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THE DEVELOPMENT AND VALIDATION OF A BACTERIOLOGICAL SCREENING TEST FOR ANTIMICROBIAL RESIDUES IN EGGS

By

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Submitted in partial fulfillment of the requirements for the degree of *Magister Scientiae* (Master of Science) in the Department of Production Animal Studies Faculty of Veterinary Science, University of Pretoria

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LIST OF ABBREVIATIONS

AbR+	:	Antimicrobial Residue positive
AbR-	:	Antimicrobial Residue negative
ADI	:	Acceptable Daily Intake
AMR	:	Anti Microbial Resistance
AR	:	Antimicrobial Residue
ATCC	:	American Type Culture Collection
APEC	:	Avian pathogenic *Escherichia coli*
AUCC	:	Animal Use and Care Committee
CAC	:	Codex Alimentarius Commission
CBD	:	Central Business District
CFU	:	Colony forming units
CI	:	Confidence Interval
CLSI	:	Clinical and Laboratory Standards Institute
CMAJ	:	Canadian Medical Association Journal
DANMAP	:	Danish Integrated Antimicrobial Resistance Monitoring and Research Programme
E	:	East
EC	:	European Commission
ELISA :	:	Enzyme-linked Immunosorbent assay
ER	:	Eastern route
EU	:	European Union
FAO	:	Food and Agricultural Organisation
FDA	:	Food and Drug Administration
g	:	Gram
GPS	:	Global Positioning System
GC	:	Gas Chromatography
HPLC	:	High Performance Liquid Chromatography
JIAC	:	Japan International Agricultural Council
Kg	:	Kilogram
ℓ	:	Litre
m^2	:	Metre square
MDC	:	Minimum Detection Concentration
MG	:	*Mycoplasma gallisepticum*
mg	:	Milligram
MMT	:	Million metric tons
mℓ	:	Millilitre
MRL	:	Maximum Residue Limits
MS	:	Mass Spectrometry
MSV	:	*Mycoplasma synoviae*

N	:	North
n	:	Number
NAFDAC	:	National Agency for Food Drug Administration and Control
NDV	:	Newcastle disease virus
NE	:	Not established
NR	:	Northern route
OIE	:	Office International *des* Epizooties
ORT	:	*Ornithobacterium rhinotracheale*
P	:	Prevalence
PCR	:	Polymerase chain reaction
pH	:	Logarithm for the reciprocal of hydrogen ion concentration in grams atom per litre, used to express the acidity or alkalinity of a solution on a scale of 0 to 14
PRU	:	Poultry Research Unit
R	:	Rand
RSA	:	Republic of South Africa
S	:	South
SM	:	Supermarket
SANAS	:	South African National Accreditation Standards
SANVAD	:	South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs
SAPA	:	Southern African Poultry Association
SR	:	Southern route
SS	:	Selected supermarkets
SVARM	:	Swedish Veterinary Antimicrobial Resistance Monitoring
TCTR	:	Tshwane Central Taxi Rank
TLC	:	Thin-Layer Chromatography
µm	:	Micrometer
USA	:	United State of America
USDA	:	United States Department of Agriculture
R_f	:	Retardation factor
µg	:	Microgram
µℓ	:	Micro litre
UV	:	Ultra-violet
VMD	:	Veterinary Medicine Directorate
W	:	West
WHO	:	World Health Organisation
WIPO	:	World Intellectual Property Organization
WR	:	Western route

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[1] http://www.tshwane.gov.za/streetmaps/Tshwane_regions.pdf. Accessed date 01/10/2008.
[2] 2011 Google - map data © 2010 AfriGIS (Pty) Ltd, Google - http://maps.google.co.uk

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SUMMARY

THE DEVELOPMENT AND VALIDATION OF A BACTERIOLOGICAL SCREENING TEST FOR ANTIMICROBIAL RESIDUES IN EGGS

By

ALEXANDER RAY JAMBALANG

Supervisor: Dr. Shahn P.R Bisschop

Co-supervisor: Dr. Jacqueline A. Picard

Department: Production Animal Studies

Degree: *Magister Scientiae* (Master of Science)

Microbiological screening of antimicrobial residues in eggs needs special attention because of the high level of naturally occurring inhibitors contained in eggs which often lead to false positive results. However, it was discovered that heating egg samples at $80^{0}C$ for 10 minutes inactivated the inhibitors. The new bacteriological screening test for antimicrobial residues in eggs which was developed during this study, contains viable spores of *Geobacillus stearothermophilus* which are sensitive to antimicrobial residues including beta-lactams, tetracycline's and macrolides. The new test method was validated based on the comparison with a reference method, namely the Kundrat micro-screening four-plate test,[1] and published literature of another standard reference method, the Premi®Test.

[1] Sigma-Aldrich SA (Pty) Ltd., P. O. Box 10434, Aston Manor 1630, South Africa.

A preliminary trial was conducted on 36 hens that were given therapeutic oral doses of over-the-counter antimicrobials daily for seven days with one of eleven antimicrobials based on the manufacturer's recommendations. Eggs were collected from the hens during and after treatment and tested for the presence or absence of antimicrobial residues. Several performance criteria and minimum detection concentrations were estimated and discussed. Some agreements and differences were found between the new and the reference tests with the new test being more sensitive to beta-lactams, tetracyclines and macrolides than the Kundrat and Premi®Test on the average. The use of florfenicol and norfloxacin in laying hens is banned and therefore there are no maximum residue limits (MRL) or published Premi®Test values. For meat, the MRL is 100mg/kg.

It was therefore concluded that the new screening test could be used for routine screening of antimicrobial residues in eggs.

A two seasonal survey was also conducted to determine the prevalence of antimicrobial residues in commercial chicken eggs in Tshwane area of Gauteng Province, South Africa. Although the season did not impact statistically on the antibiotic residues found in the eggs; eggs sold at the roadside (informal businesses), certain egg brands, and those sold at lower prices were found to be more likely to contain antibiotic residues than those obtained from formal outlets and at higher prices.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Poultry and poultry products are an important and cheap source of protein in South Africa and most other countries (Saif, Y.M., Barnes H.J., 2003; Southern African Poultry Association (SAPA), 2007; Gaudin, V., Hedou, C., Rault, A., Sanders, P. & Verdon, E., 2009); for this reason, chickens are reared as broilers for meat and or kept as layers for eggs under intensive or free range management systems. Over the years there has been a high and increasing demand for reasonably priced animal protein, especially poultry products (Tudor, F., Jordan, W. & Pattison, M., 2001; SAPA, 2007). In response to these demands, farmers have increased production by increasing the size of their enterprises, made use of genetically-improved chickens and antimicrobial performance enhancers (Gouws P. A. & Brozel V. S., 2000; Peter, L. & Fariborz, S.A., 2002; Donoghue J.D., 2003; Stolker, A.A.M., Zuidema, T. & Nielen, M.W.F., 2007). In intensive farming, opportunistic bacterial infections tend to spread rapidly causing diseases and even fatalities, often necessitating the use of antimicrobials. For practical reasons, there is often mass medication of all the animals of a particular flock in feed or drinking water (Witte, W., 1998; Donoghue, J. D. & Hairston, H., 2000; Donoghue, 2003; Dahiya, J.P., Wilkie, D.C., Van Kessel, A.G. & Drew, M.D., 2006).

Residues of drugs given to birds orally or parentally may be found in tissues, particularly when the birds are slaughtered without the observance of withdrawal periods, when eggs are harvested within the treatment or withdrawal period of the drug or if drugs are given above the recommended dose (Donoghue & Hairston, 2000; Kan & Petz, 2000; Gaudin *et al.,* 2009). Human consumers may therefore be exposed both to potentially allergenic antibiotic residues in their food products of poultry origin or find that their commensal microflora has become resistant to antibiotics that are normally reserved for the treatment of serious infections of humans. Of particular concern has been the use of the glycopeptide avoparcin® that has led to the development of resistance to its analogue vancomycin among enterococci in the intestinal tracts of chickens, which was then followed by the emergence of enteroccocal infections in hospitals that were resistant to vancomycin (Witte, 1998; Han, Y.C., Robert, L.R.H., Monica, K., Mark, W.C. & David, B., 2002; Tajick & Shoreh, 2006).

Because of the above problems, many governments, including those of the European Union (EU), have banned the use of antimicrobial performance enhancers in production animals and have set legal limits on the amount of antimicrobial residues (AR) that may be present in food of animal origin (European Commission, 1990; European Commission, 1996; European Commission, 2002; European Commission, 2006; Pig Health, 2003; Codex Alimentarius Commission/Maximum Residue Limit (CAC/MRL), 2006); these are known as the maximum residue limits for each antibiotic. A number of tests of varying sensitivity and specificity have consequently been developed to detect antimicrobial residues in order to reduce the health risks to consumers and ensure food safety; however, the tests are often expensive and thus not suited for mass testing. For this reason it was decided to develop a simple and cheap bacteriological test that could be used to screen for antimicrobial residues.
This project therefore aimed to partially validate the newly developed in-house bacteriological test for screening eggs for AR and also to screen samples of commercial chicken eggs for AR in the Tshwane area of South Africa using this relatively cheap method.

1.2 LITERATURE REVIEW

1.2.1 The Global poultry industry

The largest producer of poultry (broiler) meat in the world is the United States of America (USA) with a production of over 17 million metric tons (MMT). In 2007, more than 50% of worldwide broiler meat production occurred in only four countries viz. the USA, China, Brazil and Mexico which produced 21.7%, 13.9%, 11.8% and 3.4% respectively (SAPA, 2007).

China dominated world egg production in 2007, producing over 24 MMT or 41.3% of the 62.6 MMT of eggs produced worldwide. China is followed by the USA which produced 8.5% of the world's eggs, India at 4.3% and Japan at 4% (FAO, 2008; SAPA, 2008).

1.2.2 The South African poultry industry

In 2007, 202 countries produced 73.6 MMT of broiler meat and the Republic of South Africa (RSA) ranked 15th with a market share of 1.3%; of the 203 countries listed for shell egg production, South Africa is ranked 28th with a market share of 0.6% in the same year (SAPA, 2008). The poultry industry dominates the South African agricultural sector with turnover at producer level for 2008 of over R24.67 billion; it also provided about 61.4 % of all animal-product protein consumed in South Africa (Avi Africa, 2009). In addition, it provided employment to 77 000 people in the formal sector, which excluded smaller poultry producers, retail sales outlets and the informal sales sector (SAPA, 2007; Avi Africa, 2009).

In 2008, the South African broiler industry recorded a turnover of R18.6 billion (Avi Africa, 2009) and the Provincial distribution of broiler production, broiler grower and broiler breeder farms of members of the Southern African Poultry Association (SAPA) for 2008 are shown in Figure 1.1.

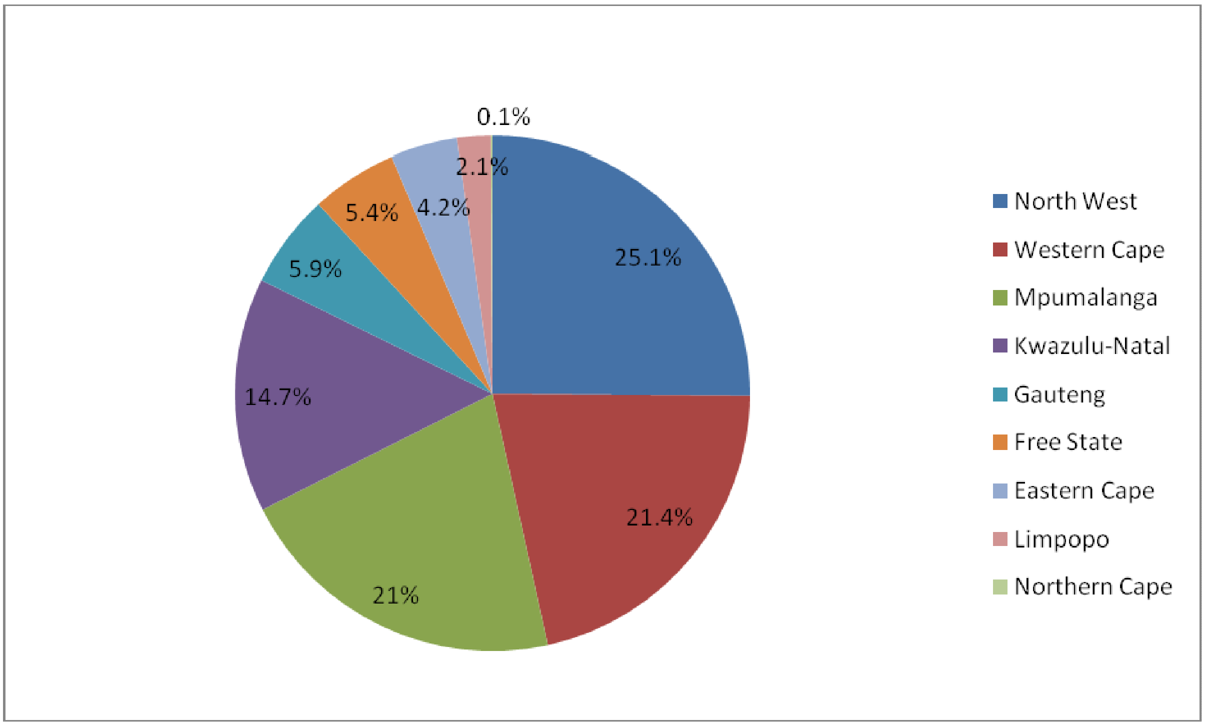

Figure 1.1: Provincial distribution of broiler production and broiler breeder farms of SAPA members in South Africa for 2008 (SAPA, 2007).

The egg industry produced and sold a total of 546 and 558 million dozen eggs in 2007 and 2008 respectively with a gross turnover of R6.04 billion at producer level for 2008 (SAPA, 2008; Avi Africa, 2009). The chick industry turnover for 2008 was R1.29 billion for chick supply to the egg and R2.97 billion for chick supply to the broiler industries (Avi Africa, 2009). The provincial distribution of egg production and egg layer farms of SAPA members amongst provinces in RSA in 2008 is shown in Figure 1.2.

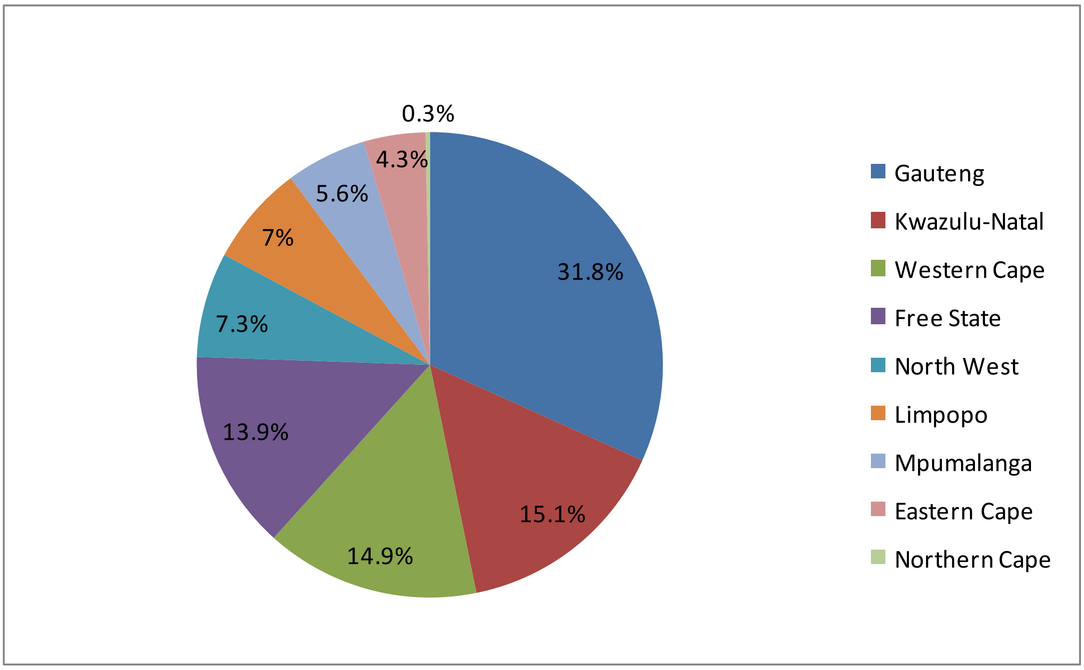

Figure 1.2: Provincial distribution of egg production amongst SAPA members in RSA for 2008 (SAPA, 2008).

1.2.3 Antimicrobial use in poultry production

An antimicrobial is a chemical substance administered orally or systemically that is able to kill or inhibit the multiplication of micro-organisms such as bacteria, viruses, fungi or protozoa, with the largest group being those that are effective against bacteria (Prescott, J. F., Baggot, J.D. & Walker, R.D., 2000). Since 1946, antimicrobials have been used by the poultry industry to enhance growth and feed efficiency and to reduce bacterial diseases (Donoghue, 2003; Moore *et al.,* 1946; Stolker *et al.,* 2007). It has been recognized that low concentrations of antimicrobials fed to food animals in feed or water lead to enhanced feed conversion efficiency and improved growth rates, especially in the early growing stages (Marrett, L. E. & Evans, R., 1999; Gouws & Brozel, 2000; Peter & Fariborz, 2002). This potential value of in-feed antimicrobials was first demonstrated in the USA, (Moore *et al.,* 1946) and later, antimicrobial usage was shown to have facilitated the efficient production of poultry. This allowed the consumer to purchase, at a reasonable cost, high quality meat and eggs as well as reduce the impact of disease outbreaks (Al-Ghamdi, M.S., Al-Mustafa, Z.H., El-Morsy, F., Al-Faky, A., Haider, I. & Essa, H., 2000; Donoghue, 2003); consequently, this has led to the use of large amounts of antimicrobials in food animals (Gouws & Brozel, 2000).

Antimicrobials commonly used in the poultry industry include: aminoglycosides (neomycin and gentamicin); tetracyclines (doxycycline, chlortetracycline and oxytetracycline); cell wall actives (amoxicillin and fosfomycin); fluoroquinolones (norfloxacin and enrofloxacin); macrolides (tylosin, tilmicosin and erythromycin); pleuromutulins (tiamulin); lincosamides (lincomycin); polypeptides (colistin); sulphonamides and potentiated sulphonamides. An amphenicol (florfenicol) was recently introduced to this industry (Stolker, A. A. M. & Brinkman, U.A.T., 2005; South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs (SANVAD), 2007; Bisschop *personal communication*, 2008).

1.2.4 Poultry layer management systems in South Africa

The types of management system employed on a farm plays an important role in the wellbeing of the birds and also have an impact on way diseases enter into the farm. It is very important that procedures such as biosecurity and cross infection between houses are prevented by adhering to standard operational procedures at all times. Rodent control and enforcement of routine feeding management without stress to the layers should be carried out as well as adhering to good hygienic measures in order to minimise contamination of feedstuffs. Monitoring systems based on proper identification and isolation of suspected or sick birds should also be enforced.

A large majority of laying hens in South Africa are kept in cages, with a small proportion (<10%) kept on the floor, either as "barn" producers or as free-range layers. However, the hens are usually crowded and stressed so respiratory disease, such as mycoplasmosis and coryza, when introduced can spread rapidly, necessitating the use of mass antimicrobial therapy.

In the deep litter management system, the birds are kept on the floor covered with wood shavings and are consequently in direct contact with their faeces. Also, their eggs are easily contaminated by faeces which can lead to penetration of the microorganism contained in the faeces into the eggs as in the case with *Salmonella enterica* and *Escherichia coli* (JIAC, 2000). In this management system, there is a higher chance of disease transmission necessitating the increased use of antimicrobials both in volume and variety in order to treat the diseases with a resultant increase of AR in eggs (JIAC, 2000; Picard personal communication, 2008).

1.2.5 Diseases in layers requiring antimicrobial therapy in South Africa

There are many diseases of laying hens found in RSA requiring the use of drugs to treat them; these diseases can be broadly categorised as mycoplasmal, bacterial, protozoal and parasitic diseases.

The important chronic respiratory disease (CRD) of poultry is caused by *Mycoplasma gallisepticum* (MG) in association with *Escherichia coli*. *Mycoplasma synoviae* (MS) may also cause respiratory disease, but more commonly causes synovitis and arthritis. Transmission of the mycoplasmas is horizontally via direct contact and respiratory aerosols and vertically through eggs while diagnosis is by serology and polymerase chain reaction (PCR) from tracheal swabs and lungs in the case of MG and joint fluid in the case of MS. Prevention is by good hygiene practices, vaccination and administration of antibiotics like the macrolides or tetracycline (JIAC, 2000; OIE Manual, 2008).

Infectious coryza is an acute respiratory disease mainly seen in layer birds caused by *Avibacterium paragallinarum*; it is characterised by nasal exudation, a swollen face and decreased egg-laying. The disease shows high incidence in non-immunised flocks and the clinical signs become serious in a mixed infection especially with MG. Diagnosis is by bacterial isolation from swabs from the nasal cavity or infraorbital sinus. Treatment is by the use of antimicrobials like tetracycline's (JIAC, 2000).

Ornithobacterium rhinotracheale (ORT) is a slow growing, pleomorphic, gram-negative, rod-shaped bacterium associated with respiratory disease in poultry – sometimes referred to a "swollen head syndrome". The organism was first described in South Africa but has since been identified in most parts of the world. Environmental factors such as poor management, inadequate ventilation, high stocking density, poor litter conditions, poor hygiene or concurrent infections influence the disease (Bosch, Van den G, 2001; Sprenger, S, J., Halvorson, D. A., Nagaraja, K. V., Spasojevic, R., Dutton, R. S and Shaw, D. P, 2000). The disease is characterized by respiratory signs of sneezing often accompanied by a slightly increased mortality and a poor performance. Bacteria such as *E. coli* and *Bordetella avium* can also trigger the disease; this leads to economic losses due to condemnations and drop in egg production. At post mortem, frothy white exudates can be seen in the air sacs which often lead to pneumonia. Treatment of *O. rhinotracheale* infection with antibiotics is very difficult because of the inconsistent sensitivity of the strains. *O. rhinotracheale* is able to acquire resistance easily against antimicrobials such as doxycycline, enrofloxacin, flumequine, lincomycin, trimethoprim sulphonamide and tylosin (Van Veen, L., Hartman, E. and Fabri, T., 2001). It is therefore important to clean and disinfect poultry houses thoroughly between placements in order to reduce disease transmission.

Other important bacterial diseases affecting poultry in RSA include salmonellosis which is an important zoonosis. Various *Salmonella enterica* serotypes are responsible for enteric and/or systemic disease manifestations (Schering-Plough Animal Health and Intervet, 2008). Although there are numerous serotypes that can be found in poultry that are transmissible to humans, *Salmonella* Enteritidis and *S*. Typhimurium are responsible for most of the outbreaks involving the eating of undercooked poultry meat and eggs (Schering-Plough Intervet, 2008). Although the abovementioned diseases can result in clinical disease in birds, they tend to act more as sub-clinical carriers. However, the non-zoonotic salmonellae *Salmonella enterica* biovar *pullorum* that causes Pullorum disease and S. *enterica* biovar *gallinarum* that causes fowl typhoid can result in high mortalities in birds (OIE notifiable disease) (JIAC, 2000; OIE Manual, 2008; Schering-Plough Intervet, 2008). Fowl typhoid is especially important in layer hens where disease is manifested by dyspnoea, depression, pericarditis, ovarian lesions, reduced egg production, diarrhoea (which often paste the vent) and increased mortality. This disease is usually controlled by vaccination and a slaughter-out policy. During an outbreak, however, antimicrobial therapy may be used to save the birds and decrease the presence of clinical signs.

Colibacillosis is a common systemic disease and is considered to be one of the leading causes of economic loss from disease in the poultry industry. It is caused by avian pathogenic *Escherichia coli* (APEC) and occurs as an acute fatal septicaemia or sub acute systemic disease affecting poultry of all ages and breeds (Dhillon, S., A. & Jack, O., K., 1996; Vandekerchove, D; Herdt, P. D; Laevens, H. & Pasmans, F., 2004). Its ubiquitous presence in poultry flocks with vast arrays of virulence and its ability to propagate rapidly contribute to its significance as an important pathogen of poultry (Dhillon, S., A. & Jack, O., K., 1996). The disease is manifested by septicaemia, white-yellowish diarrhoea, respiratory disorders, reduced egg production and immunosuppression; it occurs through vertical transmission of *E. coli* (in the eggs) and also by penetration of *E. coli* through the egg shell (faecal contamination on the surface of the eggs) (JIAC, 2000). High mortality is seen concurrent with or as a complication of other infectious agents such as Newcastle disease virus (NDV) and mycoplasmosis. Environmental factors such as exposure to chicken house dust and stress seem to contribute to the penetration of APEC into the bloodstream, which in turn leads to severe disease (colisepticaemia) and mortality. Diagnosis of the disease is by isolation of *E. coli* in pure culture from major organs like heart, liver and joints of affected chickens. Prevention and treatment is by improved hygiene measures, use of chlorinated drinking water and the use of appropriate antibiotics like oxytetracyclines (Dhillon & Jack, 1996; JIAC, 2000).

Coccidiosis is a protozoal disease caused by *Eimeria* species, it is mainly characterised by bloody diarrhoea and necrosis of the intestine leading to anaemia. Detecting the protozoa in intestinal mucosa and oocysts in faeces is used for diagnosis. Wet or damp litter favours the multiplication of the protozoan agent and prevention and treatment is by adhering to good sanitary and hygienic procedures and the use of anticoccidial drugs, ionophores, sulfa or pyrimidine drugs.

Endoparasitic diseases and helminthiasis are rare in caged birds because the birds do not usually come into contact with their faeces and intermediate hosts, but are more common in birds kept on the floor, leading to diarrhoea, anaemia and emaciation. *Ascaridia galli* may rarely be observed in chicken eggs (JIAC, 2000). Diagnosis is mainly by macroscopic observation of the intestinal contents and also by microscopy. Treatment is by use of appropriate antihelminthic like levamisole, ivermectin, or doramectin (JIAC, 2000).

The severity of most of these diseases is increased by poor management practices which result in increased antimicrobial usage to control the diseases. However, the use of antimicrobials in treating these diseases results in the secretion and accumulation of AR in eggs leading to increased public health risks (Yoshimura, H., Osawa, N., Rasa, F.S., Hermawati, D., Werdiningsih, S., Isriyanthi, N.M. & Sugimori, T., 1991; Gouws & Brozel, 2000; Frank, M.A., Anne, M.S., Hanne, D.E., Karl, P., Rene, H.S. & Fleming, B., 2001; Peter *et al.,* 2002; Omija, B., Mitema, E.S. & Maitho, T.E., 2004).

1.2.6 Antimicrobial resistance in bacteria of poultry origin

The sources of and consequences of antimicrobial resistance (AMR) especially in pathogenic bacteria were early sources of concern in human and animal health. These concerns caused the World Health Organization (WHO) to organise workshops and meetings in Geneva, Switzerland in 1994 and also in Berlin, Germany in 1997; these meetings concluded that the indiscriminate use of antimicrobials both in human and veterinary medicine could lead to increases in the resistant bacteria population with resulting adverse consequences for the prevention and treatment of diseases in humans and animals and initiated legislative restrictions banning the use of penicillin and tetracyclines without veterinary prescription (WHO, 1994; WHO, 1997).

