chain using *Campylobacter* as a case study:

The prevalence of *Campylobacter* was widespread in all farming systems, irrespective of the presence or absence of antibiotics. In fact, the industrial farms could show less prevalence if the biosecurity were improved, the current risks being increased during 'thinning', a process where the poultry stock is reduced for slaughtering and the biosecurity temporarily compromised (Allen *et al*., 2007).

Antibiotic usage on an animal production farm has the ability to create antibiotic resistant strains of bacteria that can be reduced with the prudent use of antibiotic growth promoters and therapeutic antibiotics. However, this does not guarantee the absence of antibiotic resistant strains. Even chickens from the rural areas carried antibiotic resistant *Campylobacter* strains, therefore other sources of resistant genes with conjugative properties are likely to exist.

Therapeutic treatments of poultry also pose a risk, as was found in the layer hens where resistant strains are often maintained and do not necessarily disappear when the antibiotic treatment is complete. These strains have the opportunity to accumulate during the longer lifespan of the food animal.

Considering the high prevalence of *Campylobacter* in poultry, it is peculiar that larger numbers of human cases of campylobacterioses are not reported. This might indicate that not all infections originate from consuming infected poultry products. Flies, for example, have been reported to be an important source of infection and are probably also a source of

spread to humans, hence the difficulty of tracking the infections through epidemiology and genotyping studies (Hald *et al.*, 2007).

Caution must be taken when reporting on the source of clinical antibiotic resistant foodborne bacterial strains. A number of factors, not yet fully understood by scientists, may play a role including but not limited to host specify, natural bacteria evolution, the make-up of the commensal bacteria in humans/animals and the environment.

Finally, it is evident that little data is published on the transfer of antibiotic resistance via the food chain in South Africa. The possibility that the agricultural sector and environment has many reservoirs of antibiotic resistant genes jeopardising the therapeutic treatments of clinically ill human patients needs further research. Not enough information is currently available to determine how antibiotic resistant *Campylobacter* and other foodborne bacteria impact on our health care system, and how it impacts on immune-compromised patients.

## 3.2 Limitations

This study focused on farming systems in the KwaZulu-Natal area. More farms and abattoirs from other geographical areas need to be included in an expanded study to ascertain the prevalence, antibiotic resistance, plasmid profile and strain typing country wide.

Cost constraints precluded the inclusion of all isolates in all tests. The use of representative samples may have under- and/or over-estimated results.

### 3.3 Recommendations

It is recommended that:

- Biochemical screening in tandem with advanced molecular techniques to confirm identifications at specie level is used for *Campylobacter* identification in view of the high cost of MALDI-TOF equipment and protocols.

- South Africa institutes a veterinary surveillance system to monitor antimicrobial use and susceptibility trends in selected zoonotic bacteria such as *Salmonella spp.* and *Campylobacter* spp.

- Surveillance generates the evidence base for regulations or legislations on certain agricultural practices and related occupational health and safety

- Communication between the medical and veterinary fraternities is strengthened in this regard.

One has to be cautious to state that the majority of clinical antibiotic resistant foodborne bacterial strains were disseminated via the food chain. Host specify, natural bacteria evolution, the make-up of the commensal bacteria in a human/animal and the environmental all play a huge role; not fully understood by scientists currently. The study recommends the establishment of surveillance centres to collect data on not only *Campylobacter*, but other clinically important foodborne bacteria in South Africa. This should include geographical surveillance data on clinical isolates from humans (including antibiotic susceptibility data) as well as surveillance data on isolates from animal production farms for especially poultry and swine, processed meat and the environment including water and soil sources.

Finally, it is recommended that the consequences of the use of antimicrobial growth promoters in South Africa receive more attention. Veterinarians and food animal producers need to be trained with respect to prophylactic treatment and possible human health issues related to antibiotic resistant bacteria. There is little published data on the transfer of antibiotic resistance via the food chain in South Africa. The possibility of several diverse reservoirs of antibiotic resistant genes jeopardising the antibiotic treatment of humans is thus a research area that needs attention.

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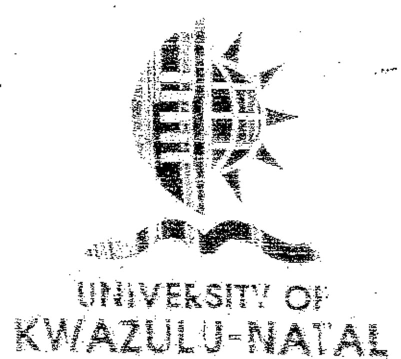
# APPENDICES

## Appendix 1 Ethical Approval




07 December 2005

Reference: AE/Bester/05

Mrs LA Bester
Biomedical Resource Unit
University of KwaZulu-Natal
WESTVILLE

Dear Mrs Bester

**Renewal: Ethical Approval of Research Project using Animals**

I have pleasure in informing you that the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for 2006 on the following project:

The prevalence and transfer of antibacterial resistance in food animals.

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

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5 February 2008

Reference: 019/08/Animal

Mrs LA Bester
Biomedical Resource Unit
University of KwaZulu-Natal
WESTVILLE CAMPUS

Dear Mrs Bester

**Renewal: Ethical Approval of Research Project using Animals**

I have pleasure in informing you that the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for 2008 on the following project:

"The prevalence and transfer of antibacterial resistance in food animals".

Yours sincerely

**Professor Theresa HT Coetzer**
**Chairperson: Animal Ethics Sub-committee**

Cc Registrar
Research Office
Head of School

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10 October 2008

Reference: 007/09/Animal

Mrs LA Bester
Laboratory Animal Technologist
Biomedical Resource Unit
University of KwaZulu-Natal
WESTVILLE

Dear Mrs Bester

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**Chairperson: Animal Ethics Sub-committee**

Cc   Registrar
     Research Office
     Head of School

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| Authors: | Bester, Linda<br>Chenia, Hafizah<br>Phulukdaree, Alisa<br>Bezuidenhout, Marian<br>Khan, Nasreen<br>Pillay, Manormoney<br>Essack, Sabiha |
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