Sweden, in 1986, legislated against the use of antimicrobials as performance enhancers in animals; this ban reduced by half the use of antimicrobials in animals in that country. In 1997, the EU banned avoparcin® as a feed additive in other member countries following bans already in place in Denmark, Norway and Sweden; and by 2006, it totally banned the use of antimicrobials for non-therapeutic purposes (Gordon D.R., 2008; Pig Health, 2003).

Where antimicrobials are widely used, the practice has been associated with increased antimicrobial resistance and where use has been restricted or banned; antimicrobial resistance has also been demonstrated to decline. A good example of this phenomenon was observed in Denmark where the use of virginiamycin increased from 1995 to 1997 and was followed by increased occurrence of virginiamycin resistance among *Enterococcus faecium* isolates from broilers from 27.3% in 1995 to 66.2% in 1997. When the use of virginiamycin was eventually banned in Denmark in January 1998, the occurrence of resistance to the drug decreased to 33.9% and later to 12.0% in 2000 (Frank *et al.*, 2001). A similar pattern was also noted with erythromycin where resistance to *E. faecium* reached a maximum of 76.3% in 1997, and then decreased to 12.0% by the year 2000 with decrease in the use the drug in Hungary (Kaszanyitzky, E.J., Tenk, M., Ghidan, A., Fehervari, G.Y. & Papp, M., 2006). In Hungary, the resistance levels of *Enterococcus* species to vancomycin in broilers reduced from 72.7% in 1995 to 5.8% in 2000 after vancomycin was banned for use in food animals in that country (Kaszanyitzky, *et al.,* 2006).

Virginiamycin is widely used as a growth promoter in the USA resulting in resistance to *Enterococcus faecium* of animal origin, whereas for avoparcin that is not being used, acquired resistance is virtually non-existent in enterococci isolated from animals (Philips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preston., R. & Waddell, J., 2004). Similarly, when the use of tetracycline supplemented feeds for prophylaxis was prohibited in the Netherlands, it produced a marked decrease in tetracycline resistant *Salmonella* infections in both humans and animals (Gouws & Brozel, 2000). In 2005, when the use of ceftiofur (cephalosporin) that was widely used in Ontario and Quebec (Canada) in chicken hatcheries was stopped, there was a resultant decrease in cephalosporin resistance in *Salmonella* samples taken from humans and retail poultry according to a report in the Canadian Medical Association Journal (re-reported by World Poultry News) (World Poultry.net, 2009). As the years went by, there was a gradual increase in the use of ceftiofur again and the latest data for Ontario released in March 2009 showed a corresponding spike in ceftiofur resistance in humans and retail chickens from 29% to 46% between 2007 and 2008 (World Poultry.net, 2009). From the above literature, it is evident that the pattern of antimicrobial occurrence correlated well with antimicrobial usage.

Interestingly, the EU ban on antimicrobial feed additives led to an increased use of antimicrobials for therapeutic reasons in countries like Denmark and Sweden (Pig Health, 2003; Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP), 2003; Philips, *et al.,* 2004; Swedish Veterinary Antimicrobial Resistance Monitoring (SVARM), 2006). This increased use of antimicrobials for therapeutic purposes was not followed by an increase of AMR in production animals; instead, there was a general decrease in the amount of AMR, perhaps it was due to a decrease in the sales of antibiotics for non-therapeutic purposes (SVARM, 2006). Therefore the possible risk of AMR in pathogens as well as commensals developing to therapeutic drugs as well as the presence of antimicrobial resistance in food of animal origin has decreased.

The use of veterinary antimicrobials for performance enhancement in South Africa is regulated by the Updated Regulations Governing Microbiological Standards for Foodstuffs and Related Matters No. R. 692, 1997. In-feed and parenteral antimicrobials may only be supplied on prescription as regulated by the Medicines and Related Substances Act No. 101 of 1965 (Act 101, 1965). However, water soluble products may be supplied directly to farmers without prescription where their use is regulated by the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act No. 36 of 1947 (Act 36, 1947). The control, sale, manufacture and importation of foodstuffs, cosmetics and disinfectants are regulated by the Foodstuffs, Cosmetics and Disinfectant Act No. 54 of 1972 as amended by Act 32 of 1981 and Transfer of Power and Duties of the State President Act 97 of 1986 (Amended Act 54, 1972). Even though there are regulations in place governing the use of veterinary antimicrobials in South Africa, there is a need to check on a regular basis whether farmers and producers are complying with the laws, because of the adverse consequences the inappropriate use of antimicrobials in animals can have on public health if neglected.

In South Africa, Manie, T.K., Veith, W., Khan, S., Brozel, V.S. & Gouws, P.A., (1998) and Oguttu J.W., (2008) reported that a large proportion of bacterial flora such as Enterobacteriaceae including *Salmonella* species and staphylococci of recently slaughtered broilers showed resistance to tetracyclines, streptomycin and oxacillin. Oguttu, (2008) noted that there was a high level of resistance in the enteric microflora of slaughtered chickens in an abattoir in RSA to tetracycline’s, fluoroquinolones, fosfomycin, sulphonamides and macrolides and that this resistance pattern correlated well with antimicrobial usage on the farms supplying the abattoir. SANVAD reported bacterial resistance to a wide range of antimicrobials in the country (SANVAD, 2007).

This serves to indicate that antimicrobials are used widely in poultry in RSA (Oguttu, 2008). Therefore, it is possible that antimicrobial residues are present in poultry products for human consumption.

1.2.7 Physiology of egg formation and factors affecting the incorporation of antimicrobials into eggs

The hen's egg is formed gradually over a period of approximately 25 hours and many organs and systems, as shown in Table 1.1, help to convert feed materials eaten and water taken in by the hen into the various substances that become part of the egg (Coutts, J. A. & Wilson, G.C., 2007). When fully formed, the average weight of a large egg is 57g and 38g of this weight (66%) is made up of water; yolk (ovum) components make up 32% of the egg's weight and are formed by the liver and transported to the ovary via the blood stream while the albumen (egg white) components make up 58% of the egg's weight and are formed by the growing follicles and oviduct; the shell makes up 10% of the egg's weight and is the last material to be formed in the process of egg formation (Kan & Petz, 2000; United States Department of Agriculture, 2000; Coutts & Wilson, 2007).

Table 1.1: Organs that form different components of an egg

ORGAN OF FORMATION	EGG COMPONENT
Liver	Yolk (Protein & Albumen)
Oviduct (Magnum)	Albumen
Shell Gland (between Isthmus & Vagina)	Shell
Shell Gland	Cuticle

Kan & Petz (2000) reported that the residues of drugs in hens accumulate first in the albumen before getting to the yolk later. They tend to closely mirror the level of drugs in the plasma. Drug residues in yolk can increase, remain constant or decrease depending on the length and timing of the drug exposure to the yolk. Also, drug residues in the yolk generally require exposure for approximately 8 to 10 days to reach a constant level.

A single exposure to a drug might be sufficient to detect the drug in yolk or albumen depending on the characteristics of the drug and the sensitivity of the analytical test used. Also, the drug clearance from the yolk and albumen depends heavily on the plasma levels of the drug tested; thus, the higher the plasma level, the longer the clearance time. Drugs that clear rapidly from the body also disappear from the yolk and albumen in two to three days after the drug withdrawal (Kan & Petz, 2000).

Some antimicrobials are designed to work systemically and if given orally, as is the case in most commercial poultry farms, they must cross the intestinal wall to exert their actions. The absorption of these antimicrobials is quite logical as they possess certain lipophilic properties in order to interact and pass through membranes. These properties are important for the antimicrobial to reach the target organs and elicit their action (Kan & Petz, 2000). During the process of blood (plasma) circulation, antimicrobials are carried along with other metabolites from the feed via the blood stream and distributed to all the organs including the egg (yolk and albumen) before the shell is finally formed (Sturkie, P.D., 1986; North, M.O. & Bell, D.D., 1990). Since the liver is both the site for the metabolism of many antimicrobial drugs as well as where the components of the yolk are manufactured, it is not surprising that large amounts of antimicrobials are found in eggs (Kan & Petz, 2000). The antimicrobial distribution in yolk or albumen will vary as it is dependent not only on how the yolk and albumen is formed, but also on their physiological properties. The distribution of antimicrobials in these two compartments as determined by several researchers is summarized in Table 1.2.

Table 1.2: Distribution of antimicrobial drug residues in poultry eggs

Antimicrobial / compound	Antimicrobial Residues found in Yolk	Antimicrobial Residues found in Albumen	Reference
Aminoglycosides			
Streptomycin	√	√	Adesiyun *et al.*, 2005.
Neomycin	√	√	Gaudin *et al.*, 2009.
Beta-lactams			
Ampicillin	√	√	Donoghue *et al.*, 1997; Adesiyun *et al.*, 2005.
Amoxicillin	√	√	Gaudin *et al.*, 2009.
Macrolides			
Tylosin	√	√	Roudaut & Moretain, 1990; Adesiyun *et al.*, 2005;Gaudin *et al.*, 2009.
Erythromycin	√	√	Gaudin *et al.*, 2009.
Tetracycline's			
Doxycycline		√	Kan & Petz, 2000; Gaudin *et al.*, 2009.
Minocycline		√	Kan & Petz, 2000.
Oxytetracycline	√	√	Yoshimura *et al.*, 1991; Mitema & Omija, 1992; Donoghue & Hairson, 1999; Zurhelle *et al.*, 2000; Omija & Mitema, 2004; Gaudin *et al.*, 2008.
Chlortetracycline	√	√	Roudaut & Moretain, 1989; Zurhelle *et al.*, 2000; Gaudin *et al.*, 2009.
Tetracycline	√	√	Roudaut & Moretain, 1989; Zurhelle *et al.*, 2000; Adesiyun *et al.*, 2005; Gaudin *et al.*, 2009.
Quinolones			
Enrofloxacin		√	Kan & Petz, 2000; McReynolds *et al.*, 2000
Ciprofloxacin		√	
Flumequin		√	
Oxolinic acid		√	
Danofloxacin	√	√	Yang *et al.*, 2006

Table 1.2 continues

Sulphonamides			
Sulfaquinoxaline	√	√	Kan & Petz, 2000; Roudant, 2002; Shaikh *et al.,* 2004; Hussein *et al.,* 2005.
Sulfadiazine	√	√	
Sulfamerazine	√	√	Gaudin *et al.*, 2009
Sulfisoxazole	√	√	
Sulfachloropyridazine	√	√	
Sulfamethizole	√	√	
Sulfathiazole	√	√	
Sulfadoxine	√	√	
Sulfadimethoxine	√	√	Skaikh & Chu, 2000; Tansakul *et al.,* 2007; Gaudin *et al.,* 2009.
sulfamethoxazole	√	√	
Sulfamethazine	√	√	
Others			
Trimethoprim	√		Kan & Petz, 2000;
Thiamphenicol	√	√	Giorgi *et al.*, 2000;
Chloramphenicol	√	√	Sisodia & Dunlop, 1972;
Coccidiostats			
Amprolium	√		Kan & Petz, 2000.
Anthelmintics			
Ivermectin	√		Kan & Petz, 2000.

1.2.8 The risks to human health associated with consumption of poultry eggs and meat and other animal products containing antimicrobial residues

Antimicrobial residues accumulate in chicken eggs and meat and in the edible tissues of food animals and when passed to humans over time can lead to a number of problems like antimicrobial resistance, anaphylactic reactions, skin allergies and some even have mutagenic potential (WHO, 1989; Teh, W.L & Rigg, A.S., 1992; National Agency for Food Drug Administration and Control (NAFDAC), 1996). Other documented harmful effects of antimicrobial residues in humans from consuming poultry and other animal products containing antimicrobial residues are summarised in Table 1.3 below.

Table 1.3: Documented health implications in humans of some antimicrobial residues in poultry and animal tissues

Antimicrobial	Health implication	Reference
Chloramphenicol	Aplastic anaemia	Paige *et al.,* 1997;
Penicillin	Allergic reaction like rashes, Urticaria, Shock, serum sickness, asthma and fever	Paige *et al.,* 1999
Streptomycin	Allergic reaction	
Sulphonamide	Rashes	
Tetracycline	Hypoplastic anaemia, discoloration of the teeth in children less than 8 years; Nausea, stomach burn, ulcerations of oesophagus; Asthma; Pseudomembraneous colitis; Neutropaenia, thrombocytopaenia, leucopaenia, aplastic anaemia, haemolytic anaemia; Anaphylactic shock; Diabetes insipidus; Fatty liver degeneration; Near-sightedness; Pigmentation of the skin; Phototoxic dermatitis; Mutagenic effect; Urticaria, erythema, exanthema and contact dermatitis. Acute toxicity, short-term and long-term toxicity, Carcinogenicity. Disturbance of the intestinal microflora	Berends *et al.,* 2001 Paige *et al.,* 1997; Paige *et al.,* 1999

Because of the documented harmful effects of antimicrobial residues in humans, internationally recognized organizations such as the WHO, FAO, Veterinary Medicine Directorate (VMD) of the European Union as well as the Food and Drug Administration (FDA) of the USA have set MRL or, Acceptable Daily Intake (ADI) for humans and withholding times for pharmacologically active substances including antimicrobial agents in foods of animal origin prior to marketing (EC, 1990; EC, 1996; Al-Ghamdi *et al.,* 2000; EC, 2002; EC, 2006; CAC/MRL, 2006).

In South Africa, the MRLs for veterinary medicine and stock remedy residues that may be present in Foodstuffs are regulated by the Updated Regulations governing the maximum limits for Veterinary Medicine and stock remedy residues that may be present in Foodstuffs (Amended Act 54, 1972); as published under Government Notice No. R. 1809 of 3 July 1992; corrected by Government Notice No. R.2376 of 28 August 1992 and amended by Government Notice No. R. 1387 of 19 November 1999 as a way of protecting the public. Tests have been developed to screen poultry meat and eggs for antimicrobial residues on a regular basis and products found to contain antimicrobial residues above the MRL, for the antimicrobial should not be allowed to reach the public in order to protect human health.

1.2.9 Factors affecting the stability of antimicrobials

Decision EC.2002/657 of the European Commission (EC) requires that stability studies should be carried out on antimicrobials during validation studies (EC, 2002). The stability of a particular drug will vary dependent on the storage matrix and its storage conditions. Literature studies showed that there is a significant lack of data on the stability of antimicrobials in stored eggs; and when looking for stability studies in other matrices (animal organs or products as listed in Table 1.4) it was observed that there was great variability in the results (Gaudin *et al.,* 2009). Also, factors such as duration and conditions of storage of eggs from point of lay to eventual purchase may affect the activity and result of the test to determine the AR status of such eggs.

Although there is little published data on the stability of antimicrobials in eggs (Gaudin *et al.,* 2009), it appears that storage at -20^{0}C or even less will allow sulphonamides to remain stable for 49 days or more and penicillins, chlorampenicol, oxytetracyclines and linocomycin even longer (Cantwell & O'Keeffe, 2006; Gaudin *et al.,* 2009). The light sensitive tetracyclines need to be stored away from light.

Table 1.4 shows the stability of different antimicrobials in different matrices at different temperatures.

Table 1.4: Stability of different antimicrobials in different animal matrices and at different storage temperatures

Antimicrobial	Matrix	Temperature	Stability (days)	Source
Neomycin	Eggs	+4^{0}C	-	1
Lincomycin	Kidney fluid	5^{0}C	49	2
Sulphadiazine	Kidney fluid	23^{0}C plus light	49	2
Sulphamethazine	Kidney fluid	23^{0}C no light	49	2
	Milk	-20°C	95	3; 4; 5
	Piglet muscle	-75°C	158	3
Sulfadimidine	Muscle, kidney, liver	-20^{0}C	350	5
Ampicillin	Muscle	-75^{0}C	253	6
Penicillin G	Kidney fluid	5^{0}C	5	2
Cephalothin;	Kidney fluid	5^{0}C	49	2
Cephalexin	Kidney fluid	5^{0}C	49	2
Chlortetracycline	Kidney fluid	5^{0}C	49	2
Oxytetracycline	Kidney fluid	5^{0}C no light	7	2
	Muscle, kidney, liver	-20^{0}C	420	5
Chloramphenicol	Kidney fluid	5^{0}C	49	2; 5
		-20^{0}C	539	2
Gentamicin	Kidney fluid	5^{0}C	49	2

Sources 1: Inglis & Katz, 1978; 2: Cantwell & O'Keeffe, 2006; 3: Papapanagiotou *et al.,* 2005; 4: Juhel-Gaugain *et al* 2005; 5: O'Brien *et al*., 1981; 6: Verdon *et al.,* 2000.

The storage of antimicrobials as well as certain testing procedures may result in them being exposed to high temperatures. In liver, muscle and kidney, ampicillin, amoxicillin, cloxacillin, penicillin G, lincomycin, spiramycin, neomycin, streptomycin, tylosin, doxycycline, enrofloxacin and flumequine are stable when subjected to 80^0C for 15 minutes (Egmond *et al*., 2000). Colistin is stable at 90^0C, chlortetracycline loses its stability at 100^0C, whereas oxytetracycline and sulphamethoxyazole will remain stable at 100^0C while sulphamethazine is stable at 122^0C (Egmond *et al*., 2000).

1.2.10 Methods used to detect antimicrobial residues in food of animal origin

Several methods have been developed to test for the presence of AR in food intended for human consumption (Aerts, M.M., Hogenboom, A.C. & Brinkman, U.A., 1995). Tests that have been used to detect AR in poultry meat and eggs and other foods of animal origin make use of microbiology, enzyme-linked immunosorbent assay (ELISA), chromatography, bio-autography, spectrophotometry (mass spectrometry) or a combination of one or two of the above mentioned methods.

Microbiological-based methods such as Delvotest®, Premi®Test, disc diffusion microbiological inhibition test (example four-plate test), FAST®, STAR® test and the One-Step-Test® can be used to detect AR in edible animal tissues and eggs (Aerts *et al.,* 1995; Kabir *et al.,* 2004; Kilinc, B., Meyer, C. & Hilge, V., 2007; Gaudin, V., Murielle, J., Jean-Pierre, M. & Sanders, P., 2008; Schneider, M.J. & Lehotay, S.J., 2008). These methods use spores of microorganisms like *Bacillus megaterium*-American Type Culture Collection (ATCC 9885); *Bacillus cereus* (ATCC 11778), *Geobacillus stearothermophillus* (ATCC 12980) and vegetative *Micrococcus luteus* (ATCC 9341) embedded in the test medium which contains an indicator and nutrients to support growth.

The microbiological tube-based tests work on the same general principle, which is that when antimicrobial compounds are present in a sample at a concentration sufficient to inhibit growth of the test organism; the colour of an acid-base indicator of the test system will stay the same. However, when no inhibition occurs (absence of antimicrobial residues), growth of the test organism occurs and there is formation of acid or reduced metabolites leading to a change in the colour of the indicator (Aerts *et al.,* 1995; Premi®Test DSM Food Specialties 1, 2008; Premi®Test DSM Food Specialties 2, 2008; World Intellectual Property Organization (WIPO), 2008). With agar diffusion methods like the four-plate test, two different micro-organisms *B. subtilis* and *M. luteus* are used as indicator microorganisms in addition, three different pH (pH 6, 7.2 and 8) of the test media inoculated with viable spores of the bacteria are used (Gaudin *et al.,* 2008; Gaudin *et al.,* 2009; Premi®Test 1, 2008; Premi®Test 2, 2008; WIPO, 2008).

The enzyme-linked immunosorbent assay (ELISA) is a rapid test that can be used to detect the presence of specific antimicrobials in tissues (Mc Glinchey, T., Rafter, P.A., Regan, F. & Mc Mahon, G.P., 2008; Wang, S., Zhang, H.Y., Wang, L., Duan, Z.J. & Kennedy, I.*,* 2006). The assay is performed by bringing cloned antibodies, either monoclonal or polyclonal, into contact with the analyte and adding an amount of radio-enzyme or fluorescent-labelled analyte, which competes with the non-labelled analyte for the available binding sites. The amount of labelled analyte bound is then determined directly or after the addition of a suitable substrate that is transformed into a selectively detectable product using an ELISA reader (Aerts *et al.,* 1995). ELISA methods using both monoclonal and polyclonal antibodies have been used to screen milk, meat and eggs for chloramphenicol and sulphachlorpyridazine at low levels (Aerts *et al.,* 1995; Spinks, C.A., Schut, C.G., Wyatt, G.M. & Morgan M.R.A., 2001; Wang *et al*., 2006).

Chromatography is the term used to describe a separation technique in which a mobile phase carrying a mixture is caused to move in contact with a selectively absorbent stationary phase. Different components of the test sample are carried forward at different flow rates by the mobile, due to their differing interactions with the stationary and mobile phases. Chromatography methods include Thin-Layer Chromatography (TLC) and Bio-autography, High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC).

Thin Layer Chromatography (TLC) uses a solid-stationary polar phase and a liquid-mobile phase. It involves spotting the sample to be analyzed near one end of the adsorbent solid phase placed in a covered developing jar containing a shallow layer of solvent. The solvent rises by capillary action up through the adsorbent and differential partitioning occurs between the components of the sample mixture dissolved in the solvent. The plate is removed from the developing chamber, dried, and the separated components of the sample are visualized straightforward or using ultra-violet (UV) lamp. It is used to support the identity of a compound in a mixture when the retardation factor (R_f = distance moved by analyte / distance moved by solvent front) of a test compound is compared with the R_f of a known compound; preferably both run on the same TLC plate (Fried, B & Sherma, J., 1999; Masoko, P., 2006; Wellesley Education, 2009).

Bio-autography additionally detects antimicrobial or antifungal activity of the test sample shown by clear zones of inhibition on TLC plates previously spread with viable broth cultures of the microorganism incubated at its optimum growth temperature (Choma, I., 2005; Masoko, P., Picard, J. & Eloff, J. N., 2005; Masako, 2006).

HPLC uses a liquid mobile phase to separate the components of a mixture; the components are dissolved in a solvent and forced to flow through a chromatographic column under high pressure. In the case of GC, the test sample is converted to vapour which is carried into a thermally-controlled column. The speed at which solutes move through the column is used to identify the solutes; from there it enters a heated detector where an electronic signal is generated upon interaction of the solute with the detector and is recorded and plotted as chromatographs (Wesley Learning, 2004; Betts, T., 2008; Global Spec, 2009).

Yuan, Y.H., Chen, Z.L., Liu, L., Zeng, Z.L., Shen, X.G. & Huang, X.H., (2004) reported the use of HPLC for the detection of chloramphenicol residues in eggs while Ruyck, D.H., Ridder, D.H., Renterghem, V.R. & Wambeke, V.F., (1999), reported the validation of the HPLC method for the analysis of tetracycline residues in eggs and broiler meat. Sulphonamide and fluoroquinolone residues were found in table eggs and animal tissues using HPLC as reported by Horii, S., Momma, C., Miyahara, K., Maruyama, T. & Matsumoto, M., (1990) and Herranz, S., Moreno-Bondi, M.C. & Marazuela, M.D., (2007). Work done on other animal products using a combination of HPLC and bioautography included that reported by Ramirez, A., Gutierrez, R., Diaz, G., Gonzalez, C., Perez, N., Vega, S. & Noa, M., (2003), who identified and quantified multiple antibiotic residues in cow's milk while Salisbury, D.C.C., Rigby, E.C. & Chan, W., (1989), detected and identified antibiotic residues in slaughtered animals in Canada.

Spectrophotometry is a technique that measures the amount of light that an analyte molecule (sample being studied) absorbs using an instrument called a spectrophotometer. The instrument operates by passing a beam of light that consists of a stream of photons through an analyte that absorbs the photons; this absorption reduces the number of photons in the beam of light, thereby reducing the intensity of the light beam reaching the detector for measurement. This technique can be applied to identify samples including AR by the difference in the intensity of light that passes through them measured by the detector (David, N. B, 2000).

Mass spectrometry (MS) is a sensitive qualitative and quantitative analytical technique that is used to identify unknown compounds, determine the isotopic composition of elements in a molecule, and determine the structure of a compound by observing its fragmentation (Wang, J., Leung, D. & Butterworth, F., 2005; Wang J. & Leung, D., 2007). The analytical principle consists of ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios. MS instruments consist of an ion source, which can convert gas phase sample molecules into ions, a mass analyzer which sorts the ions by their masses by applying electromagnetic fields and a detector for calculating the abundances of each ion present. It is the detection method of choice for the aminoglycosides (Mc Glinchey *et al.,* 2008).

Some macrolides (spiramycin, tilmicosin, oleandomycin, erythromycin, tylosin) and ionophores (lasalocid, monensin, salinomycin, narasin) have been detected in eggs, honey, liver and milk of animals using mass spectrometry alone or a combination with HPLC and other chromatographic methods (Heller, D.L. & Nochetto, C.B, 2004; Wang *et al.*, 2005; Wang & Leung, 2007).

There is no single test that can screen all AR in a large number of samples and at the same time, identify them. Each test has its advantages and limitations as summarised below. The advantages of the microbiological tests are that they can be used to test a large number of samples for AR to several antimicrobials, are simple to perform can be completed within 24 hours; (Aerts *et al.,* 1995; Kilinc *et al.,* 2007; Gaudin *et al.,* 2008, Gaudin *et al.,* 2009; Premi®Test 1, 2008; Premi®Test 2, 2008; WIPO, 2008;). A large disadvantage of many of these tests is that they cannot detect all types of antimicrobials in samples at the MRL and can be inhibited by natural inhibitors in the samples e.g. lysozyme.

ELISA tests are semi-qualitative because of the high coefficient of variation requiring an additional confirmatory method; because these test methods are highly specific, they are not well-suited to multi-residue screening (Aerts *et al.,* 1995; Gaudin *et al.,* 2009). MS is the most specific and sensitive test method as it provides structural information about an antimicrobial and allows it to be quantified at levels as low as 0.01 and 37µg/kg (Aerts *et al.,* 1995; Wang *et al.,* 2005).

Chromatography has the advantage of identifying a compound in a mixture after an initial screening with microbiological methods (Aerts *et al.,* 1995; Kilinc *et al.,* 2007; Gaudin *et al.,* 2008; Premi®Test 1, 2008; Premi®Test 2, 2008; WIPO, 2008; Gaudin *et al.,* 2009). It is however more expensive than microbiological methods but cheaper than GC and MS. TLC has the advantage of being a sensitive, fast, simple and inexpensive analytical technique but the sensititvity of the test is often poor as the amount of the test sample quantified is low (Fried, B & Sherma, J., 1999; Masoko, P., 2006; Wellesley Education, 2009).

HPLC and GC both have the advantage of having very high sensitivity and specificity as well as quantifying the samples. GC is faster than HPLC but has the problems of low and variable recovery of analytes and the use of toxic extraction solvents such as methylene chloride and acetonitrile (Wesley Learning, 2004; Wang *et al.,* 2006; Betts, T., 2008; Global Spec, 2009).

Because of the disadvantages associated with some of the techniques mentioned above, a good antimicrobial detection system will be a combination of screening and analytical techniques (Aerts *et al.,* 1995; Heller & Nochetto, 2004; Wang *et al.*, 2005; Wang & Leung, 2007; Mc Glinchey *et al.,* 2008).

1. 3 Problem statement and Objectives of the Study

The South African poultry industry is the largest in Africa and dominates the country's agricultural sector. In 2008, the industry recorded a turnover of over R24.67 billion; of this, poultry meat production was put at R18.62 billion and egg production at R6.04 billion by the Department of Agriculture of South Africa (Avi Africa, 2009).

This highly intensified poultry industry where birds are kept at high stocking densities favours the spread of diseases often necessitating the use of antimicrobials to prevent and also to control diseases. The resulting extensive use of antimicrobials favours the development of AMR as well as increases the risk of AR in eggs. The presence of AR in eggs intended for human consumption is considered to be a public health risk.

Although there are regulations governing the use of antimicrobials in poultry production in South Africa as well as allowable antimicrobial residue limits (MRL) in meat and eggs, monitoring tests are carried out irregularly and on an *ad hoc* basis. The reason being is that tests of sufficient sensitivity are based on chromatography or mass spectrophotometry and tend to have a narrow spectrum of antimicrobial detection in addition to being expensive.

For these reasons, cheaper microbiological screening tests have been developed for affordability. Although these screening tests can be done in any microbiological laboratory, they do not always detect all antimicrobials and can be expensive when large numbers of eggs are being screened for AR. Therefore, it was decided to develop a rapid, but relatively cheap in-house bacteriological test that has a wide AR screening range for the mass screening of eggs for AR and also use this test to screen eggs sold within the Tshwane area.

1.3.1 Aims of the study

The aims of this study were to:

- Develop the new in-house bacteriological screening test for AR in eggs and complete a preliminary validation of the test.
- Do preliminary screening of commercial eggs in Tshwane area to enhance the test's validation as well as to carry out a preliminary evaluation of the prevalence of AR in eggs this area.

1.3.2 Objectives of the study

The Objectives of this study were to:

- Determine the optimum test conditions that lead to repeatable results.
- Compare the performance of *Geobacillus stearothermophilus* and *Bacillus megaterium* in the detection of antimicrobials in egg samples.

- Evaluate the performance of the new test by determining the minimum antimicrobial detection limit for the different antimicrobials for this test by using eggs spiked with different antimicrobials.
- Conduct a pilot trial on thirty six laying hens treated with known concentrations of antimicrobials and screen their eggs for AR using the new in-house bacteriological screening test.
- Screen eggs from the thirty six hens in the pilot trial using a different test method (Kundrat micro screening four-plate test[1]) conducted by an independent laboratory and compare the two test results.
- Carry out a two-seasonal survey in October/November 2008 (spring) and in April/May 2009 (autumn) to determine the prevalence pattern of AR statues of commercial chicken eggs sold in Tshwane area and environs.

1.3.3 Null Hypotheses:

- The newly developed in-house bacteriological screening test cannot be used to screen for most, if not all, of the antimicrobials used in poultry production at or below the maximum threshold values or maximum residue limits (MRL) relevant in South Africa.
- None (0%) of the commercial layer chicken eggs within Tshwane area will test positive for bacterial inhibitors (AR).

1.3.4 Alternative Hypotheses

- The newly developed in-house bacteriological screening test can be used to screen for most, if not all, of the antimicrobials used in poultry production at or below the maximum threshold values or maximum residue limits (MRL) relevant in South Africa.
- About <10% commercial layer chicken eggs within Tshwane area will test positive for bacterial inhibitors (AR).

1.4 Benefits arising from the research project

- The development of a robust screening test for AR in eggs that requires the minimum of equipment and laboratory expertise and also provides a platform to allow the regular monitoring of AR in eggs.
- The results of this study will help to determine the prevalence of AR in the eggs offered to consumers and also contribute to the present level of information in the field of research on prevalence of ARs in commercial chicken eggs in Gauteng Province, South Africa.
- The results of these tests could provide food safety regulators in South Africa with scientific evidence to enforce regulations and if necessary amend existing ones.
- The relatively low cost of the test makes it an affordable and allows wider screening of eggs for residues which will potentially reduce the risk of antimicrobial residues in eggs.
- Certain epidemiological risk factors will be detected, allowing for appropriate interventions.

CHAPTER 2- MATERIALS AND METHODS

2.1 Introduction

An in-house bacteriological screening test for antimicrobial residue (AR) in eggs was developed and preliminary validation of the test was carried out. The test was also used to conduct a survey on the AR status of commercial chicken eggs in the City of Tshwane and its environs in Gauteng Province of South Africa (RSA). The test's results were compared to results obtained using a similar screening method, the Kundrat micro-screening four-plate test[1] conducted by an independent laboratory.

Initial validation tests were conducted using egg samples spiked with known doses of antimicrobials. Thereafter, thirty six (36) hens were given therapeutic doses of over-the-counter antimicrobials and their eggs were tested for AR using the in-house screening method. Once the initial bench validation test had been done, field samples of eggs were also purchased from retail sales outlets during a pilot survey in the City of Tshwane and environs were also evaluated using the test. Several performance criteria such as detection capabilities, sensitivity and relative accuracy of the screening test were determined.

2.2 Experimental Design

The project was divided into two phases:

[1] Sigma-Aldrich SA (Pty) Ltd. P. O. Box 10434, Aston Manor 1630, South Africa

Phase I- Development of an in-house bacteriological screening test for eggs

Comparison of the results of the in-house test with another commercial test (four-plate microbiological screening test)

Phase II- Survey of AR in commercial chicken eggs in the City of Tshwane and environs of Gauteng Province RSA.

2.3 Phase I

2.3.1 Checking for purity of cultures

Bacillus megaterium ATCC 9885 and *Geobacillus stearothermophilus* ATCC 12980 were streaked onto Mueller-Hinton agar[1] and incubated in an air incubator[2] for 24 hours at 35^0C and 65^0C respectively. An isolated bacterial colony from each of the cultures was sub-cultured and later Gram's stain was made to check for bacterial purity and for quality control.

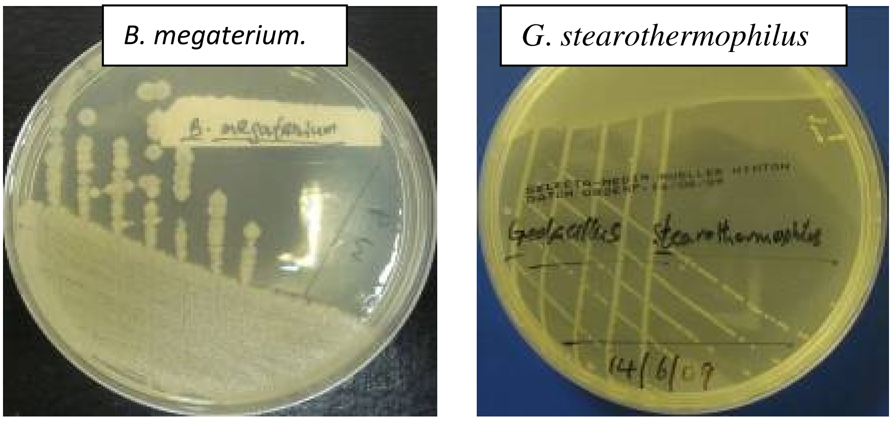

Figure 2.1: An 18-hour Culture of *B. megaterium* ATCC 9885 and *G. stearothermophilus* ATCC 12980 on Mueller-Hinton agar after incubation at 37^0C and 65^0C respectively.

[1] CM 0337 OXOID LTD Basingstoke, Hampshire England.
[2] Incothern-Labotec® 40 L Digital Incubator, Model 295, Serial No. 0100007606295, 230V-50Hz, 500W, RSA.

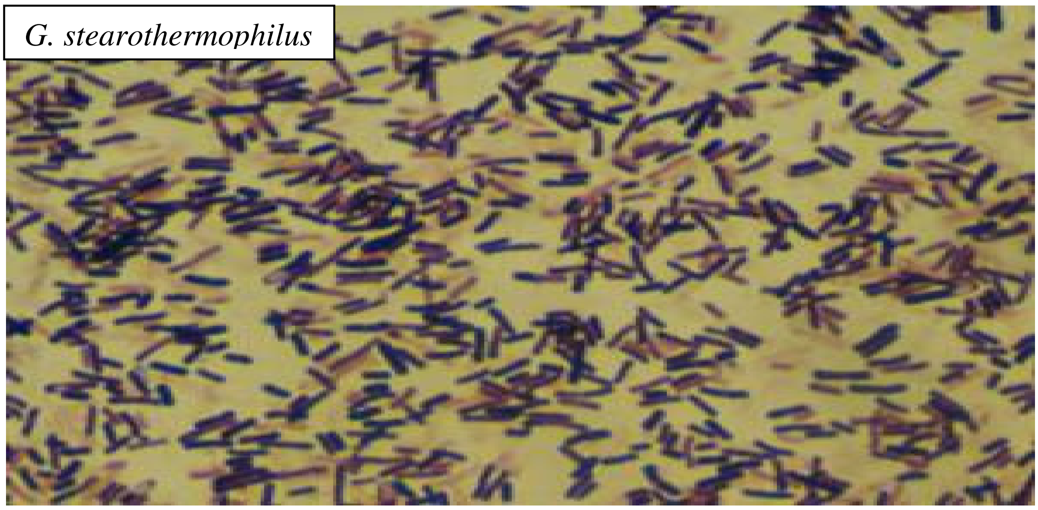

Figure 2.2: Gram stain of an isolated colony of *G. stearothermophilus* ATCC 12980 showing the variable Gram-staining as well as the sub-terminal spores typical of this genus.

2.3.2 Testing for inhibitors and inactivation of inhibitors

While running a pilot trial on homogenized egg samples spiked with known concentrations of antimicrobials to check for residues against negative control egg samples that were free from AR, it was discovered that both the spiked and negative egg controls showed growth inhibition against both test bacteria *B. megaterium* and *G. stearothermophilus* respectively. As it appeared to be a non-specific inhibitory effect, the egg yolk was carefully separated from the albumen without mixing them and each egg component was tested separately. It was later found that the non-specific inhibitory effect was present in the egg albumen but absent in the egg yolk. However, the testing of only the egg yolk could lead to false negative results, as some antimicrobials would occur in the yolk and some in the albumen. Because of this, both egg albumen and egg yolk were tested. Refer to Table 1.2 which shows the distribution of antimicrobials and other veterinary drug residues in poultry eggs.

The non-specific inhibition was thought to be due to lysozyme and peroxidase that are naturally found in eggs (WIPO, 2008). Inactivation of these proteases can be achieved either by the use of polar solvents such as methanol or by the use of heat. This means that only antimicrobials that dissolve in polar solvents would be detected. As most of the antimicrobials used in veterinary practice are stable at temperatures up to 80^0C (O'Brien *et al*., 1981; Egmond *et al*., 2000), heat inactivation was seen as a viable alternative that would allow all antimicrobials in the egg to be detected and was therefore carried out on the egg samples.

Testing for inhibitors and inactivation of inhibitors was carried out in both agar and broth samples.

i. Agar method:

1. A sterile glass suction Pasteur pipette with rubber handle was used to harvest about 100mℓ of egg albumen by carefully cracking some eggs disinfected using 70% alcohol[1] and gently pouring the content into sterile Petri dishes[2] without mixing the yolk and albumen.
2. 50mℓ of the albumen sample was put in a 100mℓ wide-mouth Pyrex® glass bottle[3] and heated at 60^0C in a water bath[4] for 60 minutes (labelled A) and another 50mℓ was not heated (labelled B) respectively, samples A & B were tested in duplicate respectively:

[1] Hayman LTD, Eastways Park, Withman, Essex, CM8 3YE, England.
[2] ORB Diagnostics, CC., P.O.Box 763, Eden vale, 1610, Johannesburg, RSA.
[3] VWR International GmbH, 76646, Bruchsal, Germany.
[4] Labotec® Model No. 132, Serial No. S.E. 6731, RSA.

3. A 2-fold serial dilution of 5mℓ egg albumen (A) previously heated plus 5mℓ of de-ionised water was made.
4. Eight serial dilutions (2^{-1} to 2^{-8}) were made in eight different sterilized transparent disposable air tight screw-cap plastic test tubes[1] labelled A1-A8 and 5mℓ of the sample from the last test tube was discarded.
5. The same procedures (steps 3-4) were repeated for albumen (B) previously not heated
6. The albumen (A)-deionised water dilutions (from step 4 above) were added to wells on Mueller-Hinton agar[2] plates seeded with viable spores of *B. megaterium* labelled as wells A1 to A8.
7. The same procedure (step 6 above) was repeated for albumen (B) and wells labelled as B1 to B8.
8. They were then incubated in an air incubator[3] for 24 hours and observed for the presence or absence of bacterial inhibition.

ii. Broth method:

1. Two sets (C & D) of eight test tubes were prepared each containing 900µℓ of heat sterilised nutrient broth[4] supplemented with 1% glucose[5] and 0.04% phenol red indicator[6] and seeded with approximately 10^7 colony forming units (CFU) /mℓ of *G. stearothermophilus.*

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[1] PlastPro Scientific, (Pty) Ltd, RSA.
[2] Oxoid products, Basingstoke, Hampshire, England.
[3] Incothern-Labotec® 40 L Digital Incubator, Model 295, Serial No. 0100007606295, 230V-50Hz, 500W, RSA.
[4] Nutrient broth 0003-01-6, Difco laboratories, Detroit, Michigan, USA.
[5] Glucose-Monohydrate, Merck Art 8342, packaged under license by Merck (Pty) Ltd, Halfway House, Unit 11 Fedlife Park, Midrand, RSA.
[6] Phenol red indicator, Unilab® 497159, Saarcham, (Pty) L, Muldersdrift, 1747, RSA.

2. 100µℓ of the heat treated albumen (B) was also added to the first test tube in set C. A 10-fold serial dilution was then carried in tubes C1 to C8.
3. The same was done to "Set D", with the exception that the untreated albumen control was used.
4. The test was done in triplicate with all the tubes being incubated at 60^0C for 24 hours.
5. The same test was repeated using instead, approximately 10^7 CFU/mℓ of *B. megaterium* and incubating these tubes in an air incubator[1] at 37^0C for 24 hours.

Both tests were repeated using 80^0C for 10 minutes as the inactivation temperature (Figure 3.4).

2.3.3 Minimum antimicrobial detection limit testing on spiked whole egg samples

1. Purchased *B. megaterium* ATCC 9885 and *G. stearothermophilus* ATCC 12980 were streaked onto Mueller-Hinton agar incubated in an air incubator for 24 hours at 37^0C and 65^0C respectively.
2. An isolated bacterial colony was sub-cultured to ensure purity and the identity confirmed using morphological and biochemical methods as described by Koneman, E.W., Allen, S.D., Janda, W.M., Schreckenberger, P.C. & Winn, W.C., (1992) and Quinn, P.J., Carter, M.E., Markey, B. & Carter, G.R., (1994).
3. Discrete colonies of *B. megaterium* ATCC 9885 and *G. stearothermophilus* ATCC 12980 were suspended in 10ml each of brain heart infusion broth[2] and distributed into ten 2 ml cryovials[3] and stored at -84^0C[1] respectively.

[1] Incothern-Labotec® 40 L Digital Incubator, Model 295, Serial No. 0100007606295, 230V-50Hz, 500W, RSA.
[2] Oxoid products, Basingstoke, Hampshire, England
[3] Simport self-standing round bottom 2 ml Cryovial® with silicone washer seal and external threads, 2588 Bernard-Pilon, Beloeil, QC J3G 4S5, Canada.

4. The minimum antimicrobial detection limits or minimum detection concentration (MDC) of *B. megaterium* and *G. stearothermophilus* were determined using the broth microdilution test (CLSI, 2008) for various antimicrobials. The following analytical grade antimicrobials, purchased from Sigma-Aldritch[2] were tested: enrofloxacin, norfloxacin, neomycin, tylosin, chlortetracycline, florfenicol, sulfadiazine, sulfamethoxyzole, trimethoprim, spectinomycin, ampicillin, gentamicin, fosfomycin, lincomycin, tiamulin, colistin, oxytetracycline and doxycycline. These cover the common therapeutic antimicrobials used in the South African poultry industry, shown in Table 3.1 in Chapter 3 (Stolker & Brinkman, 2005; Bisschop, *personal communication*, 2009).

5. The test was repeated using a two-fold serial dilution of the antimicrobials in 10% beaten egg contents in Mueller-Hinton-phenol-red glucose broth as earlier described in section 2.3.2. Since lysozyme may be present, in especially the egg albumen, it was necessary to heat inactivate the mixture. In the case of *G. stearothermophilus*, heat inactivation was done after adding the bacterial suspension because *G. stearothermophilus* is thermophilic, and was able to withstand the temperature of 80^0C without dying. Furthermore, its spores require temperatures greater than 70^0C to stimulate germination (Nazina, T.N., Tourova, T. P., Poltaraus, A.B., Novikova, E.V., Grigoryan, A.A., Ivanova, A.E., Lysenko, A.M., Petrunyaka, V.V., Osipov, G.A., Belyaev, S.S. And Ivanov, M.V., 2001). In the case of *B. megaterium,* heat inactivation at 80^0C was done before adding the bacterial suspension because the bacteria grow best at 37^0C and cannot withstand 80^0C. The minimum antimicrobial

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[1] Forma Scientific inc., -86°C Freezer Model 938 208/230V, 50/60HZ, 12.0 A, 1PH. HI STAGE: R-134A, 27.0 OZ, Ohio 45750, USA.

[2] Sigma-Aldrich SA(Pty) Ltd. P. O. Box 10434, Aston Manor 1630, South Africa

detection limits test was then repeated weekly over a 5-week period to ensure repeatability and reproducibility of results.

6. The tests (new in-house bacteriological screening test) had positive growth controls containing the bacteria in the broth culture and a negative control containing broth only (Figure 3.4). The bacterial suspension (100µℓ) was also plated out onto Mueller-Hinton agar to ensure purity and that the correct inoculum size had been used.
7. The tests were considered to be complete when there was a colour change of the broth from red to yellow after incubation, indicating glucose fermentation in the growth controls.
8. Photographs of the test results were taken to show the colours of positive and negative results and to also compare them with that of the control (Figures 3.3 and 3.4).
9. The MDC of the antimicrobials in µg/ℓ was recorded (Figure 3.5 and Table 3.1) and was considered to be the lowest concentration of antimicrobial at which growth was not detected or there was no colour change from red to yellow after two to four hours of incubation.

2.3.4 Detection of antimicrobial residues in eggs of treated hens using the test bacterium *Geobacillus stearothermophilus*

1. The following over-the-counter antimicrobials were purchased: amoxicillin[1], trimethoprim[2], lincomycin[3], fosfomycin[4], tylosin[5], tiamulin[6], ciprofloxacin[7], doxycycline[8], enrofloxacin[9], oxytetracycline[10] and sulphachloropyrazine[11]. These represent several classes of antimicrobials and are often used in poultry production.
2. A total of 36 laying hens were fed a layer ration containing no antimicrobials and given water *ad libitum.* These hens were randomly divided into twelve groups of three each and each group was treated daily for seven days by oral gavage with one of the antimicrobials, based on the manufacturer's recommended dosage and the hens' body weights. One group received only water and served as a control group (see Table 2.1). All the eggs were collected a day before dosing and also on the day of dosing (just before dosing) and thereafter collected daily during dosing for seven days as well as for another seven days after the antimicrobial treatments had been halted.
3. A pool of the eggs collected on a day per group was tested using the in-house bacteriological screening test for bacterial inhibitors (AR) in eggs.

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[1] Avimox 10%, Bremer Pharma, GMBH 27540, Bremerhaven, Germany.
[2] Trimethoprim, V-tech Veterinary solutions pharmacy, Y53159. www.V-tech.co.za. RSA
[3] Lincocin,® Pharmacia South Africa (Pty) Ltd., Unit G, Alphen Square West, George Str., Midrand 1685, RSA.
[4] Fosbac, Bedson® Africa (Pty) Ltd., Willow Business Park, Silverton X52, Pretoria, RSA.
[5] Tylovet o-s, V.M.D. nv/sa- Berendonk, 74-b-2370 Arendonk, Belgium.
[6] Tiamutin 10% premix, Divpharm manufacturing and packaging (pty) Ltd-In-house sample, RSA.
[7] Ciprotab® 500, V.S International Pvt Ltd., Plot no. J/76, M.I D.C., Tarapur, Thane-401 506, India.
[8] Doxybiotic, MEDPET (Pty) Ltd., Unit 7A, Droste Industrial Park, Benrose, Johannesburg 2094, RSA.
[9] Bartril 10%, Bayer (Pty) Ltd., Animal Health Division, Bayer AG, Germany.
[10] Terramycin, Pfizer Laboratories (Pty) Ltd., 102 Rivonia Road , Sandton 2196, RSA.
[11] ESB3, Norvatis South Africa (Pty) Ltd., P.O Box 92, Isando 1600, RSA.

Table 2.1: Weights of hens, dosages and withdrawal periods of antimicrobials administered to hens.

Cage no	Antimicrobial	Dosage mg/kg of body mass	Weight of bird (kg)	Dose administered (mg) in 5mℓ water	Withdrawal period[1] (days)
1	Water (control)	5 mℓ water	2.0	5mℓ	-
			1.5	5mℓ	
			2.0	5mℓ	
2	Amoxicillin	250	2.4	600	1
			1.9	475	
			2.4	600	
3	Trimethoprim	100	1.9	190	10
			2.0	200	
			2.0	200	
4	Lincomycin	100	1.8	180	2
			1.6	160	
			1.6	160	
5	Fosfomycin	160	2.1	336	7
			1.7	272	
			2.1	336	
6	Tylosin	50	2.0	100	3
			1.3	65	
			2.0	100	
7	Tiamulin	30	2.1	63	3
			2.0	60	
			2.3	69	
8	Ciprofloxacin	5	2.3	11.5	7
			1.8	9	
			2.1	10.5	
9	Doxycycline	50	2.0	3.3	3
			2.0	3.3	
			2.0	3.3	
10	Enrofloxacin	5	2.0	10	7
			2.0	10	
			2.0	10	
11	Oxytetracycline	50	2.0	100	4
			2.0	100	
			2.0	100	
12	Sulphachloropyrazine	24	2.0	48	3
			1.8	43.2	
			2.0	48	

[1] Withdrawal periods claimed by the various manufacturers.

2.3.5 Test Procedure

The eggs collected from the hens were subjected to the in-house bacteriological screening test for bacterial inhibitors in eggs:

1. Using a Class II Biological Safety Cabinet[1] to avoid contamination, the eggs were arranged with the pointed ends facing downwards in a plastic egg crate that was cleaned and the eggs disinfected by spraying with 70% alcohol[2] and allowed to air-dry.
2. A hole of approximately 1 to 2cm in diameter on the top part (air sac end) of the eggs was made using a sterile pair of scissors and forceps.
3. The egg contents were poured into a Sterilized Nasco plastic whirlpak® bag[3], sealed and homogenized for 60 seconds using a stomacher lab-blender- 400[4] to mix the contents thoroughly.
4. Each test was conducted in triplicate (for reproducibility); a positive growth control was included with each test, furthermore purity checks and a bacterial count check were done with each test run by spreading 100µℓ of seeded-broth on Mueller Hinton agars and incubating at 37^0C and 80^0C overnight.
5. Sterilized nutrient broth containing 1 % glucose and 0.04 % phenol red was seeded with approximately 10^7 CFU/mℓ of *G. stearothermophilus* (equivalent to a 0.5 Mac Farland standard). Volumes of 900µℓ were then pipetted into sterile 3mℓ capacity plastic screw cap tubes. The egg mixture was added at a volume of 100µℓ.

[1] Scientific Engineering, Industrial North, Model 650, 1500W, RSA
[2] Hayman LTD, Eastways Park, Withman, Essex, CM8 3YE, England.
[3] Sterilized Nasco plastic whirlpak® bag with puncture proof tabs, USA.
[4] Model No. BA 6021, single phase, Seward Medical, UAC House, Black Friars Road, London,95 W, Great Britain.

6. The samples in the test tubes were allowed to stand on the bench for 15 minutes to allow the bacteria and egg contents to mix with each other (pre-diffusion) and then heated at 80^0C for 10 minutes in a water bath to inactivate the natural inhibitors and to allow the bacterial spores to germinate.
7. The temperature of the water bath was reduced from 80^0C to 65^0C and test samples incubated further for 2 to 4 hours, until the colour of the growth control changed from red to yellow.
8. A colour change of the test sample from red to yellow indicated that antimicrobial compounds were absent (positive for bacterial fermentation), and where the colour remained red, it indicated the presence of antimicrobials.
9. The duration of the test and the results were recorded on a record sheet and photographic records also made.

2.3.6 Comparison of the In-house bacteriological screening test with Kundrat micro screening four-plate test

The principle of the Kundrat micro screening four-plate test[1] is similar to other agar-plate tests like the STAR® test (Gaudin *et al.,* 2008). A microorganism (*Bacillus subtilis* BGA) that is sensitive to antimicrobial residues is seeded into the agar medium in a Petri dish; the test samples are then placed on the surface (or wells made in the agar) of the seeded agar and incubated at the optimal growth temperature of the test microorganism (37^0C). After diffusion of the AR contained in the test samples into the agar medium, zones of inhibition

[1] Sigma-Aldrich SA(Pty) Ltd., P. O. Box 10434, Aston Manor 1630, South Africa.

around the samples are produced by inhibiting the growth of the test microorganism. These zones of inhibition are measured, recorded and results calculated.

A total of forty egg samples, previously screened for AR using the in-house method were also sent to an independent laboratory for screening in order to compare and enhance the new in-house test validation. The test samples were made up of ten egg samples previously spiked with the following known antimicrobials, avimox, trimethoprim, fosfomycin, tylosin, tiamulin, ciprofloxacin, doxycycline, enrofloxacin, oxytetracycline and sulphachloropyrazine and another thirty field samples collected during the two surveys.

2.4 Phase II

2.4.1 Survey of antimicrobial residues in commercial chicken eggs in Tshwane area of Gauteng Province, RSA.

- Estimation of the sample number (n = number) was done using Epi Info™[1] software from the internet and calculated as follows:
    - Estimated prevalence P = 5% ± 3% (precision or error limits)
    - 95% confidence level

    The formula used assumes independent observation without clustering, an infinite population and is based on the normal approximation to the binomial distribution:

[1] Epi Info™ version 6.04d for Windows, Centers for Disease Control and Prevention, Atlanta Georgia. http://www.cdc.gov/epiinfo/Epi6/ei6.htm. Accessed date 01/10/2008.

$$n = \frac{t^2 \times P\,(1-P)}{m^2}$$ (FAO, 1990; Fosgate, G.T., 2009)

n = required sample size

t = confidence level 95% (1.96)

P = estimated prevalence 5% (0.05)

m = margin of error 3% (0.03)

$$n = \frac{1.96^2 \times 0.05\,(1-0.05)}{0.03^2}$$

$$n = \frac{0.182476}{0.0009}$$ = 203 samples per survey (total of 406 samples for surveys 1 and 2)

- Based on the above calculation, an observational study was conducted to collect a total of approximately 406 samples in two surveys (six eggs per sample). However, a total number of 422 samples were finally collected. Included in the 422 samples were 81 samples from twenty selected supermarkets which were determined by using the Random Number Generator Software[1] from 168 listed supermarkets in the Yellow Pages[2] and the Phone Book for Tshwane and surrounding areas for 2007/2008[3] (Table 2.2).

[1] Software on the internet for generating random numbers www.random.org/integers. Accessed date 01/10/2008.
[2] List if shops and business addresses for the city if Tshwane and surrounding areas for 2007/2008 www.yellowpages.co.za. Accessed date 01/10/2008.
[3] Telephone directory and addresses of business location in the City of Tshwane and surrounding areas for 2007/2008 www.telcom.co.za. Accessed date 01/10/2008.

Table 2.2: Twenty selected supermarkets which were determined by using the Random Number Generator Software.

SUPER MARKET	NUMBER	AVERAGE	RANDOM NUMBER PICKED
Fruit & Veg City	9	1	5
Checkers	12	1	12
Pick ' N Pay	37	4	21, 31, 16, 3
Spar	57	7	41, 36, 17, 44, 31, 43, 2
Shoprite	14	2	8, 12
Makro Superstore	3	1	3
Woolworths	36	4	16, 28, 22, 30
TOTAL	**168**	**20**	**20**

- The remaining 341 field survey samples were purchased along the major taxi-routes whose original sales outlet locations and numbers could not be pre-determined. This was because they were not listed in the Yellow Pages and the Phone Book for Tshwane and surrounding or any other directory; samples were however purchased along the routes as were available as at the time of sampling.
- The major taxi road-routes running respectively from North to South and East to West of Tshwane were used to buy egg samples from shops and supermarkets situated along the route (see Section 2.4.2).
- Sampling was carried out in two seasons in the year to control variation over the samples; the first survey was in October/November 2008 (spring) and second survey was in April/May 2009 (autumn).
- The second survey sampling, sample processing and result analysis was conducted in the same way as was done for the first survey.

The following definitions were assigned to the following sales outlets:

- Supermarkets were retail sales-outlets where products were housed in covered buildings with high standards of care maintained; the owners claim to have residue control programmes in place. Included in this category were the twenty selected supermarkets because they were responsible for a very large proportion of the eggs sold and some of them were also situated along the routes driven while others were randomly selected from the Yellow Pages[1] and Phone Book for Tshwane and surrounding areas 2007/2008[2] using Random Number Generator Software[3].
- Garage shops were shops located in the fore-court of branded petrol stations situated along road-sides where groceries and fast food items were sold alongside eggs.
- Butcheries were meat and meat products retail outlets where eggs were also sold along with these products.
- Roadside / informal shops were sales-outlets where eggs were displayed on tables exposed to the sun without cover or shelter and were mainly found along the roads/ at taxi ranks/train stations and in township areas.
- Other-shops were any sales outlets other than those listed above, for example, small covered buildings with low standards of care maintained and no residue control programmes in place.

[1] List if shops and business addresses for the city if Tshwane and surrounding areas for 2007/2008 www.yellowpages.co.za. Accessed date 01/10/2008.
[2] Telephone directory and addresses of business location in the City of Tshwane and surrounding areas for 2007/2008 www.telcom.co.za. Accessed date 01/10/2008.
[3] Software on the internet for generating random numbers www.random.org/integers. Accessed date 01/10/2008.

2.4.2 Sampling locations

The following road routes were used and all retail outlets selling eggs found along a particular route were sampled. The routes are shown highlighted in pink lines in Figure 2.3.

The starting point was the Tshwane Central Taxi Rank (TCTR) located along the corners of van der Walt and Bloed Streets.

- SOUTH: From the TCTR movement was South along Andries Street to Tshwane Central Train Station (Bosman Street Train Station) and return to PCTR via van der Walt Street.
- NORTH: From TCTR movement was west along Bloed to Paul Kruger streets; then north along Paul Kruger and Soutpan Road to Soshanguve.
- EAST: From TCTR movement was east on Church Street to Silverton then to Mamelodi.
- WEST: From TCTR movement was along Church Street and then west to Atteridgeville.
- In addition, 20-supermarkets were randomly selected from one hundred and sixty eight supermarkets listed in the Yellow Pages[1] and Phone Book for Tshwane and surrounding areas 2007/2008[2] using Random Number Generator Software[3]. This was done in order to have samples close to the estimated 406 in case there were not enough samples found along the road routes.

[1] List if shops and business addresses for the city if Tshwane and surrounding areas for 2007/2008 www.yellowpages.co.za
[2] Telephone directory and addresses of business location in the City of Tshwane and surrounding areas for 2007/2008 www.telcom.co.za. Accessed date 01/10/2008.
[3] Software on the internet for generating random numbers www.random.org/integers. Accessed date 01/10/2008.

2.4.3 Sampling procedures

- The positional co-ordinates of each sampling site were recorded on an e-Trex Legend ™ Global Positioning System-GPS[1]. The names and address of each retailer were also recorded (see Appendix 2A). The pink lines highlight the roads where eggs for testing were purchased for the two surveys Figures 2.3 to 2.7.
- A maximum of six undamaged eggs of each of the different brands available in each retail outlet were purchased, clearly labeled and stored in a cooler box away from sunlight.
- Large sized eggs were preferred, where they were not available, extra-large followed by smaller sized eggs were purchased.
- Any refusal to sell eggs by the retailer was recorded with an explanation.
- On reaching the laboratory, usually within 6 hours, all the eggs were stored in the refrigerator[2] at ±5^{0}C until all the eggs had been collected. This was over a period of ten days, for each of the two surveys.

[1] E-Trex Legend ™ Global Positioning System-GPS[1] www.garmin.com. Accessed date 01/10/2008.
[2] Club refrigeration, Manufactured by RECAM International, model SU 70, Serial No. 90789, Motor Watts 34, 220 V, Fan 2067, RSA.

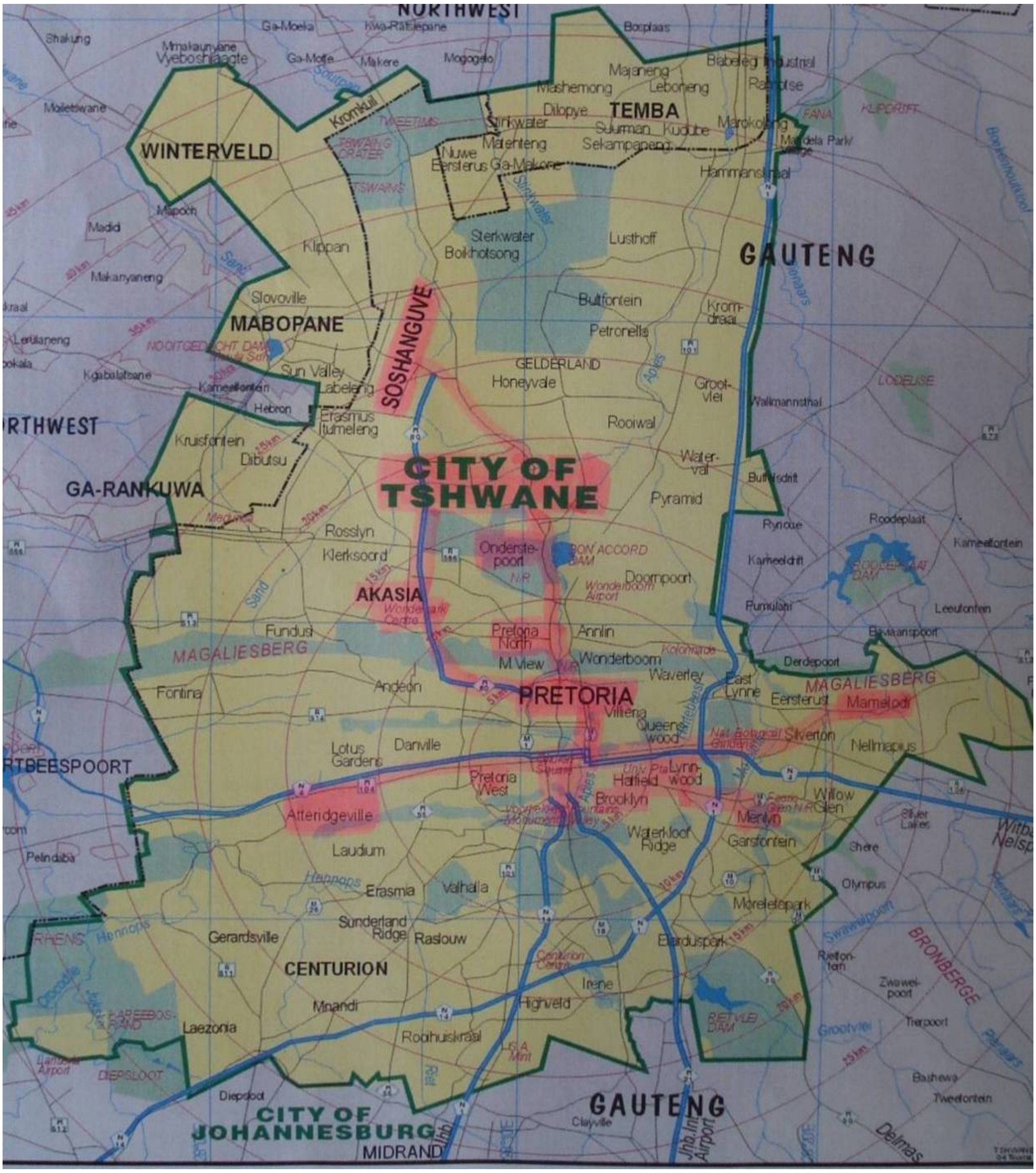

Figure 2.3: Map[1] of the City of Tshwane (Pretoria) showing Atteridgeville, Mamelodi, Tshwane Metropolis (Pretoria CBD), Soshanguve and other locations where egg samples were purchased for the two surveys. The pink lines highlight the roads where eggs for testing were purchased.

[1] http://www.tshwane.gov.za/streetmaps/Tshwane_regions.pdf. Accessed date 01/10/2008.

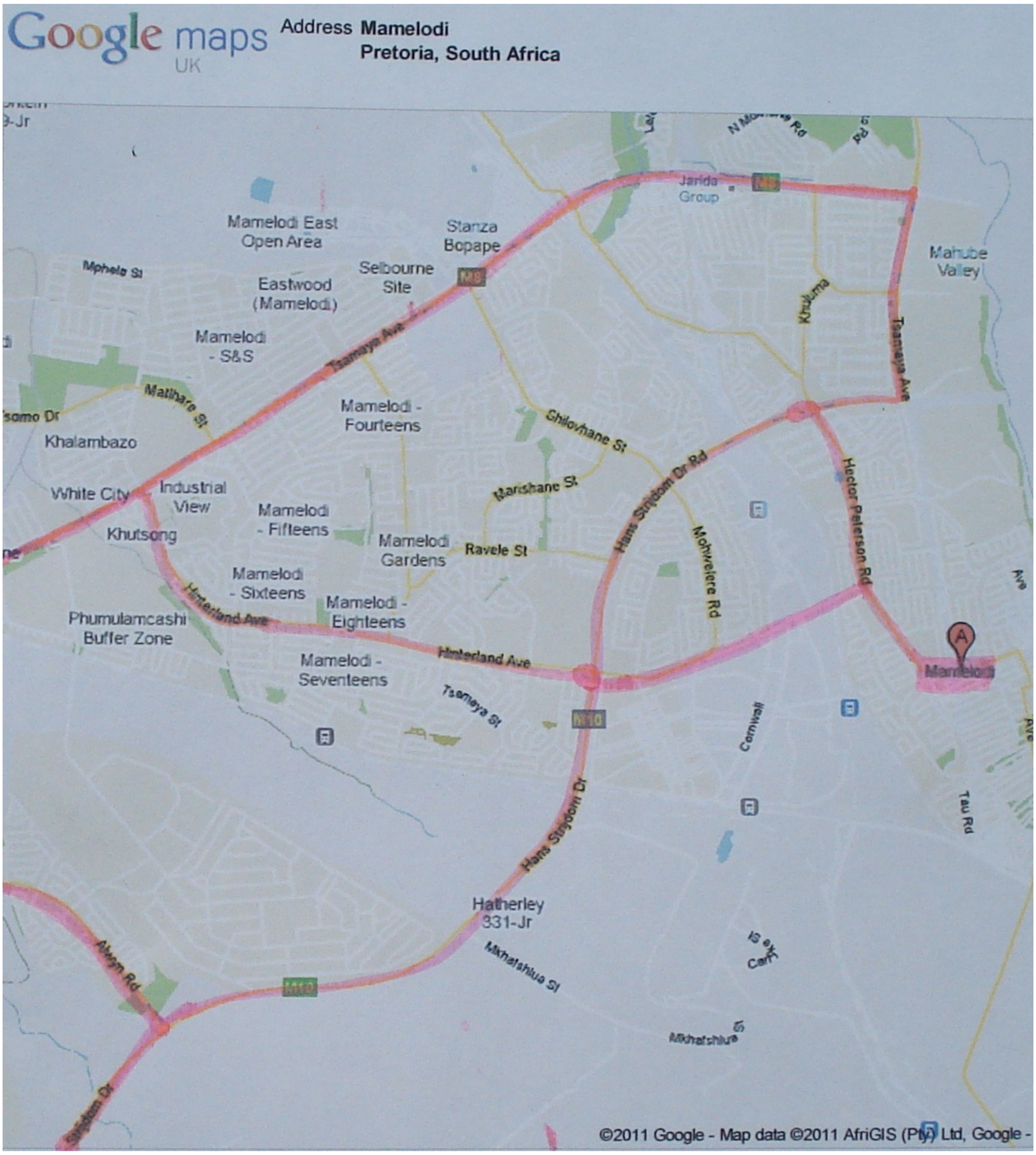

Figure 2.4: Street map[1] of Mamelodi (RSA) the pink lines highlight the roads where eggs for testing were purchased from different sales outlets for the first and second surveys (eastern road routes).

[1] 2011 Google - map data © 2010 AfriGIS (Pty) Ltd, Google - http://maps.google.co.uk

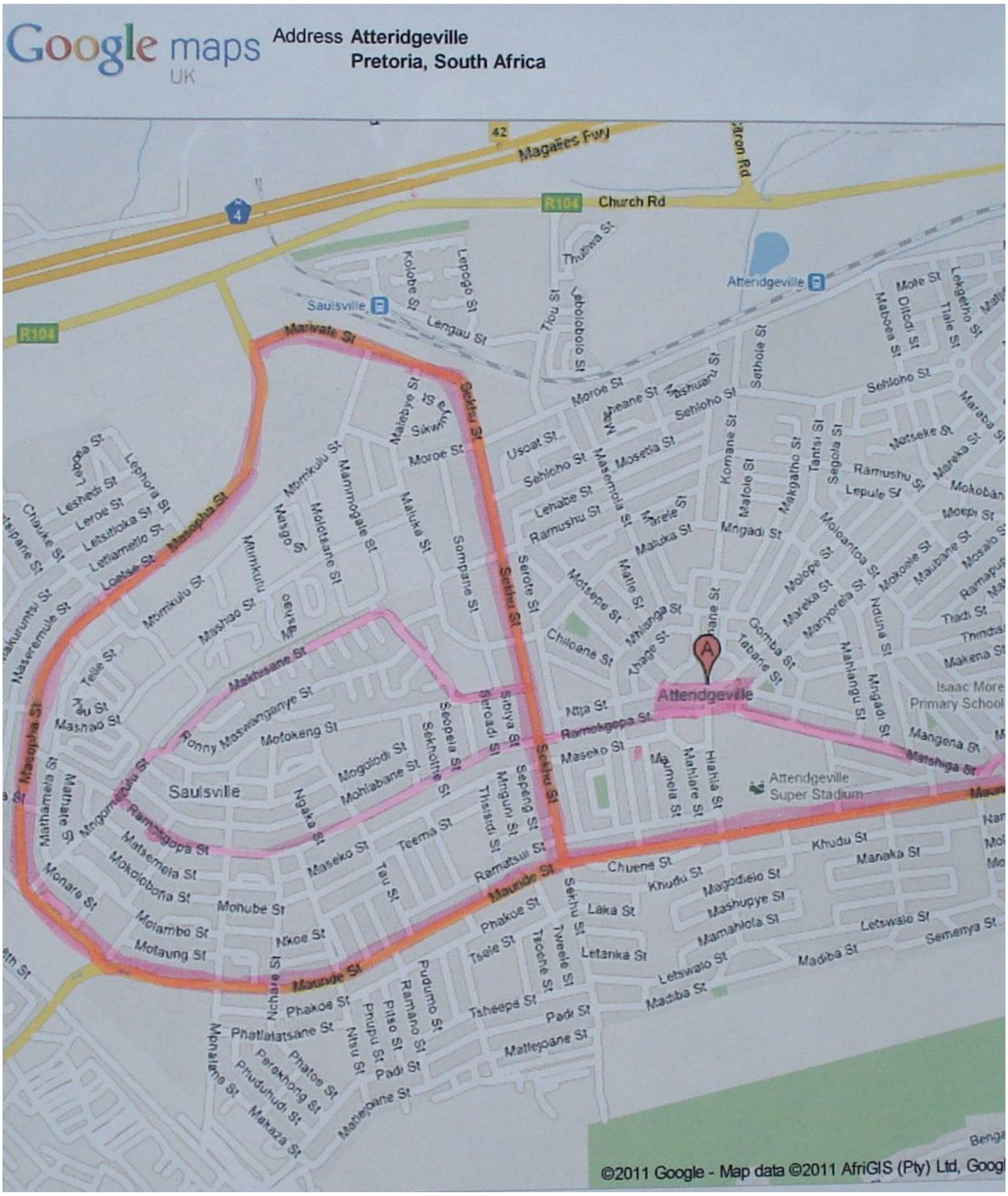

Figure 2.5: Street map[1] of Atteridgeville (RSA) the pink lines highlight the roads where eggs for testing were purchased from different sales outlets for the first and second surveys (western road routes).

[1] 2011 Google - map data © 2010 AfriGIS (Pty) Ltd, Google - http://maps.google.co.uk

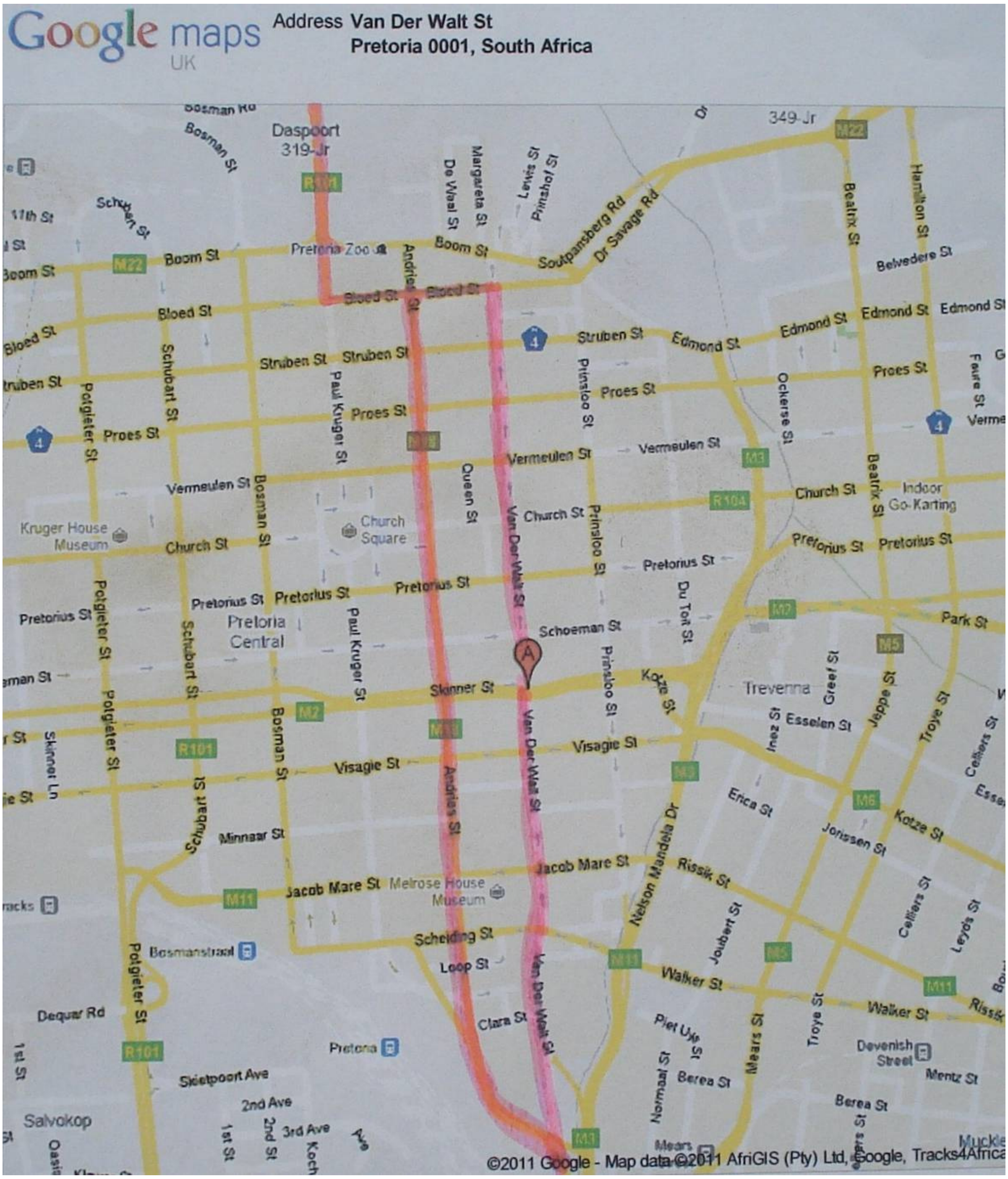

Figure 2.6: Street map[1] of Tshwane (RSA) the pink lines highlight the roads where eggs for testing were purchased from different sales outlets for the first and second surveys (southern road route)

[1] 2010 Google - map data © 2010 AfriGIS (Pty) Ltd, Google - http://maps.google.co.uk

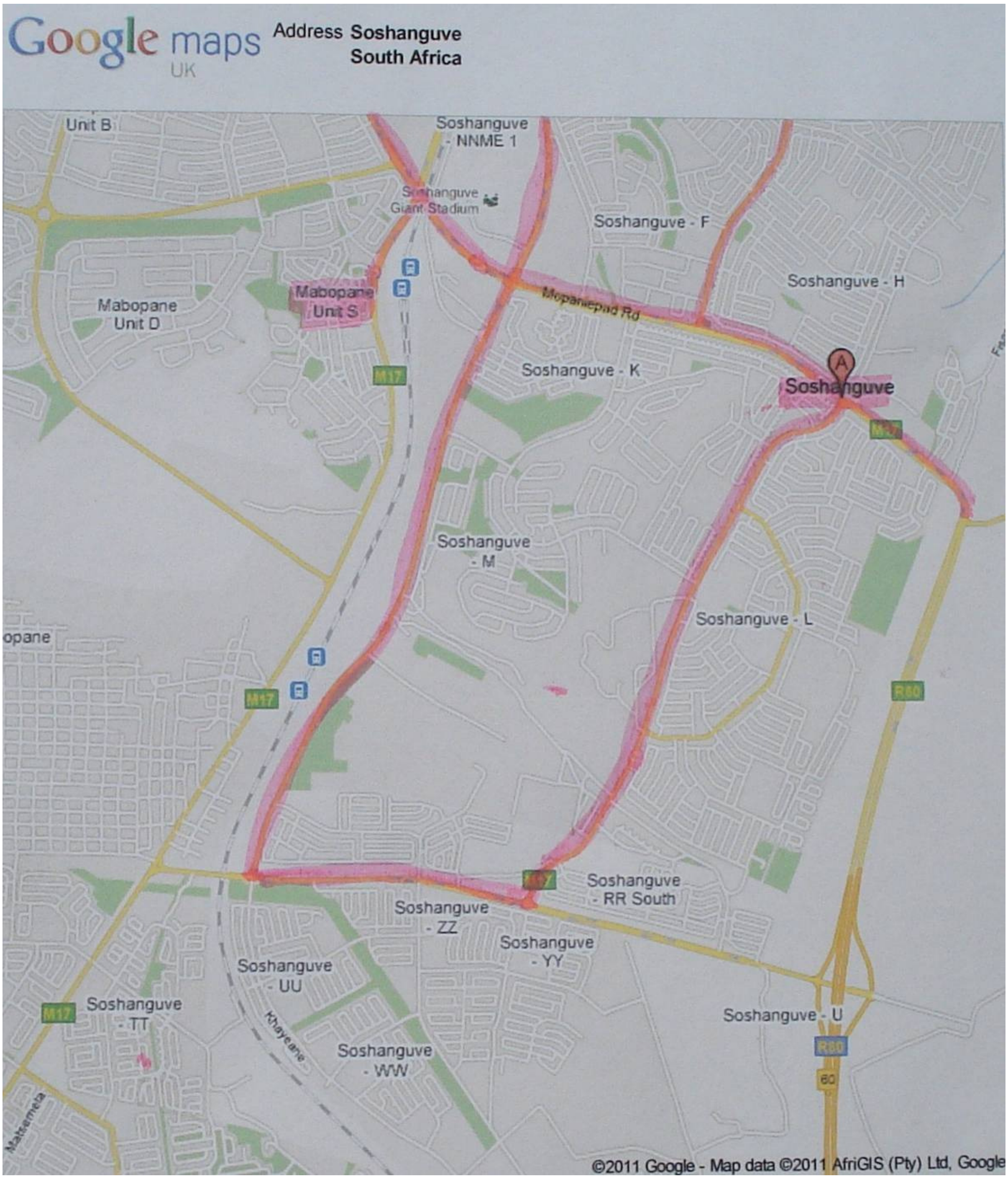

Figure 2.7: Street map[1] of Soshanguve (RSA) the pink lines highlight the roads where eggs for testing were purchased from different sales outlets for the first and second surveys (northern road route).

[1] 2010 Google - map data © 2010 AfriGIS (Pty) Ltd, Google - http://maps.google.co.uk

2.4.4 Screening test procedure

The microbiological screening test as described in section 2.3.5 was carried out on all the egg samples. The test was conducted in triplicate within a sample, and testing repeated three times on different days for reproducibility within and between samples. After testing, egg samples were stored at -20^{0}C.

2.4.5 Methodology used for statistical analyses

Multivariate regression was used to estimate the association of factors with AR. The percent of samples positive for AR, the confidence interval (CI), and the significance test (set at $P < 0.05$) were determined. Graphs to show association of egg prices with AR were determined (Wald, A., 1947; Seely & El-Bassiouni, 1983).

CHAPTER 3 – RESULTS

3.1 Introduction

Results of the preliminary studies conducted using the in-house screening method on spiked egg samples and also from eggs from 36 hens that were housed in the Poultry Research Unit (PRU) and given therapeutic doses of over-the-counter antimicrobials are presented. Also presented are the results from the field survey from egg retail sales-outlets in Tshwane area, RSA.

3.2 Experimental Results

3.3 Phase I

3.3.1 Testing for inhibitors and inactivation of inhibitors

i. Agar method

The albumen samples in wells A1- A4 and B1- B4 (dilution 2^{-1} to 2^{-4}) of Mueller-Hinton agar seeded with *B. megaterium* and *G. stearothermophilus* inhibited the growth of these bacteria. This was indicated by clear zones of inhibition around the edges of the wells (Figure 3.1). The same effect (inhibition) was noted when the albumen was heated to 60^0C for 60 minutes.

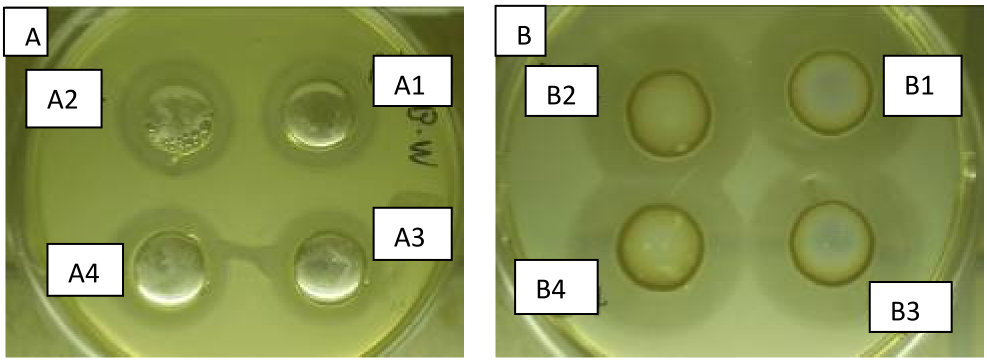

Figure 3.1: Clear zones of inhibition due to the presence of albumen in wells A1-A4 and B1-B4 seeded with *B. megaterium* and *G. stearothermophilus* respectively on Mueller Hinton agar plates.

However the bacteria were no longer inhibited by the albumen at a dilution of 2^{-5} or more (see Figure 3.2).

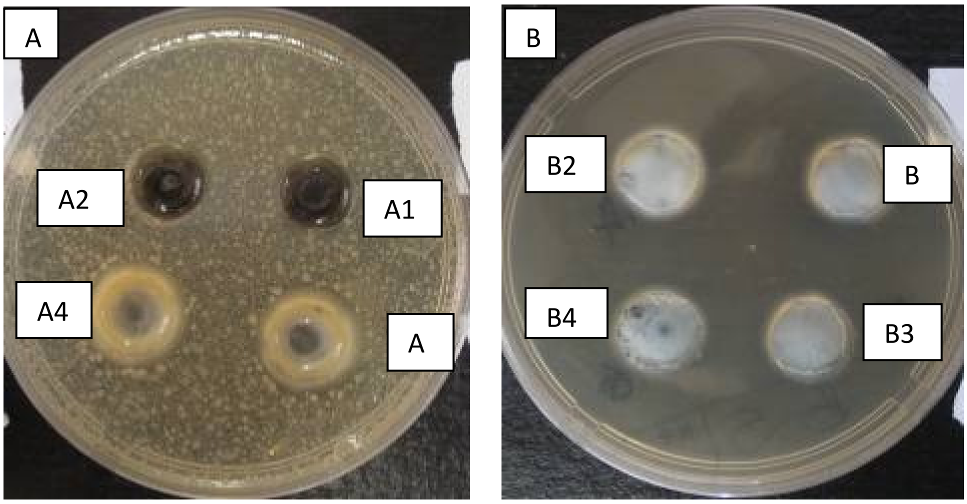

Figure 3.2: Growth of *B. megaterium* (A) A1 to A4 and *G. stearothermophilus* (B) A1 to A4 on Muller Hinton agar plates indicated by the absence of zones of inhibition at low dilutions (2^{-5}-2^{-8}) of egg albumen.

ii. Broth method:

After heating the albumen in an air incubator[1] to 60^0C for 60 minutes, one of the eight albumen samples (see Figure 3.3 tube 8 below-arrow) that were labelled as tubes 1- 8 and diluted 10^{-1} inhibited the growth of *B. megaterium* and *G. stearothermophilus* in their respective nutrient broths after 24 hours of incubation. However, there was no growth inhibition noted in the further dilutions 10^{-2} - 10^{-8} (shown in tubes 19-25 in Figure 3.3) for both *B. megaterium* and *G. stearothermophilus* respectively.

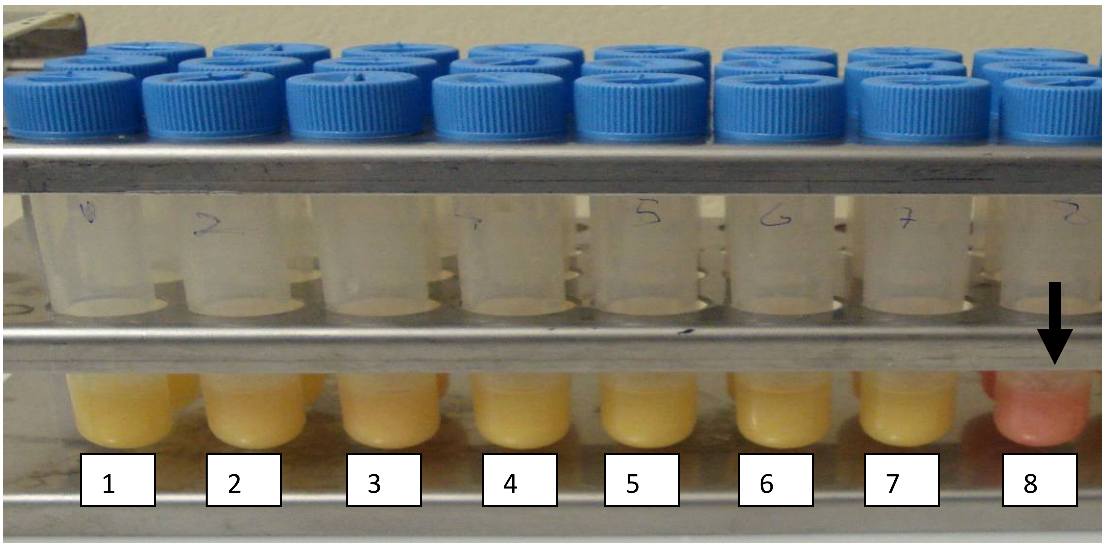

Figure 3.3: Broth cultures showing growth inhibition of *G. stearothermophilus* in tube 8 (arrow) indicated by red colour due to the presence of albumen.

Both tests (agar and nutrient broth) were repeated using the temperature of 80^0C for 10 minutes as the inactivation temperature. This short temperature treatment at 80^0C

[1] Incothern-Labotec® 40 L Digital Incubator, Model 295, Serial No. 0100007606295, 230V-50Hz, 500W, RSA.

for 10 minutes was able to effectively inactivate the non-specific inhibitors present in all the albumen dilutions of both the agar and broth. The results of a broth dilution series is shown in Figure 3.4.

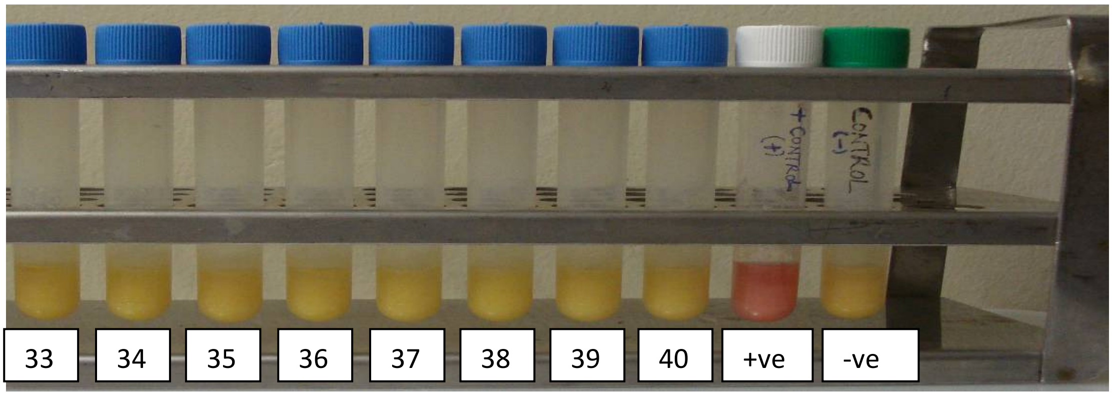

Figure 3.4: 24-hour cultures of a 2-fold dilution series of albumen showing growth of *G. stearothermophilus* indicated by yellow colouration of all the test samples (tubes 33-40) due to the inactivation of albumen (lysozyme). Red-coloured Ab +ve (negative bacterial growth control) and yellow Ab -ve controls are included for comparison.

3.3.2 Minimum antimicrobial detection limit testing on spiked whole egg samples

The minimum detection concentration (MDC) of the antimicrobials in µg/ℓ was considered to be the lowest concentration of antimicrobial at which growth was not detected; or there was no colour change from red to yellow after two to four hours of incubation (Figure 3.5). The mean MDC for each of the tested antimicrobial using *B. megaterium* and *G. stearothermophilus* is shown in Table 3.1 and compared to its MRL and minimum detection concentration of the Premi®Test. These results varied within one dilution of the test where a two-fold dilution series was made of antimicrobials in nutrient broth only. It was not possible to compare them to spiked eggs samples without heating due to the presence of naturally occurring bacterial inhibitors in egg albumen. Note that this result was not unexpected as I had checked the material data sheets of each antimicrobial as well as publications on these products to ascertain their heat-stability (Egmond *et al*., 2000; Ramirez *et al.,* 2003; Aerts *et al.,* 1995; Premi®Test 1, 2008; Premi®Test 2, 2008; WIPO, 2008.

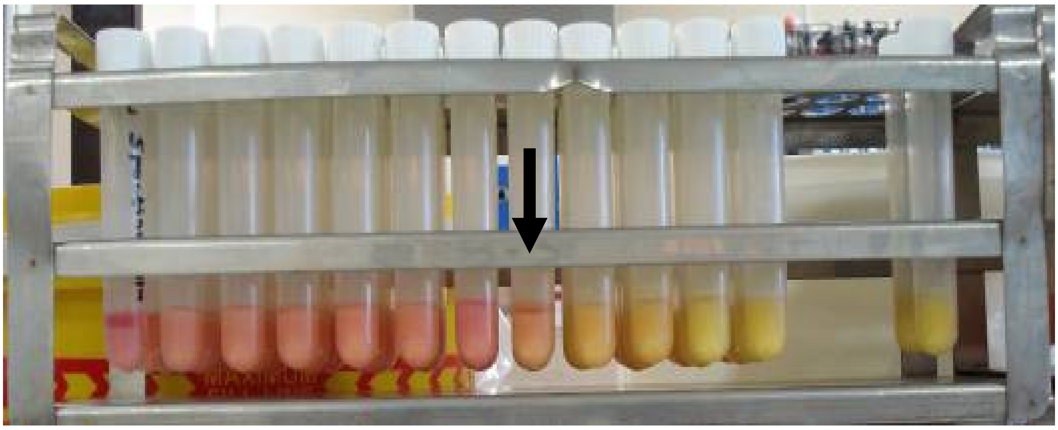

Figure 3.5: Broth cultures showing the minimum detection concentration of spectinomycin indicated by growth inhibition of *G. stearothermophilus* in tube 8 (indicated by arrow).

3.3.3 Results of detected antimicrobial residues in eggs of treated hens

Different antimicrobials were assigned and administered to the eleven different groups of hens comprising of three hens per group for seven days based on the calculated body masses (Table 2.1). Eggs were collected from each of the eleven groups of hens on a daily basis and recorded as shown in Tables 3.2 and 3.3.

Table 3.1: Average of In-house sensitivity test results using either *B. megaterium* or *G. stearothermophilus* detection systems and compared with published Premi®Test and E.U maximum residue limits-MRL (European Commission, 1990)

Antimicrobial	***B. megaterium* µg/ℓ**	***G. stearothermophilus* µg/ℓ**	**Std. Deviation µg/ℓ**	**Premi®Test µg/ℓ**	**MRL µg/ℓ**
Enrofloxacin	30	23	2.35	250	100
Norfloxacin	1 000	247	0.33	-	NE[1]
Neomycin	63	3.8	0.58	600	500
Tylosin	250	3	0.50	50	200
Chlortetracycline	1 028 000	163	1.51	600	200
Florfenicol	8 000	90	1.34	-	NE[1]
Sulfadiazine	1 028 000	88	2.44	25	100
Sulphamethoxyzole	1 028 000	126	1.19	25	100
Trimethoprim	8 000	39.8	1.66	50	50
Spectinomycin	16 000	263.6	0.58	-	200
Ampicillin	2 000	2.34	0	5	50
Gentamicin	15	2.34	0	100	100
Fosfomycin	128 000	4 953	2.61	-	100
Lincomycin	64 000	4.6	1.15	150	50
Tiamulin	>64 000	367.9	1	-	1 000
Colistin	>32 000	10.5	0.58	-	300
Oxytetracycline	8 000	69.4	0.58	400	200
Doxycycline	1 028 000	6.1	1.31	200	200

[1] NE, not established

Table 3.2: Daily number of eggs collected (day -1) prior to treatment, days of treatment (day 0 - 6), and days after treatment of hens with different antimicrobials (days 7 – 13)

Cage no	Antimicrobial	Number of eggs collected before and during treatment								Number of eggs collected after treatment						
		Day -1	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13
1	Water (control)	2	2	2	2	2	2	1	2	2	2	1	2	1	2	2
2	Amoxicillin	2	2	1	2	2	1	1	1	2	2	1	2	2	1	2
3	Trimethoprim	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4	Lincomycin	2	2	1	2	2	1	1	2	2	2	1	2	2	1	1
5	Fosfomycin	2	2	1	2	2	2	0	1	2	2	2	2	2	2	2
6	Tylosin	2	2	3	1	1	2	1	1	1	0	1	2	0	2	2
7	Tiamulin	1	1	0	1	1	1	0	1	1	1	0	1	1	1	1
8	Ciprofloxacin	1	1	1	0	1	1	1	2	2	1	2	1	3	2	1
9	Doxycycline	2	2	1	0	1	0	1	0	1	0	1	1	1	0	0
10	Enrofloxacin	3	3	3	1	3	2	2	1	1	3	0	1	2	3	3
11	Oxytetracycline	1	1	1	1	1	0	1	0	1	1	0	1	1	1	0
12	Sulphachloropyrazine	1	1	0	0	1	1	1	0	1	1	1	1	0	1	1

Eggs collected from each of the groups were subjected to the new in-house test method and results shown in Table 3.3.

Table 3.3: Results of the In-house bacteriological method for AR on pooled egg samples from the 36 treated hens, a day prior to treatment (day -1) days of treatment (days 0 - 6) and days after treatment of hens (days 7 – 13).

Group No	Antimicrobial	Day -1	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13
	Pre-treatment					Treatment							Post	Treatment		
1	Water (control)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	Amoxicillin	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-
3	Trimethoprim	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
4	Lincomycin	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-
5	Fosfomycin	-	-	+	+	+	+	0	+	-	-	-	-	0	-	-
6	Tylosin	-	-	+	+	+	+	+	+	+	0	-	-	-	-	-
7	Tiamulin	-	-	0	+	+	+	0	+	-	-	0	-	-	-	-
8	Ciprofloxacin	-	-	+	0	+	+	+	+	+	-	-	-	-	0	0
9	Doxycycline	-	-	+	0	+	0	+	0	-	0	-	-	-	-	-
10	Enrofloxacin	-	-	+	+	+	+	+	+	-	-	0	-	-	-	-
11	Oxytetracycline	-	-	+	+	+	0	+	0	-	-	0	-	-	-	0

0 = No eggs collected for that day
+ = Presence of inhibitors (presence of antimicrobial residues or no bacterial growth). Each sample (+) was tested in triplicate.
- = No inhibitors (absence of antimicrobial residues or presence of bacterial growth). Each sample (-) was tested in triplicate.

Results in Table 3.3 above showed complete absence of antimicrobial residue in all the egg samples collected for all the hen groups during the pre-treatment period and on day 0. Eggs from the hen control group (given water only) were also free from AR throughout the trial. Most of the eggs collected during the treatment period showed presence of inhibitors (AR+ or no bacterial growth) while eggs collected during the post treatment period showed a decrease and later absence of AR- or presence of bacterial growth from day 7-13. Each egg sample was tested in triplicate for reproducibility of result.

3.3.4 Results of the comparison of the In-house bacteriological screening test for AR in eggs with the Kundrat micro screening four-plate test

The Kundrat micro-screening four-plate test[1] for screening antimicrobial residues in eggs (another microbiological screening method) was used by an independent laboratory that has been accredited by the South African National Accreditation Standards (SANAS) to screen 40 treated hens and field sample eggs. Results of the in-house bacteriological screening test for AR in eggs were compared with that of the Kundrat micro-screening four-plate test for antimicrobial residues in eggs and the results are shown in Table 3.4.

Some results that previously tested as doubtful (+/-) were later found to test either as positive or negative for AR, the doubtful results were later found to be as a result of improper mixing of the egg albumen and yolk (for equal distribution of the AR) rather than from the test procedure. This was because when a stomacher lab-blender- 400[2] was later used to mix the egg contents thoroughly for 60 seconds, and the samples re-tested using the same procedure as previously done, the problem was resolved.

[1] Sigma-Aldrich SA(Pty) Ltd., P. O. Box 10434, Aston Manor 1630, South Africa.
[2] Model No. BA 6021, single phase, Seward Medical, UAC House, Black Friars Road, London 95 W, Great Britain.

Table 3.4: Comparison of the results of the bacteriological in-house screening test for antimicrobial residues in eggs with that of the Kundrat micro screening four-plate test

Sample Number	Sample identification	In-house test AR positive(+) or AR negative (-)	Kundrat four-plate test[1]
1	Avimox	+	-
2	**Trimethoprim**	+	+
3	Fosfomycin	+	-
4	Tylosin	+	-
5	Tiamulin	+	-
6	Ciprofloxacin[1]	+	-
7	Doxycycline	+	-
8	Enrofloxacin	+	-
9	Oxytetracycline	+	-
10	Sulphachloropyrazine	+	-
14	NR 45	+	-
15	ER 4	+	-
16	ER 37	+	-
17	WR 32	+	-
18	WR 33	+	-
19	SM 18	+	-
20	NR 69	+	-
23	NR 61	-	-
24	ER 32	-	-
29	SM 23	-	-
31	S 1	+	-
32	S 5	+	-
36	E 40	+	-
37	W 2	+	-
38	**W 34**	+	+
39	W 37	+	-
40	E 2	+	-
46	E 10	-	-
49	W 10	-	-
50	W 7	-	-
51	NR 35	+	-
52	ER 38	+	-
53	WR 19	+	-
54	WR 31	+	-
55	WR 35	+	-
56	W 29	-	-
57	E 35	-	-
58	E 36	-	-
59	SS 6	-	-
60	SS 37	-	-

Note that samples 51-60 previously tested as doubtful (+/-)

[1] Ciprotab® 500 V.S international Pty Ltd., Plot no. J/76, M.I D.C, Tarapur, Thane-401 506, India (not approved for use in South Africa, it was however considered because of its use in other countries including Sudan).

3.4. Phase II:

3.4.1 Results of the survey of antimicrobial residues in commercial chicken eggs in Tshwane area of Gauteng Province, RSA.

The survey of antimicrobial residues in commercial chicken eggs in the Tshwane Metropolitan Area of Gauteng Province, South Africa was carried out in two seasons in the year; the first survey was in October/November 2008 (spring) and the second survey was in April/May 2009 (autumn).

3.4.2 Results of the screening test procedure

- The microbiological screening test as described in section 2.3.3 was carried out on all the eggs samples and results shown in Table 3.5.
- 406 egg samples were originally estimated to be collected to comprise 203 samples for each of the 2 surveys; however, 341 field samples were eventually collected that were made up of 186 samples for the first survey and 155 samples for the second survey. This was because only 341 field samples out of the estimated 406 samples could be found and purchased on the road routes used for sample collection.
- An additional 81 egg samples were also purchased from 20 selected supermarkets. These in addition to the 341 field samples gave a final total number of 422 egg samples collected.
- The 20 selected supermarkets had 5 AR positive eggs out of 81 egg samples, the informal/roadside outlets had 10 out of 53, butcheries 1 out of 37, garage shops 2 out

of 39, "supermarkets" 5 out of 67 and other shops had 13 AR positive out of 145 egg samples.

- There were 36 AR positive egg samples out of 422 samples representing about 8.5% in the entire survey whereas about 1.2% of the samples from the selected supermarkets were AR positive.

3.4.3 Statistical analyses of potential factors associated with AR in eggs

The overall results for the presence of AR for all the sampled categories are presented for the two surveys. The percent of samples positive for AR, the confidence interval (CI), and the significance test (set at $P < 0.05$) were determined. Multivariable logistic regression as well as histograms and graphs were done to demonstrate whether there was an association of egg characteristics, retailers and sales outlet with the presence of AR. These are presented in Tables 3.5 to 3.7 and Figures 3.6 and 3.7. Note that six eggs comprised an egg sample or egg unit. Also, the two different medians in egg unit price were taken into account and the egg price below the median price was statistically more significant than that above the median.

Risk factors that proved to have an association with the presence of AR included; eggs less than the median price ($P = 0.059$), eggs sold by roadside shops ($P = 0.016$) as well as Brands 9 ($P = 0.047$), 22 ($P < 0.001$) and 27 ($P = 0.017$). Eggs purchased from the 20 selected supermarkets which comprised of large supermarket chain stores proved to have very low levels of AR which was significantly different to the other retailers ($P = 0.016$).

Table 3.5: Percent of egg samples positive for antimicrobial residues based on descriptive factors.

Factor (sample size)	Factor level 1[a]		Factor level 2[b]		P-value*
	Percent %	**95% CI**	**Percent %**	**95% CI**	
Overall (n=341)	9.1	6.4-12.5	N/A	N/A	N/A
Survey 1 (186) Versus Survey2 (155)	11.3	7.3-16.5	6.5	3.3-11.2	0.122
Large size eggs (271) versus other sizes (70)	9.6	6.5-13.5	7.1	2.7-15.1	0.525
Egg price below median (165) versus other prices (176)	**12.1**	**7.8-17.8**	**6.3**	**3.3-10.6**	**0.059**
Niche eggs (53) versus other eggs (288)	7.5	2.4-17.2	9.4	6.4-13.2	0.800
Locations: Tshwane (Pretoria) (157) versus other locations (184)	7.0	3.7-11.8	10.9	7.0-16.0	0.216
Soshanguve (78) versus other locations (263)	11.5	5.8-20.1	8.4	5.5-12.2	0.392
Mamelodi (52) versus other locations (289)	7.7	2.5-17.5	9.3	6.4-13.1	1.00
Atteridgeville (54) versus other locations (287)	13.0	5.8-24.0	8.4	5.6-12.0	0.301
Sales outlets: Butchery eggs (37) versus other outlets (304)	2.7	0.1-12.6	9.7	6.9-13.6	0.226
Informal /road-side shops (53) versus other outlets (288)	**18.9**	**10.0-31.0**	**7.3**	**4.7-10.7**	**0.016**
Garage shops (39) versus other outlets (302)	5.1	0.9-15.9	9.6	6.6-13.3	0.554
Other shops (145) versus other outlets (196)	9.0	5.1-14.5	10.1	6.2-15.2	0.945
Supermarkets (67) versus other outlets (274)	7.5	2.8-15.8	9.5	6.4-13.4	0.605
20 selected supermarkets (81) versus other outlets (341)	**1.2**	**0.1-5.9**	**9.1**	**6.4-12.5**	**0.016**

*Based on Pearson chi-square or Fisher exact test; factor that is compared to; the other (sum) factor[b] being compared to.
Numbers highlighted in bold indicate variables with significant associations with antimicrobial residues.

Table 3.6: Percentage of egg samples positive for antimicrobial residues by brand.

Egg brand#	Sample size	No. positive for AR	Percentage %	95% CI	P- Value*
1	40	2	5.0	0.8-15.6	0.537
2	8	0	0	0-31.2	0.418
3	18	0	0	0-15.3	0.224
4	21	2	9.5	1.6-28.1	0.737
5	17	3	17.6	4.7-40.9	0.117
6	8	0	0	0-31.2	0.418
7	4	0	0	0-52.7	0.567
8	25	3	12.0	3.1-29.3	0.404
9	**37**	**6**	**16.2**	**6.8-30.7**	**0.047**
10	18	0	0	0-15.3	0.224
11	15	0	0	0-18.1	0.267
12	6	0	0	0-39.3	0.483
13	10	0	0	0-25.9	0.365
14	36	3	8.3	2.2-21.0	0.865
15	14	0	0	0-19.3	0.284
16	46	5	10.9	4.1-22.5	0.400
17	11	1	9.1	0.5-37.3	0.850
18	6	0	0	0-39.3	0.483
19	8	0	0	0-31.2	0.418
20	14	0	0	0-19.3	0.284
21	1	0	0	0-95.0	0.775
22	**6**	**3**	**50**	**14.7-85.3**	**<0.001**
23	5	0	0	0-45.1	0.522
24	1	0	0	0-95.0	0.775
25	5	0	0	0-45.1	0.522
26	16	1	6.3	0.3-27.2	0.840
27	**6**	**2**	**33.3**	**6.0-73.8**	**0.017**
28	1	0	0	0-95.0	0.775
29	3	1	33.3	1.7-86.8	0.092
30	3	0	0	0-63.2	0.620
31	2	0	0	0-77.6	0.685
32	1	0	0	0-95.0	0.775
33	3	0	0	0-63.2	0.620
34	3	0	0	0-63.2	0.620
35	1	0	0	0-95.0	0.775
36	2	0	0	0-77.6	0.685
37	1	0	0	0-95.0	0.775

*Based on a Z test comparing percentages to the mean value of 7.6%.

Unique numerical identifier of brands to avoid test bias as well as protecting the identity of producers.

Numbers highlighted in bold indicate egg brands with significant associations with antimicrobial residues.

Table 3.7: Multivariable logistic regression results to estimate associations with antimicrobial residues

Variable	**Parameter estimate ($\hat{\beta}$)**	**P-value (Wald)**	**Odds ratio**	**(95% CI)**
Survey 1versus survey 2	0.631	**0.122**	1.9	0.8-4.2
20 selected supermarkets	1.853	**0.095**	6.4	0.7-56
Brands 9, 22, 27	1.297	**0.004**	3.7	1.5-8.9
Outlet category	—	0.222	—	—
Garage shop	Referent	—	—	—
Supermarkets	0.655	0.455	1.9	0.3-11
Butcheries	-0.883	0.485	0.4	0.04-4.9
Informal/roadside shops	1.237	**0.135**	3.4	0.7-17
Other shops	0.533	0.503	1.7	0.4-8.1

Boldly highlighted numbers indicate variables with significant associations with AR

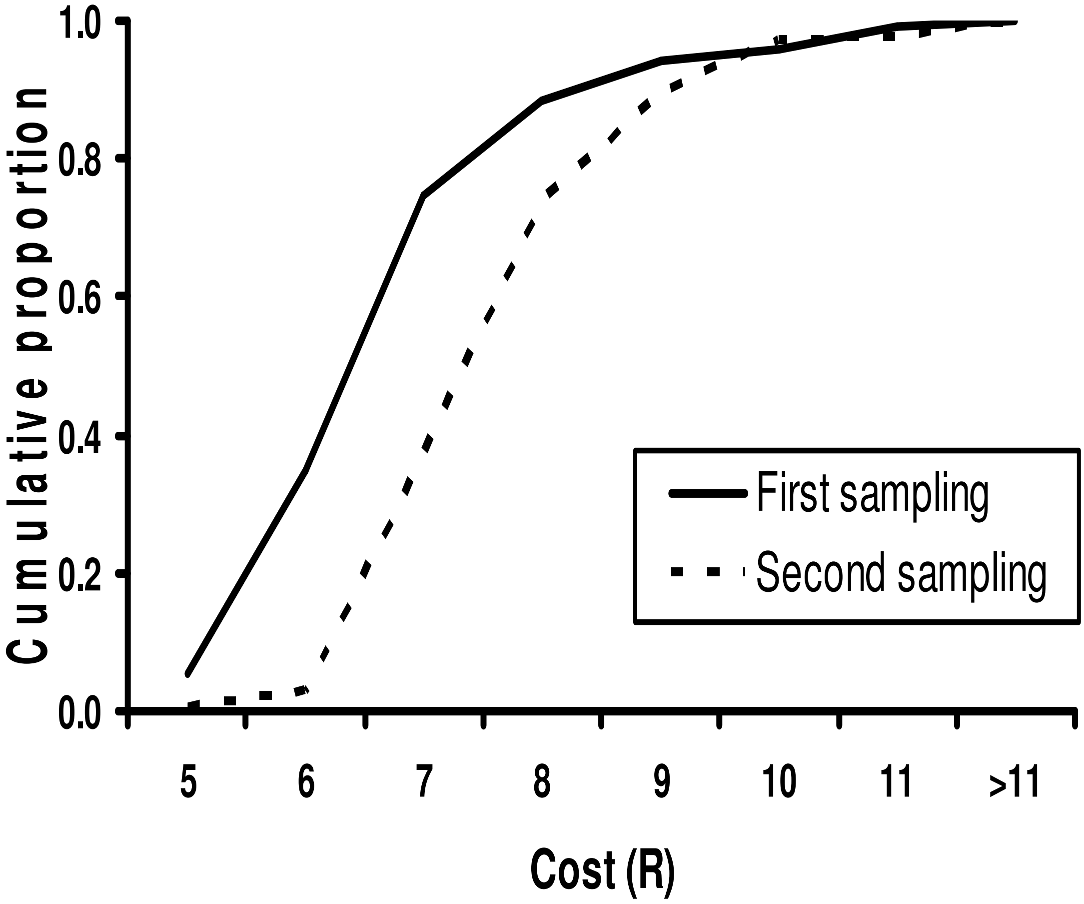

Figure 3.6: Cumulative proportions of samples at different costs (Rand) for the two sampling periods.

Figure 3.6 shows a slight increase in the cost of eggs of about R1 per dozen during the second survey (dotted curve) over the first survey (bold curve).

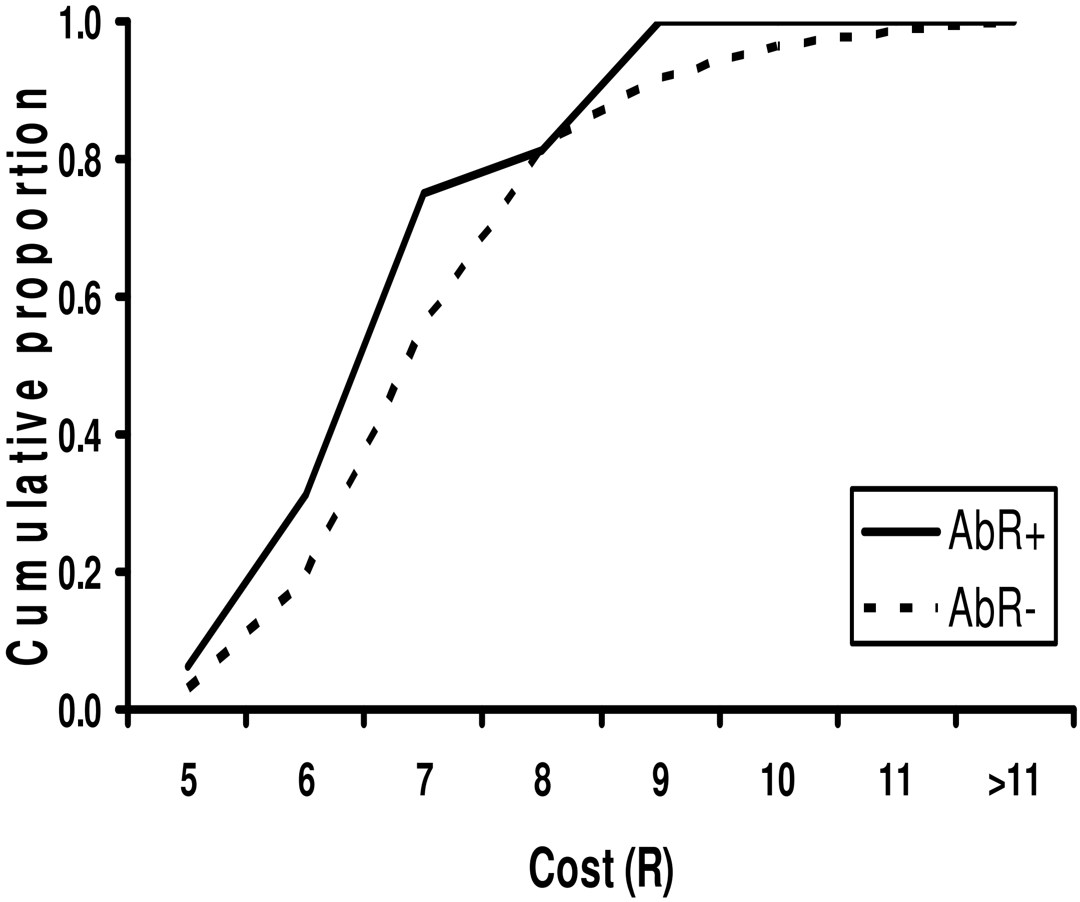

Figure 3.7: Cumulative proportions of samples at different costs (Rand) for antimicrobial residue negative (AbR-) and antimicrobial residue positive (AbR+) eggs

Figure 3.7 shows a close association between cost of eggs and the presence of antimicrobial residues in such eggs, with the cheaper eggs (bold curve) having more positive antimicrobial residues than the more expensive eggs (dotted curve).

CHAPTER 4: DISCUSSION

4.1 Introduction

Poultry and poultry products including eggs are comparatively cheap sources of protein in RSA and most other countries, for this reason, many people who cannot afford the more expensive beef, pork or fish resort to buying poultry and their products as an alternative source of protein (Saif, Y.M. & Barnes H.J., 2003; SAPA, 2008; Gaudin, V., Hedou, C., Rault, A., Sanders, P. & Verdon, E., 2009). Because most people in the RSA consume eggs, it is very important that these eggs are free from AR. Consumers may suffer from health risks like skin allergies, anaphylactic reactions or even toxicity or find that their commensal micro-flora has become resistant to antimicrobials doses that are normally reserved for the treatment of serious infections of humans. It is therefore very important that eggs sold to the consumers are free from AR. (Witte, 1998; Donoghue & Hairston, 2000; Kan & Petz, 2000; Han, *et al*., 2002; Kabir *et al.,* 2004; Tajick & Shoreh, 2006; Gaudin *et al.,* 2009). Because of these concerns, maximum residue limits (MRLs) have been set for ARs in food items to safeguard the consumer (EC, 1990; EC, 1996; EC, 2006; Pig Health, 2003; Kabir *et al.,* 2004; CAC/MRL, 2006; Gaudin *et al.,* 2008).

Several screening methods for the detection of AR in animal products have been developed, most of which are based on microbial susceptibilities. For a screening method to be of use it must be capable of detecting most if not all possible bacterial inhibitors at the MRL in animal products. Furthermore, the tests must be cheap and easy to perform so that they can be applied to a large number of samples. Thereafter positive samples can be subjected to more expensive specific tests such as HPLC and MS. However, in Africa, even the cheapest tests can prove to be too expensive for mass application and robust tests are required that can be used reliably with the minimum of equipment and expertise. For this reason, the new in-house bacteriological screening test was developed to screen eggs for AR as previously described in chapter two with results shown in chapter three.

4.2 Quality Control on bacterial cultures and test procedures

Bacterial culture, bacterial count checks, morphological identification, biochemical tests and Gram stains were carried out on the sub-cultured *Bacillus megaterium* ATCC 9885 and *Geobacillus stearothermophilus* ATCC 12980 to ensure viability and purity of cultures. These were conducted in order to ensure that the correct bacteria were used and to avoid the use of contaminated bacteria (Koneman *et al.,* 1992 and Quinn *et al.,* 1994); see also section 2.3.1, Figures 2.1 and 2.2. There were no problems encountered when quality control was carried out on the cultures and the two bacteria were thereafter used for the test.

For an assay or test method to be initially or completely validated, it is important for the test to consistently produce the same result (reproducibility) and there must be repeatability of result as well; that is, an agreement between replicate samples within-runs (intra-tube and inter-tube respectively) and between-runs of the assay and they must be independent of each other (OIE, 2008). The OIE recommends at least four runs of quadruplicate samples for an assay to determine within-runs, that is, a sample has to be tested at least twenty times (OIE, 2008). This ensures that the test procedure and methods used are correct and can be carried out (reproduced) in any other laboratory (OIE, 2008). It was for this reason that each of the test samples were run five times in triplicate for reproducibility of result within the same test sample and also between different samples in separate racks; testing of samples were performed on different days and different time as a check (OIE, 2008).

While testing for AR in egg samples collected from the 36 hen trial, it was surprisingly noticed that on the first day of testing, eggs from seven groups of hens that were treated with the following antimicrobials: lincomycin, fosfomycin, tylosin, tiamulin, enrofloxacin, oxytetracycline and sulphachloropyrazine, were inconsistent; some of the triplicate within-samples for a particular group tested as positive while others tested as negative. It was expected that all the triplicate within-samples for a particular group or antimicrobial group should have the same result. Because of the variations that were noticed, the test procedure was repeated in order to overcome the inconsistency of within-samples. It was discovered that the inconsistency was as a result of inadequate mixing of the egg albumen and yolk (the eggs were initially mixed in a sterile beaker using a sterile glass rod) rather than from the test procedure or other sources. When a stomacher lab-blender- 400[1] was later used to mix the egg contents thoroughly for 60 seconds, and the samples re-tested using the same procedure as previously done, all the triplicate samples within a run produced a consistent result of either being positive or negative, therefore, the problem was resolved, see Table 3.3. It is therefore very important to homogenize the egg samples properly before testing in order to avoid the problem of uneven distribution of egg contents and inconsistency of results.

Similarly, some results that previously tested as doubtful (+/-) later tested either as positive or negative for AR; the doubtful results were also found to be as a result of improper mixing of the egg albumen and yolk. The problem was however resolved in the same way as was done

[1] Model No. BA 6021, single phase, Seward Medical, UAC House, Black Friars Road, London 95 W, Great Britain.

also for the 36 hen trial and some of the samples that previously tested as doubtful now tested either as negative or positive for AR with no doubtful results any longer as indicated in Table 3.4.

4.3 Inactivation of inhibitors

Initially, due to fears that some antimicrobials would be heat-inactivated, it was decided to test the eggs without any form of prior heat treatment. The eggs proved to contain non-specific bacterial growth inhibitors. With repeated testing, it was found that the inhibitors contained in the eggs were in the albumen and were most likely lysozyme or peroxidase. As the MRLs are very low, presence of bacterial growth inhibitors even at low concentrations are considered unacceptable. Also, testing either the yolk or albumen alone would result in some antimicrobials (which are incorporated only in either the albumen or yolk) not being detected (refer to Table 2.1). Therefore there was a need to inhibit the non-specific inhibitors in the albumen.

There are two ways to destroy non-specific bacterial growth inhibitors; by the use of chemicals or by heat. Chemicals have the disadvantage that they affect the pH and polarity of the antimicrobials, resulting in a smaller spectrum of antimicrobials being detected while heat-inactivation can result in the destruction of some heat-sensitive antimicrobials. For that reason, it was decided to test whether heat treatment at 60^0C would be effective in inactivating lysozyme; it was not. Temperatures of 65^0C, 70^0C and 75^0C were also tried, but they did not also work (Ramirez *et al.,* 2003; Aerts *et al.,* 1995) until 80^0C was tried; this temperature of 80^0C for 10 minutes was effective and could inactivate all the natural inhibitors in the eggs; this was later found to also be in agreement with reports by

Premi®Test-1 (2008); Premi®Test-2 (2008) and WIPO (2008). Fortunately, the temperature of 80^{0}C and short treatment time of 10 minutes did not cause a drop in the activity of the antimicrobials when spiked and later field egg samples were tested over a period of five weeks; this was later seen to be in agreement with other reports (Egmond *et al*., 2000; Premi®Test 1, 2008; Premi®Test 2, 2008; WIPO, 2008). When the heat treated eggs (80^{0}C) were tested alone without spiking with antimicrobials, they did not show any form of bacterial inhibition, indicating inactivation of inhibitors see Tables 3.1 to 3.3.

This temperature and time treatment at 80^{0}C for 10 minutes was therefore established for the new in-house bacteriological screening method.

4.4 Prevalence of antimicrobial residues in eggs in different countries

The literature review showed that the incidence of AR residues differed between countries. Sudan is a country with no legislation on antibiotic residues in eggs and has >60 % AR in egg samples surveyed in the country as reported by Sirdar, M, M., 2010. The prevalence in Trinidad is about 16.1% and 15% in eggs found in malls and supermarkets respectively as reported by Adesiyun *et al*., 2005; while that of China and Saudi Arabia are 12.2% and 14.4% as reported by Wang *et al.,* 2007 and Al-Ghamdi *et al*., 2000 respectively.

4.5 Minimum antimicrobial detection concentration on spiked whole egg samples

It took between two and four hours of incubation in a water bath at the temperature of 65^{0}C for *G. stearothermophilus* to change the colour of the test broth from red to yellow for the different antimicrobials used (example is spectinomycin in Figure 3.5) compared to a longer incubation time of over 4 hours at 37^{0}C for the test to be completed using *B. megaterium*. Thus *G. stearothermophilus* produced a more rapid result than *B. megaterium;* also, any bacterial contaminants in the sample will usually not grow at 65^{0}C.

G. stearothermophilus was able to detect all the tested antimicrobials, that is, enrofloxacin, norfloxacin, neomycin, tylosin, chlortetracycline, florfenicol, sulfadiazine, sulfamethoxyzole, trimethoprim, spectinomycin, ampicillin, gentamicin, fosfomycin, lincomycin, tiamulin, colistin, oxytetracycline and doxycycline at lower concentrations than *B. megaterium*. Because of this marked difference in the antimicrobial sensitivity between the two bacteria, *B. megaterium* was considered a poor candidate and tested only once in order to cut down on antimicrobial wastage. *G. stearothermophilus* with very good antimicrobial sensitivity was chosen and tested five times for repeatability and the sensitivity level average minimum detection level (MDC) determined (see Table 3.1). The MDC of the antimicrobials by the bacterium in µg/ℓ was considered to be the lowest concentration of antimicrobial at which bacterial growth was not detected.

Based on the facts given above *G. stearothermophilus* was selected as the test bacteria for the new in-house bacteriological test method (see Table 3.1).

4.6 Comparison of minimum detection concentration, MRL and Premi®Test values

When the results of the average MDC limits of the various antimicrobials to *G. stearothermophilus* were compared to that of the European Union's MRL and Premi®Test values, it showed that on the average, the new in-house test method is more sensitive to all but three (fosfomycin, spectinomycin and sulphamethoxyazole) of the eighteen antimicrobials tested which are above the European Union's MRL values (see Table 3.1). Two of the eighteen antimicrobials tested (florfenicol and norfloxacin) have no established MRL or published Premi®Test values, therefore, their MDC and sensitivity could not be compared to that obtained by the new bacteriological in-house test method (Table 3.1). However, the new test method was able to detect and establish MDC values for the two antimicrobials (florfenicol and norfloxacin) that did not have MRL values (Table 3.1). Similarly, six (colistin, florfenicol, fosfomycin, norfloxacin, spectinomycin and tiamulin) of the eighteen antimicrobials tested have no published Premi®Test values (but of the six antimicrobials with no Premi®Test values, colistin, fosfomycin, spectinomycin and tiamulin have MRL values) ; therefore, their detection values could also not be compared to that obtained by the new test method (Table 3.1). The new test method therefore has a wider published detection range for the eighteen tested antimicrobials than the European Union's MRL and Premi®Test.

When the MDC values of the new bacteriological in-house test method were compared to the published detection values of Premi®Test alone, it was discovered that the MDC values for the new test method were lower (still more sensitive) to all but two (Sulfadiazine and Sulphamethoxyzole) of the compared antimicrobial (Table 3.1). Thus Premi®Test has more sensitive detection levels for sulfadiazine and sulphamethoxyzole than the new bacteriological in-house test method. The above mentioned values showed that the new bacteriological in-house test method was able to detect AR in egg samples at or below most European Union's MRL and set Premi®Test values; this also made it a more sensitive test method than the Premi®Test in addition to detecting a wider range of the compared antimicrobials.

As a screening test, it is better for a test method to be more sensitive in order to include most samples than lack in sensitivity; since the results will be further subjected to a more specific test method for confirmation.

4.7 Antimicrobial residues detected in eggs of treated hens using the test bacterium *Geobacillus stearothermophilus*

As expected, none of the eggs collected a day before and on the day of treatment had any antimicrobial inhibitors in them. This indicated that measures taken to ensure that the feed had no bacterial inhitors were effective and that false positive results were highly unlikely; at this stage the test specificity appears to be 100%. Reasons for possible false positive results are from feed incorporated with antimicrobials from manufacturers or water or wood shavings that have been contaminated with antimicrobials.

All the eggs collected a day after treatment and during the period of treatment at therapeutic concentrations indicated the presence of inhibitors. This was expected because the hens' egg is formed gradually over a period of about 25 hours and spends approximately 15 minutes in the funnel (infundibulum), 3 hours in the magnum, 1 hour in the isthmus, 21 hours in the shell gland (uterus) and less than 1 minute in the vagina/cloaca. If the feed or water taken in by the hen contains antimicrobials, they are absorbed and incorporated into the various substances that become part of the egg as shown in Table 1, (Coutts, J. A. & Wilson, G.C., 2007). AR in eggs tend to closely mirror the level of drugs in the plasma and a single exposure to a drug might be sufficient to detect it in egg (Kan & Petz, 2000); also, the drug clearance from the egg depends heavily on the plasma levels of the drug tested; thus, the higher the drug's plasma level as well as the longer the exposure time, the longer the clearance time (Kan & Petz, 2000). The new test was able to detect AR in all (100%) the eggs collected during treatment (days 1 to 6) which was in agreement with earlier reports by Kan & Petz, 2000; Coutts, J. A. & Wilson, G.C., 2007. The untreated control group tested negative for anti-microbial inhibitors throughout the trial.

Antimicrobial residues could not be detected in eggs of some groups (lincomycin[1], fosfomycin[2], tiamulin[3], doxycycline[4], enrofloxacin[5], oxytetracycline[6] and sulphachloropyrazine[7]) by one day after the end of treatment. This result was unexpected as it had been assumed that ARs would still be incorporated into eggs for at least 24 hours after the end of treatment and also based on the drug withdrawal periods as indicated in Table 2.1. This finding needs further investigation. Eggs from hens treated with amoxicillin[8], tylosin[9] and ciprofloxacin[10] still had AR in them one day after the end of treatment and the group that was treated with trimethoprim[11] still had AR in their eggs up to the end of the trial which was 9 days after the end of treatment. It was not surprising to find AR in the hens eggs one day after the end of treatment or more as antimicrobials often remain in the plasma after treatment and eggs harvested within the drugs' withdrawal periods usually have the drug residues in them (Sturkie, P.D., 1986; North, M.O. & Bell, D.D., 1990; Donoghue & Hairston, 2000; Kan & Petz, 2000; Kabir *et al.,* 2004; Gaudin *et al.,* 2009). The longer period of AR secretion seen in trimethoprim justifies its long withdrawal period of about ten days

[1] Lincocin,® Pharmacia South Africa (Pty) Ltd., Unit G, Alphen Square West, George Str., Midrand 1685, RSA.
[2] Fosbac, Bedson® Africa (Pty) Ltd., Willow Business Park, Silverton X52, Pretoria, RSA.
[3] Tiamutin 10% premix, Divpharm manufacturing and packaging (pty) Ltd-In-house sample, RSA.
[4] Doxybiotic, MEDPET (Pty) Ltd., Unit 7A, Droste Industrial Park, Benrose, Johannesburg 2094, RSA.
[5] Bartril 10%, Bayer (Pty) Ltd., Animal Health Division, Bayer AG, Germany.
[6] Terramycin, Pfizer Laboratories (Pty) Ltd., 102 Rivonia Road , Sandton 2196, RSA.
[7] ESB3, Norvatis South Africa (Pty) Ltd., P.O Box 92, Isando 1600, RSA.
[8] Avimox 10%, Bremer Pharma, GMBH 27540, Bremerhaven, Germany.
[9] Tylovet o-s, V.M.D. nv/sa- Berendonk, 74-b-2370 Arendonk, Belgium.
[10] Ciprotab® 500, V.S International Pvt Ltd., Plot no. J/76, M.I D.C., Tarapur, Thane-401 506, India.
[11] Trimethoprim, V-tech Veterinary solutions pharmacy, Y53159. www.V-tech.co.za. RSA

(see Table 2.1). The new test method was able to detect all the antimicrobials used for the hens' trial (see Table 3.1) and is therefore a useful initial screening method for AR in eggs. Prophylactic and high antimicrobial concentrations were not tested in this trial because during the 36 hen trial, the hens were only given therapeutic and not prophylactic doses of the antimicrobials based on their body weights. It was expected that the hen's eggs would contain AR based on the drug's concentration; this was seen with all the drugs administered to all the hens at therapeutic doses during the 36 hen trial. On the other hand, prophylactic doses of drugs are usually lower in concentration than the therapeutic concentrations, based on this; it was assumed that their concentration will not be high enough to be detected in the eggs. Also the withdrawal periods of the drugs were those of the therapeutic and not for prophylactic doses, therefore, the lower prophylactic concentrations were not expected to be detected in eggs, hence they were not tested. Since high concentrations were not also given to the hens and AR in eggs tend to closely mirror the level of drugs in the plasma (Kan & Petz, 2000), it was expected that high concentrations of antimicrobials in eggs will be detected if present, hence no need to test it separately.

4.8 The new In-house bacteriological screening test for AR in eggs was relatively better and cheaper than the Kundrat four-plate microbiological screening method

All (100%) the samples that were negative with the in-house test also proved to be negative with the Kundrat test. This indicates that false positive results for this test are very unlikely; therefore the calculated specificity is 100%. Of the 10 eggs originating from the 36 treated hens, all were positive for AR using the in-house test (100%), only the one (1) treated with trimethoprim proved to be positive with the Kundrat micro-screening four-plate test[1]; that is, 1 out of 10 which also represents only 10% of the 10 tested antimicrobials. As it was expected that the treated hens' eggs were all (100%) positive for ARs, it was surprising that there was only 1 positive result using the Kundrat test; this result indicates that the in-house test is 9 out of 10 times (90%) more sensitive than the commercial Kundrat test for the tested antimicrobials. The results from the field samples gave a similar pattern of results with only 1 of the 19 positive field samples giving a positive result on the Kundrat test (Table 3.4). This shows that the in-house method test is 18 out of 19 times (97%) more sensitive than the commercial Kundrat test (average 94%); again reinforcing the assertion that the in-house test is more sensitive than the Kundrat micro-screening four-plate test for AR in eggs since it screened a wider range of egg samples for AR.

While it cost about R140 to screen an egg sample using the Kundrat micro-screening four-plate test and about and R100 using the Premi®Test, it cost only about R15.00 with the new test method. 80 triplicate samples (320) can be screened at a time with the new in-house test method and results read in less than 4 hours compared to the Premi®Test which can screen only 10 samples at a time (Premi®Test-1, 2008) within 4 hours, thus saving time, effort and money. It is a good screening test for multi-residue testing unlike the other commercial tests that are mostly suitable for targeted or single-residue testing (Aerts *et al.,* 1995; Gaudin *et al.,* 2009). The new in-house test only requires basic laboratory equipment such as a water bath, blender, incubator and reagents plus media & test tubes. Other tests like ELISA, HPLC and MS are expensive to purchase (thousands of rands) and run and also require skilled personnel

[1] Sigma-Aldrich SA (Pty) Ltd., P. O. Box 10434, Aston Manor 1630, South Africa.

to manipulate and read results compared to this cheap method that does not require skilled personnel to perform it.

4.9 Antimicrobial residues were found in commercial chicken eggs in Tshwane area of Gauteng Province, South Africa

Methods

Multivariable logistic regression was used to estimate associations of a descriptive factor(s) with antimicrobial residues (AR). The brand of eggs, cost for sampled eggs (6 eggs /sample unit) for the 2 different sampling periods and outlet category among other categories were analysed for the percentage of samples with AR in them. The confidence interval (CI = 95%), and the significance test (set at $P < 0.05$) were determined. Histograms and graphs to show association with AR are also presented.

4.9.1 Which combination of independent variables best explains AR status?

Eggs were categorised according to prices between R5 and 5.99 to above R10 per half dozen and there was a negative correlation between price and AR status ($p=0.059$). The group costing between R5 and R5.99 per half dozen eggs was used as the reference group, and the likelihood of finding AR in eggs in each one of the other categories that were included in the model was compared to that of the reference group. Result on Table 3.5 and graphs in Figures 3.6 and 3.7 showed that eggs that are more expensive costing R10 and above to had lower likelihood of having AR than the cheaper eggs costing R5.99 or less. This association was significantly related to AR statues and therefore considered useful.

It is possible that farmers who used antimicrobials on their birds during egg laying more easily sell such eggs to distributors and informal/roadside retailers at cheaper prices in order to cut down on losses they would have incurred if they destroyed such eggs (these small retailers do not carry out quality control on their eggs). This practice leads to larger numbers of cheaper egg samples found containing AR in informal/roadside outlets than those sold in conventional big supermarkets (that are more expensive) and who claim to have residue quality control measures in place. This is clearly seen in the results on Table 3.5, here, the informal/roadside outlets were found to sell cheaper eggs in this study than any other sales-outlet, some even costing R5 and below per half dozen of eggs compared to the higher price of R10 and above (with some costing R18) per half dozen sold in the big super market chains. The cheaper eggs were found to contain more AR than the expensive ones, which is a significant association between egg prices and AR.

There were five sales-outlet types: selected supermarkets, butcheries, garage shops, informal/roadside shops and “other” shops. The survey revealed that outlet type affected AR status because 18.9% of eggs in the informal/roadside outlets were AR positive compared to 1.2% AR positive eggs from the 20 selected big chain supermarkets. From the above findings, we can also see that there is a close link between informal/roadside outlets being associated with low egg prices, this in turn explains the correlation between price and AR found in eggs earlier seen where cheaper eggs were found to contain more AR than the expensive ones (Table 3.5). It is therefore very important to educate the populace on the dangers of buying and consuming cheap exposed eggs; rather, egg consumers should buy the more expensive eggs which are safer.

Results from eggs purchased from the informal/roadside outlets were compared with those purchased from other outlets (butcheries, garage shops, and "other" shops) and the selected big chain supermarket was taken as the reference category. Association with AR was thereafter determined and results showed that the likelihood of finding AR in the informal/roadside outlets was highly significantly. The informal/roadside outlets had 18.9% AR positive compared to 1.2% AR positive eggs for the 20 selected big chain supermarkets. The butcheries, garage shops, and "other" shops outlets on the other hand had similar odds of supplying AR positive eggs (amongst themselves) but had no statistical significance when compared with the supermarkets. From the above findings, we can also see that there is a significant association between informal/roadside outlets with AR.

Thirty seven different egg brands were represented in the study and given coded numbers in order to protect their owner's identity. 12 out of 37 brands contained AR while the remaining 25 did not, of these, 3 brands (9, 22 and 27) had a significantly higher proportion of AR positive samples as compared to the others (Table 3.6). These same brands (9, 22 and 27) were also found in the 20 selected large chain supermarkets, but with lower AR level. Brands 1, 9 and 16 appeared to be the most widely distributed egg brands in the surveyed areas because even the most rural location had them and different brands of eggs were simply purchased (with no bias) based on their availability compared to the fewer numbers for the other brands.

Therefore, the factors that proved to significantly increase the risk of ARs included prices less than the median, informal/roadside retail outlets and certain brands purchased. Eggs purchased from the large supermarket chains proved to contain significantly lower levels of AR than other retail outlets.

4.9.2 Other categories that were analyzed but no AR differences were found

The percentage of the entire egg samples (including the 20 supermarkets) positive for AR for the 2 survey seasons were compared, the first survey was in October/November 2008 (spring) and the second survey was in the autumn, April/May 2009 (South African tourism, 2008). The result indicated that there was no significant difference in the proportion of samples that were positive for AR between the two sampling periods with the first survey having 45% and the second survey having 54% (section 3.4.2). Although the number of samples positive for AR for the 2 surveys were similar, there may have been a trend for a higher prevalence for AR positive eggs during the second survey (spring, September to November 2008) compared to the first survey as earlier indicated; the 2 surveys were considered similar.

The reason there was little difference in the results for spring and autumn in this study might be because of the similarities between the 2 seasons, therefore, increasing the period (seasons) for the survey to include spring, summer, autumn and winter will ensure that all weather conditions are considered and this will give a true representation of the seasons, hence, a good result.

The location of purchase of eggs from Tshwane CBD, Atteridgeville, Soshanguve and Mamelodi were compared with that of other locations to see if there was an association with AR as widely speculated. It was surprising to find that there was no statistical significance in terms of AR with the locations of purchase of eggs.

Niche-marketed eggs sampled in this study included organic, pasteurised and free range eggs, these eggs were considered more likely to be free from AR than eggs not marketed as niche. The niche eggs were compared to eggs not marketed as niche for the absence of AR as claimed by their producers, but the result showed that there was no significant difference between the 2 categories. Therefore, niche eggs and eggs not marketed as niche have similar likelihood for AR in this study.

There were four categories of egg sizes in the study (large, extra large, medium and small) and category 1 (large eggs) was taken as the reference category (Table 3.5). The likelihood of finding AR in any egg size category was not significantly different to the likelihood of finding ARs in large eggs (p=0.525) as shown in Table 3.5.

It was surprising to find that the storage temperature of -20^{0}C was able to keep the egg samples in good condition (as there is hardly any literature reports on storage and stability of eggs at -20^{0}C) and the AR also remained stable for 15 days during the duration of the 36 hen trial. Similarly, because of the earlier problem of improper mixing of egg that was encountered, the egg samples were retested after 60 days of storage at -20^{0}C and were also found to be in good condition without loss of antimicrobial stability; this is a reportable finding in this study.

4.10 Factors for consideration

Eggs are contaminated with antimicrobial residues before they are laid as a result of birds being treated during lay and the patterns according to which these eggs acquire drug residues prior to lay is logical; for example, certain producers may use antimicrobials and others may not. Producers who use antimicrobials in their laying hens may do so for certain farms but not for others and in farms where antimicrobials are used, they may be used during certain seasons only, or in certain age groups only, or only in the face of infection. Also, the extent to which producers adhere to withdrawal periods may also vary; all of the above mentioned factors contribute and also affect the extent to which AR are found in eggs if strict compliance to laid out rules are not followed.

CHAPTER 5- CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

5.1.1 The new bacteriological in-house test

- The new bacteriological in-house method is easy to perform and is appreciably cheaper than similar test kits that are available commercially such as the Kundrat and Premi®Test tests; it does not require skilled personnel to manipulate the test and interpret results.
- The relatively low cost of the test makes it affordable and allows wider screening of eggs which potentially decrease the risk of antimicrobial residues in eggs.
- The new test was able to determine the optimum test conditions that led to repeatable results which are:
  - Proper mixing of egg contents in a blender for homogenization before testing
  - Inactivation of lysozyme at a temperature of 80^0C for 10 minutes was established and used for the new in-house bacteriological screening method.
  - Results can be obtained in about 2-3 hours making it faster than other microbiological methods.
- The performance of *Geobacillus stearothermophilus* and *Bacillus megaterium* in the detection of antimicrobials in egg samples was tested using the new test; here, *Geobacillus stearothermophilus* performed better than *Bacillus megaterium*.
- Screened eggs for AR using the new test method that was carried out during the pilot trial on thirty six laying hens treated with known concentrations of antimicrobials.

- The minimum antimicrobial detection limit for the different antimicrobials were determined using the new test by using eggs spiked with different antimicrobials (Table 3.1)
- The new bacteriological in-house test method was able to detect residues of enrofloxacin, norfloxacin, neomycin, tylosin, chlortetracycline, trimethoprim, spectinomycin, ampicillin, gentamicin, lincomycin, tiamulin, colistin, oxytetracycline and doxycycline in egg samples at or below most EU MRL and the set Premi®Test values. This also made it a more sensitive test method than the EU MRL and the set Premi®Test in addition to detecting a wider range of the compared antimicrobials in this study. The new in-house bacteriological screening test for AR in eggs proved to be more sensitive than the Kundrat micro-screening four-plate test[1] for screening AR in eggs. (Table 3.4).
- The storage temperature of -20^{0}C for 60 days was established for egg samples in this study.
- Was used to carry out a two-seasonal survey in October/November 2008 (spring) and in April/May 2009 (autumn) and determined the prevalence pattern of AR statues of commercial chicken eggs sold in Tshwane area and environs.
- Initial screening test results using the new in-house bacteriological method should be further subjected to the more expensive specific tests like the HPLC and MS for confirmation.

[1] Sigma-Aldrich SA (Pty) Ltd., P. O. Box 10434, Aston Manor 1630, South Africa.

5.1.2 Field samples

- Even though there was a slightly higher prevalence for AR during the second survey in spring than the first survey in autumn, results from the two seasons were considered similar.
- The cheaper the egg the more likely it was to contain AR.
- Eggs sold by informal/roadside shops had significant association with AR ($p = 0.016$). Eggs bought from big chain supermarkets had a slightly reduced likelihood of containing AR.
- Egg brands 9 ($P = 0.047$), 22 ($P < 0.001$) and 27 ($P = 0.017$) had significant chances of having AR in them, therefore, other brands should be preferred to them.
- <10% (7.5%) of the total samples were positive for AR in commercial chicken eggs sold in Tshwane area and environs. Even though this figure seem to be on the low side compared with what obtains in countries like Sudan >60 %, Trinidad 16.1%, Saudi Arabia 14.4% and China 12.2% (Al-Ghamdi *et al*., 2000, Adesiyun *et al*., 2005, Wang *et al.,* 2007 and Sirdar, M, M., 2010), because of the public health significance of AR in humans, it is important that the relevant authorities should check this.

5.2 Recommendations

- Overall, egg price, brand and type of sales-outlet are important determinants of the likelihood of finding AR in eggs and further studies are indicated to more specifically investigate these factors.

- There are regulations in place in RSA to check for AR in eggs, but there is an overall reliance on the retailers to do the testing – which they are doing - hence good quality control at large chain supermarket level, but this very important procedure is lacking in the smaller retailers. The government should therefore put its own active surveillance in place in order not to over rely on retailers.
- Egg brands 9, 22 and 27 were purchased from both large and small retailers and it was expected that they should have the same results; but surprisingly, these same brands that were purchased from the larger retailers contained very little AR while those purchased from smaller retailers contained more AR. This strongly suggests that some farms are using the smaller retailer's to sell eggs that they cannot distribute through the larger supermarkets; or some distributors commingle eggs having AR at labeling points and sell them to smaller retailers while they sell AR free eggs to the larger supermarkets. This is because the smaller retailers do not conduct residue control and such eggs can easily pass through them while they sell AR free eggs to the bigger retailers; knowing that the bigger retailers carry residue checks and may detect and reject any AR eggs. This calls for government funded testing and enforcement of regulations, and if necessary, amend existing ones to address the situation before it gets out of control.
- Individual labeling of eggs on the farm will allow for trace-back and should be included in any AR testing programme.
- Because of the relatively low cost of the test which also makes it affordable and allows wider screening of eggs for residues, it should be employed for use in RSA with the aim of potentially reducing the risk of antimicrobial residues in eggs.

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APPENDICES

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Ref: V029/09

FACULTY OF VETERINARY SCIENCE
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24 November 2009

Dear Dr Jambalang
Department of Production Animal Studies

PROTOCOL V029/09: THE DEVELOPMENT AND VALIDATION OF A BACTERIOLOGICAL SCREENING TEST FOR ANTIMICROBIAL RESIDUES IN EGGS

I am pleased to inform you that the abovementioned protocol was approved by the Research Committee.

Kindly note that the protocol has to be approved by the Animal Use and Care Committee as well before you may commence with the project.

Please take note of the attached document.

Kind regards,

Lunga Mengu
SECRETARY: RESEARCH COMMITTEE

Cc: Ms Elmarie Mostert, Animal Use and Care Committee, (elmarie.mostert@up.ac.za)
Prof P Thompson, Research Co-ordinator, (peter.thompson@up.ac.za)
Dr S Bisschop, Supervisor, (shahn.bisschop@up.ac.za)
Mrs D Marais, Student Administration, (denise.marais@up.ac.za)

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ANIMAL USE AND CARE COMMITTEE
Private Bag X04
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Tel: 012-529 8434
Fax: 012-529 8300

Ref: V029-09

01 October 2009

Dr SPR Bisschop
Department of Production Animal Studies
Faculty of Veterinary Sciences
(shahn.bisschop@up.ac.za)

Dear Dr Bisschop

V029-09: The development and validation of a bacteriological screening test for antimicrobial residues in eggs *(Dr AR Jambalang)*

The application for ethical approval, dated 13 July 2009 was approved by the Animal Use and Care Committee at its meeting held on 28 September 2009.

Best regards

E Mostert

Elmarie Mostert
AUCC Coordinator

Copy Dr AR Jambalang

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Dear Dr Bisschop

It is my pleasure to inform you that student Jambalang's title:

The development and validation of a bacteriological screening test for antimicrobial residues in eggs has been approved by the Post Graduate Committee.

Please inform all relevant parties.

Kind Regards
Tuesday, July 21, 2009 7:58 AM

Denise Marais
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Record of egg samples purchased for antimicrobial residue (AR) screening

S/NO	DATE	CODE	GPS POINT (DEGREES)	EGG SIZE	PRICE(6 EGGS)	EGG BRAND	ADDRESS/LOCATION
1	2008/10/14	SR 1	S25 44'41.90" E028 11'44.50"	X-LARGE	R 6.50	THORN TREE EGGS	SUPERAND CENTRAL SHOP ANDRIES STREET
2	2008/10/14	SR 2	S25 44'56.70" E028 11'27.40"	LARGE	R 6.00	WOHLFAHRT GRAINFED EGGS	L.M SUPERMARKET NO. 76 ANDRIES STREET
3	2008/10/14	SR 3	S25 45'34.75" E028 11'32.31"	LARGE	R 7.00	ZEEKOEGAT EGGS	4.U SUPERMARKET, ANDIRES STREET
4	2008/10/14	SR 4	S25 45'11.60" E028 11'46.50"	LARGE	R 6.50	THORN TREE EGGS	NO NAME
5	2008/10/14	SR 5	S25 45'25.61" E028 11'29.77"	LARGE	R 8.50	MORIA EGGS	CALTEX GARAGE, ANDRIES STREET
6	2008/10/14	SR 6	S25 45'24.20" E028 11'19.20"	LARGE	R 7.00	WOHLFAHRT GRAIN FED EGGS	LOW SIDE SUPERMARKET SHOP NO 7, PRETORIA TRAIN STATION
7	2008/10/14	SR 7	S25 45'05.21" E028 11'27.27 "	LARGE	R 5.00	WOHLFAHRT EGGS	TRAIN STATION PRETORIA
8	2008/10/14	SR 8	S25 45'24.32" E028 11'15.18"	LARGE	R 6.00	ZEEKOEGAT EGGS	TRAIN STATION PRETORIA
9	2008/10/16	SR 9	S25 44'56.44" E028 11'35.48"	X-LARGE	R 7.50	WELDHAGEN EGGS	REX CAFE' & TAKE AWAY STORE, CNR SCHOEMAN/V.WALT STR
10	2008/10/16	SR 10	S25 44'54.77" E028 11'36.62"	LARGE	R 8.00	NULAID GRAIN FED EGGS	PICK 'N PAY SUPERMARKET, V.WALT STREET
11	2008/10/16	SR 11	S25 44'54.77" E028 11'36.62"	X-LARGE	R 7.50	P 'N P NO NAME	PICK 'N PAY SUPERMARKET, V.WALT STREET
12	2008/10/16	SR 12	S25 44'36.72" E028 11'33.26"	LARGE	R 7.00	KIEPERSOL EGGS	ROYAL EXPRESS SHOP, V. WALT STREET
13	2008/10/16	SR 13	S25 44'49.16" E028 11'34.43"	LARGE	R 7.50	TOP LAY COUNTRY EGGS	WALTLOO MEAT & CHICKEN SHOP OPP. ABSA BANK V.WALT STR
14	2008/10/16	SR 14	S25 44'45.41" E028 11'35.21"	X-LARGE	R 13.00	WOOLWORTHS FREE RANGE EGGS	WOOL WORTHS SUPERMARKET SAMMY MARKS SQUARE
15	2008/10/16	SR 15	S25 44'42.36" E028 11'34.04"	LARGE	R 5.50	FAIR ACRES EGGS	JAKARANDA BUTCHERY, V. WALT STREET
16	2008/10/16	SR 16	S25 44'68.20" E028 11'58.00"	MIXED	R 7.00	FARM FRESH EGGS	CENTRAL SUPERLINER SUPER MARKET
17	2008/10/16	SR 17	S25 44'31.03" E028 11'32.65"	LARGE	R 5.99	NO BRAND EGGS	MODERN MEATS, NO. 35, V.WALT STREET
18	2008/10/16	SR 18	S25 44'25.33" E028 11'33.33"	LARGE	R 5.00	NO BRAND	SUPER SAVE BUTCHERY V. WALT STREET
19	2008/10/16	SR 19	S25 44'25.58" E028 11'33.26"	LARGE	R 6.99	VLEIVIEW EGGS	NO NAME
20	2008/10/16	SR 20	S25 45'05.57" E028 11'37.37"	LARGE	R 7.00	FAIR ACRES GRAIN FED EGGS	PRIME STAR STORE V. WALT STREET CORNER STRUBEN STREET
21	2008/10/16	SR 21	S25 44'53.70" E028 11'56.00"	LARGE	R 7.00	FAIR ACRES EGGS	STAR BUTCHERY STORE, NO. 78 V. WALT STREET
22	2008/10/16	SR 22	S25 44'34.86" E028 11'34.15"	LARGE	R 8.00	THORN TREE GRAIN FED EGGS	UNION MEAT MARKET, V. WALT STREET
23	2008/10/16	SR 23	S25 44'24.56" E028 11'32.47"	LARGE	R 8.00	FAIR ACRES GRAIN FED EGGS	ASTOR EXPRESS SUPERMARKET 17, NO. 45 V. WALT STREET
24	2008/10/16	SR 24	S25 44'24.56" E028 11'32.47"	LARGE	R 5.39	MORESON EGGS	ASTOR EXPRESS SUPERMARKET 17, NO. 45 V. WALT STREET

25	2008/10/16	NR 1	S25 44'24.44" E028 11'37.64"	MEDIUM	R 8.00	WELDHAGEN EGGS	CHERRY QUEEN SHOP BLOED STREET
26	2008/10/16	NR 2	S25 44'24.44" E028 11'37.64"	LARGE	R 8.00	QUANTUM EGGS	CHERRY QUEEN SHOP BLOED STREET
27	2008/10/16	NR 3	S25 44'24.29" E028 11'36.74"	LARGE	R 7.00	NO BRAND	RANAMATE STORE BLOED STREET
28	2008/10/16	NR 4	S25 44'24.91" E028 11'31.20"	MEDIUM	R 7.00	WELDHAGEN EGGS	TOP SCORE MEAT SHOP BLOED STREET
29	2008/10/16	NR 5	S25 44'25.20" E028 11'30.60"	LARGE	R 7.00	ALZU GRAIN FED EGGS	SCORE SUPERMARKET BLOED STREET CORNER V.WALT STREET
30	2008/10/16	NR 6	S25 44'25.20" E028 11'30.60"	LARGE	R 7.59	NULAID EGGS	SCORE SUPERMARKET BLOED STREET CORNER V.WALT STREET
31	2008/10/16	NR 7	S25 44'25.50" E028 11'28.83"	MEDIUM	R 7.00	RIETFONTEIN EGGS	OBC CHICKEN, NO. 318, BLOED STREET
32	2008/10/16	NR 8	S25 44'41.62" E028 11'52.00"	LARGE	R 5.99	TOP LAY COUNTRY EGGS	SHOPRITE U-SAVE SUPERMARKET, 271, BLOED STREET
33	2008/10/16	NR 9	S25 43'42.69" E028 11'20.39"	LARGE	R 8.00	BUFFELSDRIFT EGGS	MACEDONIA CAFE', PARK TOWN, PAUL KRUGER STREET
34	2008/10/16	NR 10	S25 43'34.29" E028 11'17.35"	LARGE	R 6.85	THORN TREE EGGS	PAX CONVINIENCE STORE, 324, PAUL KRUGER STREET
35	2008/10/16	NR 11	S25 43'27.87" E028 11'14.82"	MEDIUM	R 9.00	WELDHAGEN EGGS	CALTEX GARAGE SHOP, CAPITAL PARK, PAULKRUGER STREET
36	2008/10/16	NR 12	S25 42'48.53" E028 11'05.65"	LARGE	R 6.90	BUFFELSDRIFT EGGS	LOOTS CAFE' & TAKE AWAY, PARK TOWN PAUL KRUGER STREET
37	2008/10/16	NR 13	S25 42'36.53" E028 11'04.83"	MEDIUM	R 9.00	WELDHAGEN EGGS	CALTEX GARAGE SHOP, BY ELOFFDAL MOTORS,647 P.KRUGER STR
38	2008/10/16	NR 14	S25 42'33.17" E028 11'06.68"	LARGE	R 7.00	FARM HOUSE GRAIN FED EGGS	SPAR SUPERMARKET, PARK TOWN SHOPPING CTR,KRUGER STR
39	2008/10/16	NR 15	S25 42'33.17" E028 11'06.68"	LARGE	R 7.50	SPAR EGGS	SPAR SUPERMARKET, PARK TOWN SHOPPING CTR,KRUGER STR
40	2008/10/16	NR 16	S25 42'27.88" E028 11'05.92"	MEDIUM	R 7.00	WELDHAGEN EGGS	MR. R5 STORE PARK TOWN, PAUL KRUGER STREET
41	2008/10/16	NR 17	S25 42'23.94" E028 11'06.26"	MEDIUM	R 9.00	NULAID EGGS	MADERS FOOD STORE, PAUL KRUGER STREET
42	2008/10/16	NR 18	S25 42'03.51" E028 11'09.87"	LARGE	R 5.50	WELDHAGEN EGGS	MCC CAFE', PAUL KRUGER STREET
43	2008/10/16	NR 19	S25 42'01.30" E028 11'10.21"	LARGE	R 8.00	NO BRAND	MAYVILLE CAFE' PAUL KRUGER STREET
44	2008/10/16	NR 20	S25 39'58.87" E028 11'64.60"	LARGE	R 6.50	NO BRAND	TEX FARM AROUND WONDERBOOM TAXI RANK
45	2008/10/16	NR 21	S25 36'52.96" E028 10'27.48"	LARGE	R 7.00	GROOT EGGS	ONDERSTEPOORT SUPERMARKET, SOUTPAN ROAD
46	2008/10/16	NR 22	S25 36'22.18" E028 10'05.96"	LARGE	R 9.00	FAMILY FAVOURITE EGGS	CALTEX GARAGE SHOP, SOUTPAN-SOSHANGUVE ROAD
47	2008/10/16	NR 23	S25 34'05.38" E028 09'18.33"	LARGE	R 6.50	BUFFELSDRIFT EGGS	HAAKDOORN SHOP, SOUTPAN ROAD
48	2008/10/16	NR 24	S25 31'39.26" E028 06'34.11"	LARGE	R 6.50	WELDHAGEN EGGS	TOTAL GARAGE SHOP, MATLANTA ROAD SOSHANGUVE
49	2008/10/16	NR 25	S25 31'08.03" E028 05'32.99"	LARGE	R 7.00	WOHLFAHRT GRAIN FED EGGS	CHIPHILE SUPER SAVE S'MARKET, SOSHANGUVE SHOPPING CTR
50	2008/10/16	NR 26	S25 31'23.36" E028 06'01.16"	X-LARGE	R 7.00	J.C PIENER EGGS	CHILLIES ROAD-HOUSE SHOP BY TOTAL GARAGE SOSHANGUVE
51	2008/10/20	NR 27	S25 28'42.28" E028 06'00.79"	LARGE	R 7.50	EGGBERT EGGS	SPAR SUPERMARKET BLOCK FF, SOSHANGUVE
52	2008/10/20	NR 28	S25 28'42.28" E028 06'00.79"	LARGE	R 6.00	SPAR EGGS	SPAR SUPERMARKET BLOCK FF, SOSHANGUVE

53	2008/10/20	NR 29	S25 28'42.28" E028 06'00.79"	LARGE	R 6.49	SPAR BRAND EGGS	SPAR SUPERMARKET BLOCK FF, SOSHANGUVE
54	2008/10/20	NR 30	S25 28'57.57" E028 04'01.00"	LARGE	R 7.00	FARMERS FAVOURITE EGGS	U SAVE SHOPRITE MAGOVENI SHOPPING CENTRE STAND 759
55	2008/10/20	NR 31	S25 28'57.57" E028 04'01.00"	LARGE	R 5.99	TOP LAY COUNTRY EGGS	U SAVE SHOPRITE MAGOVENI SHOPPING CENTRE STAND 759
56	2008/10/20	NR 32	S25 30'23.10" E028 06'26.17"	LARGE	R 6.50	QUANTUM EGGS	PHAAHLA SUPERMARKET FALALA COMPLEX SOSHANGUVE
57	2008/10/20	NR 33	S25 30'23.10" E028 06'26.17"	MIXED	R 9.00	MAGNUM EGGS	TUCKS SHOP, BLOCK F, SOSHANGUVE (INFORMAL SHOP)
58	2008/10/20	NR 34	S25 30'28.50" E028 06'48.30"	LARGE	R 8.00	KIEPERSOL EGGS	ENGEN GARAGE SHOP, SOSHANGUVE
59	2008/10/20	NR 35	S25 29'49.89" E028 05'37.64"	MIXED	R 6.50	MAGNUM EGGS	OBC SUPERMARKET, SOSHANGUVE PLAZA
60	2008/10/20	NR 36	S25 29'49.89" E028 05'37.64"	LARGE	R 7.00	GROMER EGGS	SHOPRITE SUPERMARKET SOSHANGUVE PLAZA
61	2008/10/20	NR 37	S25 29'50.40" E028 05'52.30"	MIXED	R 5.99	RIETFONTEIN EGGS	NIZAMS SUPERMARKET SOSHANGUVE PLAZA
62	2008/10/20	NR 38	S25 29'44.60" E028 05'27.89"	MIXED	R 6.50	MAGNUM EGGS	BP GARAGE MABOPANE, BUITEKANT STREET SOSHANGUVE
63	2008/10/20	NR 39	S25 29'44.60" E028 05'27.89"	LARGE	R 7.50	KIEPERSOL GRAIN FED EGGS	MABOPANE TRAIN/TAXI STATION
64	2008/10/20	NR 40	S25 29'44.60" E028 05'27.89"	LARGE	R 5.00	NO BRAND EGGS	MABOPANE TRAIN/TAXI STATION
65	2008/10/20	NR 41	S25 29'43.73" E028 05'07.31"	LARGE	R 6.50	TOP LAY GRAIN FED EGGS	PICK 'N PAY SUPERMARKET SOSHANGUVE FAMILY SOUP
66	2008/10/20	NR 42	S25 29'43.73" E028 05'07.31"	LARGE	R 5.99	WELDHAGEN EGGS	PICK 'N PAY SUPERMARKET SOSHANGUVE FAMILY SOUP
67	2008/10/20	NR 43	S25 29'51.20" E028 05'49.60"	LARGE	R 8.00	POLANI FRESH FARM EGGS	EXPRESS MEAT BUTCHERY, SHOP 10, PNP SHOPPING CENTER
68	2008/10/20	NR 44	S25 29'51.20" E028 05'49.60"	LARGE	R 4.95	NO BRAND EGGS	MEAT AND CHICKEN SHOP 1, MAPONYA COMPLEX SOSHANGUVE
69	2008/10/20	NR 45	S25 29'19.20" E028 05'56.50"	LARGE	R 8.70	QUANTUM EGGS	SHELL GARAGE SERVICE CENTER NO. 2, BLOCK FF SOSHANGUVE
70	2008/10/20	NR 46	S25 29'43.73" E028 05'07.31"	MIXED	R 7.00	MAGNUM EGGS	OBC CHICKEN, MABOPANE CENTRAL
71	2008/10/20	NR 47	S25 29'43.73" E028 05'07.31"	LARGE	R 7.00	GROMER EGGS	SHOPRITE SUPERMARKET MABOPANE CENTRAL CITY
72	2008/10/20	NR 48	S25 29'43.73" E028 05'07.31"	LARGE	R 6.49	TOP LAY COUNTRY EGGS	PICK 'N PAY SUPERMARKET MABOPANE
73	2008/10/20	NR 49	S25 29'43.73" E028 05'07.31"	LARGE	R 8.00	WOHLFAHRT GRAIN FED EGGS	FRUIT AND VEG STORE MABOPANE
74	2008/10/20	NR 50	S25 31'17.59" E028 05'33.13"	MIXED	R 9.00	MAGNUM EGGS	BP GARAGE SOSHANGUVE
75	2008/10/20	NR 51	S25 31'19.49" E028 07'53.30"	X-LARGE	R 6.00	NULAID EGGS	ROAD-SIDE, SOSHANGUVE MAIN ENTRANCE OFF-SOUTPAN
76	2008/10/21	NR 52	S25 30'56.73" E028 04'55.98"	LARGE	R 6.50	WELDHAGEN EGGS	TOTAL GARAGE SHOP, SOSHANGUVE
77	2008/10/21	NR 53	S25 30'12.34" E028 04'38.90"	MEDIUM	R 9.00	TOP LAY EGGS	TEMBISA CAFE' SMALL SHOP SOSHANGUVE
78	2008/10/21	NR 54	S25 29'19.20" E028 04'18.50"	LARGE	R 6.00	THORN TREE EGGS	ALBARAKA SUPERMARKET SOSHANGUVE
79	2008/10/21	NR 55	S25 28'57.58" E028 04'01.00"	LARGE	R 5.99	TOP LAY COUNTRY EGGS	U SAVE SHOPRITE SUPERMARKET SOSHANGUVE
80	2008/10/21	NR 56	S25 28'59.28" E028 02'57.63"	LARGE	R 7.00	THORN TREE GRAIN FED EGGS	MAGGIES SUPERMARKET, BEIRUT ROAD, SOSHANGUVE

81	2008/10/21	NR 57	S25 29'42.95" E028 03'20.02"	LARGE	R 7.60	NULAID GRAIN FED EGGS	SCORE SUPERMARKET BEIRUT ROAD SOSHANGUVE
82	2008/10/21	NR 58	S25 30'35.47" E028 03'33.12"	MIXED	R 7.50	MAGNUM EGGS	MARY'S CONVENIENT STORE SOSHANGUVE
83	2008/10/21	NR 59	S25 30'46.38" E028 02'40.88"	LARGE	R 6.10	KIEPERSOL GRAIN FED EGGS	OK GROCER S'MARKET MABOPANE 1148 BLOCK X EXTENSION N
84	2008/10/21	NR 60	S25 31'24.09" E028 02'08.34"	LARGE	R 7.00	GROMER EGGS	SHOPRITE SUPERMARKET MARULA SHOPPING CENTRE SO'GUVE
85	2008/10/21	NR 61	S25 32'50.20" E028 06'01.05"	LARGE	R 6.50	NO BRAND	VIVA TAKALANI SUPERMARKET SOSHANGUVE
86	2008/10/21	NR 62	S25 29'42.95" E028 03'20.02"	MEDIUM	R 8.00	KIEPERSOL EGGS	ENGEN GARAGE SHOP, SOSHANGUVE
87	2008/10/21	NR 63	S25 31'19.40" E028 07'53.30"	X-LARGE	R 6.00	NULAID EGGS	ROAD-SIDE SELLER, SO'GUVE MAIN ENTRANCE
88	2008/10/21	NR 64	S25 29'44.60" E028 05'27.89"	LARGE	R 5.00	TOP LAY COUNTRY EGGS	TAXI/TRAIN STATION MABOPANE CENTRAL
89	2008/10/21	NR 65	S25 29'44.60" E028 05'27.89"	X-LARGE	R 6.00	NULAID EGGS	TAXI/TRAIN STATION MABOPANE CENTRAL
90	2008/10/21	NR 66	S25 29'44.60" E028 05'27.89"	LARGE	R 5.00	POLANI FRESH FARM EGGS	TAXI/TRAIN STATION MABOPANE CENTRAL
91	2008/10/21	NR 67	S25 29'44.60" E028 05'27.89"	LARGE	R 5.00	TOP LAY COUNTRY EGGS	TAXI/TRAIN STATION MABOPANE CENTRAL
92	2008/10/21	NR 68	S25 29'44.60" E028 05'27.89"	LARGE	R 5.00	POLANI FRESH FARM EGGS	TAXI/TRAIN STATION MABOPANE CENTRAL
93	2008/10/16	NR 69	S25 39'14.82" E028 10'54.04"	LARGE	R 28.00	NULAID EGGS (30 EGGS)	ROAD-SIDE VENDOR, FRONT OF FAC. VET. SCIENCE O'POORT
94	2008/10/22	ER 1	S25 44'46.03" E028 11'31.67"	X-LARGE	R 9.00	NULAID GRAIN FED EGGS	SHOPRITE SUPERMARKETCHURCH STREET CNR V. WALT STREET
95	2008/10/22	ER 2	S25 44'46.74" E028 11'30.54"	LARGE	R 7.95	NULAID GRAIN FED EGGS	JET MART CHURCH STREET
96	2008/10/22	ER 3	S25 44'46.74" E028 11'30.54"	LARGE	R 7.50	TOP LAY COUNTRY EGGS	JET MART CHURCH STREET
97	2008/10/22	ER 4	S25 44'45.64" E028 11'47.57"	MIXED	R 8.00	MAGNUM EGGS	XPRESS SUPER MARKET OPP. RESERVE BANK CHURCH STREET
98	2008/10/22	ER 5	S25 44'44.70" E028 11'46.50"	LARGE	R 6.50	THORN TREE EGGS	G.E STORE CHURCH STREET
99	2008/10/22	ER 6	S25 44'42.75" E028 12'17.76"	LARGE	R 7.00	NULAID GRAIN FED EGGS	SHOPRITE SUPERMARKET ARCADIA
100	2008/10/22	ER 7	S25 44'40.80" E028 13'54.62"	LARGE	R 8.00	WELDHAGEN EGGS	NADA MINI MARKET ARCADIA
101	2008/10/22	ER 8	S25 44'40.80" E028 13'54.62"	MIXED	R 6.50	MAGNUM EGGS	NADA MINI MARKET ARCADIA
102	2008/10/22	ER 9	S25 44'40.56" E028 13'02.11"	LARGE	R 8.60	QUANTUM EGGS	ETING STORE ARCADIA
103	2008/10/22	ER 10	S25 44'40.56" E028 13'02.11"	LARGE	R 6.80	QUANTUM EGGS	ETING STORE ARCADIA
104	2008/10/22	ER 11	S25 44'34.40" E028 14'29.13"	LARGE	R 8.50	WELDHAGEN EGGS	ENGEN GARAGE SHOP, ARCADIA
105	2008/10/22	ER 12	S25 44'32.11" E028 14'47.66"	MEDIUM	R 8.50	WELDHAGEN EGGS	STAR ENGEN MART ARCADIA
106	2008/10/22	ER 13	S25 43'56.73" E028 17'03.02"	LARGE	R 8.50	FAIR ACRES	ENGEN GARAGE OPP. SILVERTON VILLAGE SHOPPING CENTRE
107	2008/10/22	ER 14	S25 43'58.39" E028 17'02.49"	LARGE	R 8.50	NO BRAND	TIP TOP MEAT SILVERTON VILLAGE SHOPPING CENTRE
108	2008/10/22	ER 15	S25 43'57.37" E028 17'44.22"	LARGE	R 7.50	RIETFONTEIN EGGS	SILVERTON SPAR SUPERMARKET PRETORIA STREET

109	2008/10/22	ER 16	S25 43'57.37" E028 17'44.22"	LARGE	R 7.00	WOHLFAHRT GRAIN FED EGGS	SILVERTON SPAR SUPERMARKET PRETORIA STREET
110	2008/10/22	ER 17	S25 43'59.58" E028 17'44.22"	MIXED	R 8.50	MAGNUM EGGS	SILVERTON SHELL GARAGE OPP. SAP STATION, SILVERTON
111	2008/10/22	ER 18	S25 43'59.45" E028 17'02.00"	LARGE	R 6.50	WELDHAGEN EGGS	DOLLARS SUPERMARKET SILVERTON, PRETORIA
112	2008/10/22	ER 19	S25 43'59.47" E028 17'58.68"	LARGE	R 8.00	NULAID GRAIN FED EGGS	JET SILVERTON, PRETORIA STREET
113	2008/10/22	ER 20	S25 43'59.73" E028 18'06.30"	LARGE	R 7.00	NULAID GRAIN FED EGGS	SHOPRITE SUPERMARKET SILVERTON
114	2008/10/22	ER 21	S25 43'58.50" E028 18'04.70"	LARGE	R 6.40	VLEIVIEW EGGS	POLLIES CAFE' & TAKE AWAY SILVERTON
115	2008/10/22	ER 22	S25 44'00.76" E028 18'55.28"	LARGE	R 9.00	VLEIVIEW FARM GRAIN FED EGGS	COUNTRY FRESH /VIVA SUPER SAVE SILVERTON
116	2008/10/22	ER 23	S25 43'51.80" E028 19'02.74"	LARGE	R 8.70	ZEEKOEGAT EGGS	WALTLOO TOTAL GARAGE
117	2008/10/22	ER 24	S25 43'13.94" E028 19'54.26"	LARGE	R 7.00	TOP LAY EGGS	WALTLOO MEAT & CHICKEN, 349 ZASM STREET WALTLOO
118	2008/10/22	ER 25	S25 43'08.37" E028 19'53.41"	LARGE	R 7.50	WOHLFAHRT GRAIN FED EGGS	SUPER SEVEN SUPERMARKET WALTLOO
119	2008/10/27	ER 26	S25 43'03.73" E028 20'14.51"	LARGE	R 7.60	NULAID GRAIN FED EGGS	SCORE SUPERMARKET DENNEBOOM MAMELODI
120	2008/10/27	ER 27	S25 43'03.73" E028 20'14.51"	LARGE	R 6.39	TOP LAY COUNTRY EGGS	SCORE SUPERMARKET DENNEBOOM MAMELODI
121	2008/10/27	ER 28	S25 43'03.73" E028 20'14.51"	LARGE	R 6.00	WELDHAGEN GRAIN FED EGGS	SEGWAGWA SUPERMARKET METROPOLITAN C25 DENNEBOOM
122	2008/10/27	ER 29	S25 43'03.73" E028 20'14.51"	LARGE	R 7.00	NO BRAND	JUICE AND DAIRY METROPOLITAN DENNEBOOM
123	2008/10/27	ER 30	S25 43'14.30" E028 20'32.80"	LARGE	R 5.99	QUANTUM EGGS	NIZAMS SUPERMARKET DENNEBOOM
124	2008/10/27	ER 31	S25 43'03.73" E028 20'14.51"	LARGE	R 6.50	WELDHAGEN GRAIN FED EGGS	PROBITUM STORE MAMELODI
125	2008/10/27	ER 32	S25 43'03.01" E028 22'02.92"	LARGE	R 7.00	ALZU GRAIN FED EGGS	SCORE SUPERMARKET MAMELODI TSAMAYA AVENUE
126	2008/10/27	ER 33	S25 42'48.60" E028 22'31.38"	MEDIUM	R 6.00	TOP LAY EGGS	KHULAMI MEAT & CHICKEN 18874, TSAMAYA RD MAMELODI
127	2008/10/27	ER 34	S25 42'49.58" E028 22'29.39"	LARGE	R 7.65	FARM EGGS	TOTAL GARAGE BESIDE KHULAMI MEAT & CHICKEN TSAMAYA RD
128	2008/10/27	ER 35	S25 42'51.40" E028 22'52.00"	LARGE	R 8.00	NO BRAND EGGS	SHELL GARAGE OPP. KHULAMI MEAT & CHICKENTSAMAYA ROAD
129	2008/10/27	ER 36	S25 42'43.32" E028 22'40.74"	MIXED	R 7.45	FARM FRESH EGGS	LE BAMBA SUPERMARKET 19683, TSAMAYA ROAD MAMELODI
130	2008/10/27	ER 37	S25 42'00.97" E028 25'30.29"	LARGE	R 5.99	RIETFONTEIN EGGS	NIZAM SUPERMARKET, MAXCITY SHOPPING CENTRE MAMELODI
131	2008/10/27	ER 38	S25 42'00.97" E028 25'30.29"	LARGE	R 5.99	WELDHAGEN EGGS	NIZAM SUPERMARKET, MAXCITY SHOPPING CENTRE MAMELODI
132	2008/10/27	ER 39	S25 41'55.64" E028 25'21.24"	LARGE	R 7.50	TOP LAY EGGS	PICK 'N PAY SUPERMARKET MAXCITY SHOPPING CTRMAMELODI
133	2008/10/27	ER 40	S25 41'55.64" E028 25'21.24"	LARGE	R 6.50	WELDHAGEN GRAIN FED EGGS	PICK 'N PAY SUPERMARKET MAXCITY SHOPPING CTR MAMELODI
134	2008/10/27	ER 41	S25 41'55.64" E028 25'21.24"	LARGE	R 6.49	TOP LAY COUNTRY EGGS	PICK 'N PAY SUPERMARKET MAXCITY SHOPPING CTR MAMELODI
135	2008/10/27	ER 42	S25 43'18.16" E028 24'05.57"	LARGE	R 8.00	TOP LAY EGGS	ROAD-SIDESTORE BESIDE UNIV. PRETORIA MAMELODI CAMPUS
136	2008/10/27	ER 43	S25 43'19.29" E028 23'21.59"	LARGE	R 6.40	TOP LAY EGGS	B.P MAMELODI, HINTERLAND AVENUE MAMELODI

137	2008/10/27	ER 44	S25 43'29.20" E028 23'11.80"	LARGE	R 5.99	NO BRAND EGGS	DLAMINI & SON SUPERMARKET, 18680, HINTERLAND AVENUE
138	2008/10/27	ER 45	S25 42'40.96" E028 22'25.51"	LARGE	R 7.50	NO BRAND	OSIZWANI SUPERMARKET MAMELODI
139	2008/10/27	ER 46	S25 42'40.96" E028 22'25.51"	LARGE	R 7.50	NO BRAND	OSIZWANI BUTCHERY MAMELODI
140	2008/10/27	ER 47	S25 42'60.01" E028 20'50.00"	LARGE	R 6.00	NO BRAND EGGS	ROAD-SIDE SHOP KUBONE ROAD MAMELODI WEST
141	2008/10/27	ER 48	S25 42'48.69" E028 20'04.83"	LARGE	R 7.00	FAMILY FAVOURITE EGGS	SSS SUPER STORE MAMELODI CROSSING SHOP NO. 5
142	2008/10/27	ER 49	S25 42'59.65" E028 20'20.80"	LARGE	R 6.99	SUCCESS EGGS	ROAD-SIDE SHOP BY MAMELODI CROSSING
143	2008/10/27	ER 50	S25 42'49.17" E028 20'06.81"	LARGE	R 9.00	NULAID GRAIN FED EGGS	WALTLOO MEAT & CHICKEN MAMELODI CROSSING
144	2008/10/27	ER 51	S25 42'53.37" E028 19'58.23"	LARGE	R 7.00	TOP LAY EGGS	SHOPRITE SUPERMARKET MAMELODI
145	2008/10/27	ER 52	S25 42'49.68" E028 20'20.80"	X-LARGE	R 6.79	TOP LAY COUNTRY EGGS	NO NAME MAMELODI CROSSING
146	2008/10/28	WR 1	S25 44'47.85" E028 11'13.47"	LARGE	R 8.00	ZEEKOEGAT EGGS	M.O.T AFRICAN MARKET SHOP, CHURCH SQUARE
146	2008/10/28	WR 2	S25 44'51.85" E028 10'04.21"	MIXED	R 8.00	MAGNUM EGGS	STEVE'S CAFE' CHURCH STREET, ATTERIDGEVILLE ROAD
148	2008/10/28	WR 3	S25 44'53.91" E028 09'43.22"	X-LARGE	R 9.00	MORIA EGGS	DELI QUEEN FRUITS AND VEG SHOP ATTERIDGEVILLE ROAD
149	2008/10/28	WR 4	S25 44'53.93" E028 09'43.19"	X-LARGE	R 9.50	WELDHAGEN EGGS	ATHINA FRUITS & VEG, CHURCH STREET, ATTERIDGEVILLE ROAD
150	2008/10/28	WR 5	S25 44'53.93" E028 09'43.19"	X-LARGE	R 8.00	NULAID EGGS	ATHINA FRUITS & VEG, CHURCH STREET, ATTERIDGEVILLE ROAD
151	2008/10/28	WR 6	S25 44'54.90" E028 09'28.08"	MIXED	R 9.95	FARM FRESH EGGS	NAPOLI CAFE' CHURCH STREET, ATTERIDGEVILLE ROAD
152	2008/10/28	WR 7	S25 44'54.90" E028 09'28.08"	LARGE	R 7.00	WELDHAGEN EGGS	FONS MODERN TRADING CENTRE CHURCH STREET, A'VILLE
153	2008/10/28	WR 8	S25 44'52.19" E028 09'38.38"	LARGE	R 6.50	NO BRAND EGGS	DINO'S TAKEAWAY, CHURCH STREET, ATTERIDGEVILLE ROAD
154	2008/10/28	WR 9	S25 44'56.17" E028 09'09.52"	LARGE	R 9.95	WELDHAGEN EGGS	M.K SUPERMARKET CHURCH STREET, ATTERIDGEVILLE
155	2008/10/28	WR 10	S25 44'45.39" E028 09'36.09"	LARGE	R 6.50	ZEEKOEGAT EGGS	B.P GARAGE, CHURCH STREET, ATTERIDGEVILLE
156	2008/10/28	WR 11	S25 44'45.39" E028 09'36.09"	LARGE	R 6.50	QUANTUM EGGS	B.P GARAGE, CHURCH STREET, ATTERIDGEVILLE
157	2008/10/28	WR 12	S25 44'35.30" E028 09'86.10"	LARGE	R 6.50	NO BRAND EGGS	AL-MEDINA SUPERMARKET, CHURCH STREET, ATTERIDGEVILLE
158	2008/10/28	WR 13	S25 44'53.20" E028 08'53.73"	LARGE	R 7.00	GROMER EGGS	SHOPRITE SUPERMARKET QUAGGA CENTRE, ATTERIDGEVILLE RD
159	2008/10/28	WR 14	S25 44'53.20" E028 08'53.73"	LARGE	R 8.89	P 'N P FREE RANGE EGGS	PICK 'N PAY SUPERMARKET, QUAGGA SHOPPING CENTRE
160	2008/10/28	WR 15	S25 44'53.20" E028 08'53.73"	LARGE	R 6.50	P 'N P NO NAME	PICK 'N PAY SUPERMARKET, QUAGGA SHOPPING CENTRE
161	2008/10/28	WR 16	S25 45'01.69" E028 07'56.74"	LARGE	R 8.50	QUANTUM EGGS	SHELL GARAGE CHURCH STREET, ATTERIDGEVILLE ROAD
162	2008/10/28	WR 17	S25 45'41.85" E028 05'07.02"	LARGE	R 7.70	QUANTUM EGGS	SHELL GARAGE, SEEISO ROAD, ATTERIDGEVILLE
163	2008/10/28	WR 18	S25 45'54.99" E028 04'54.86"	LARGE	R 8.00	QUANTUM EGGS	AFRICAN SUPPLY BUTCHERY STORE, ATTERIDGEVILLE
164	2008/10/28	WR 19	S25 45'54.99" E028 04'54.86"	LARGE	R 8.00	J.C PIENAAR EGGS	AFRICAN SUPPLY STORE 2, ATTERIDGEVILLE

165	2008/10/28	WR 20	S25 46'06.29" E028 04'40.14"	LARGE	R 7.00	THORN TREE GRAIN FED EGGS	LOBAY GAP SUPERMARKET, ATTERIDGEVILLE
166	2008/10/28	WR 21	S25 46'13.83" E028 05'23.98"	LARGE	R 8.00	KIEPERSOL EGGS	ROYAL BUTCHERY, ATTLYN SHOPPING CENTRE, ATTERIDGEVILL
167	2008/10/28	WR 22	S25 46'13.83" E028 05'23.98"	LARGE	R 7.50	RIETFONTEIN EGGS	NIZAMS SUPERMARKET, ATTLYN SHOPPING CENTRE, A'VILLE
168	2008/10/28	WR 23	S25 46'13.83" E028 05'23.98"	X-LARGE	R 7.70	GROMER EGGS	SHOPRITE SUPERMARKET, ATTLYN SHOPPING CENTRE, A'VILLE
169	2008/10/28	WR 24	S25 46'57.40" E028 04'54.90"	MEDIUM	R 6.00	WELDHAGEN EGGS	KHAHLA BUTCHERY MAUNDE STREET, ATTERIDGEVILLE
170	2008/10/28	WR 25	S25 46'47.90" E028 04'00.48"	LARGE	R 6.50	WELDHAGEN EGGS	ROAD SIDE SHOP NO NAME BESIDE ENGEN GARAGE SAULSVILLE
171	2008/10/28	WR 26	S25 47'15.55" E028 02'56.28"	LARGE	R 6.00	TOP LAY COUNTRY EGGS	EUREKA SUPERMARKET, BLOCK W127 VERGENOEG, SAULSVILLE
172	2008/10/28	WR 27	S25 47'15.55" E028 02'56.28"	LARGE	R 6.50	NO BRAND EGGS	ABIO SUPERMARKET, MAUNDE ROAD, SAULSVILLE
173	2008/10/28	WR 28	S25 47'29.20" E028 02'28.50"	LARGE	R 6.50	QUANTUM EGGS	SOM SUPERMARKET, MAUNDE ROAD, SAULSVILLE
174	2008/10/28	WR 29	S25 46'17.84" E028 04'18.50"	LARGE	R 8.00	QUANTUM EGGS	N.V SUPERMARKET, ATTERIDGEVILLE
175	2008/10/28	WR 30	S25 45'02.90" E028 07'35.80"	MIXED	R 8.00	MAGNUM EGGS	SASOL GARGE SHOP, CHURCH SQUARE, ATTERIDGEVILLE ROAD
176	2008/10/29	WR 31	S25 47'10.98" E028 02'29.27"	LARGE	R 7.00	NO BRAND	SHONGWE SUPERMARKET,CHURCH STREET, A'VILLE
177	2008/10/29	WR 32	S25 47'10.98" E028 02'29.27"	LARGE	R 7.50	QUANTUM EGGS	ROAD-SIDE SHOP MAUNDE STREET ATTERIDGEVILLE
178	2008/10/29	WR 33	S25 47'10.98" E028 02'29.27"	LARGE	R 7.50	WELDHAGEN EGGS	ROAD-SIDE SHOP MAUNDE STREET ATTERIDGEVILLE
179	2008/10/29	WR 34	S25 47'10.98" E028 02'29.27"	MEDIUM	R 6.50	WELDHAGEN EGGS	ROAD-SIDE SHOP MAUNDE STREET ATTERIDGEVILLE
180	2008/10/29	WR 35	S25 47'10.98" E028 02'29.27"	LARGE	R 6.50	NO BRAND EGGS	ROAD-SIDE SHOP MAUNDE STREET ATTERIDGEVILLE
181	2008/10/29	WR 36	S25 46'21.90" E028 02'55.40"	LARGE	R 6.50	NO BRAND EGGS	SIBASA WHOLE SELLERS, CHURCH STREET, A'VILLE
182	2008/10/29	WR 37	S25 47'10.98" E028 02'29.27"	LARGE	R 7.50	RIETFONTEIN EGGS	ROAD-SIDE SHOP ATTERIDGEVILLE EXTENSION
183	2008/10/29	WR 38	S25 47'10.98" E028 02'29.27"	LARGE	R 7.00	FAIR ACRES EGGS	ROAD-SIDE SHOP ATTERIDGEVILLE EXTENSION
184	2008/10/29	WR 39	S25 47'10.98" E028 02'29.27"	LARGE	R 9.00	ZEEKOEGAT EGGS	ROAD-SIDE SHOP ATTERIDGEVILLE EXTENSION
185	2008/10/29	WR 40	S25 47'10.98" E028 02'29.27"	X-LARGE	R 9.00	J.C PIENAAR EGGS	ROAD-SIDE SHOP ATTERIDGEVILLE EXTENSION
186	2008/10/29	WR 41	S25 47'10.98" E028 02'29.27"	LARGE	R 8.50	RIETFONTEIN EGGS	ROAD-SIDE SHOP ATTERIDGEVILLE EXTENSION
187	2008/11/20	SM 1	S25 40'46.03" E028 14'24.38"	LARGE	R 8.00	FARM HOUSE GRAIN FED EGGS	MONTANA SUPERSPAR SHOP 1 MONTANA CORNER ZAMBAZI DR
188	2008/11/20	SM 2	S25 40'46.03" E028 14'24.38"	LARGE	R 10.00	BOSCHVELD FREE RANGE EGGS	MONTANA SUPERSPAR SHOP 1 MONTANA CORNER ZAMBAZI DR
189	2008/11/20	SM 3	S25 40'46.03" E028 14'24.38"	LARGE	R 7.00	THORN TREE GRAIN FED EGGS	MONTANA SUPERSPAR SHOP 1 MONTANA CORNER ZAMBAZI DR
190	2008/11/20	SM 4	S25 40'46.03" E028 14'24.38"	LARGE	R 9.40	THORN TREE FREE RANGE EGGS	MONTANA SUPERSPAR SHOP 1 MONTANA CORNER ZAMBAZI DR
191	2008/11/20	SM 5	S25 43'05.26" E028 13'05.49"	LARGE	R 6.50	WELDHAGEN GRAIN FED EGGS	PICK 'N PAY SUPERMARKET GEZINA
192	2008/11/20	SM 6	S25 43'05.26" E028 13'05.49"	LARGE	R 6.50	P 'N P NO NAME	PICK 'N PAY SUPERMARKET GEZINA

193	2008/11/20	SM 7	S25 43'05.26" E028 13'05.49"	LARGE	R 8.99	WELDHAGEN FREE RANGE EGGS	PICK 'N PAY SUPERMARKET GEZINA
194	2008/11/20	SM 8	S25 43'05.26" E028 13'05.49"	LARGE	R 7.00	TOP LAY GRAIN FED EGGS	PICK 'N PAY SUPERMARKET GEZINA
195	2008/11/20	SM 9	S25 43'05.26" E028 13'05.49"	LARGE	R 8.00	ALZU FREE RANGE EGGS	PICK 'N PAY SUPERMARKET GEZINA
196	2008/11/20	SM 10	S25 43'05.26" E028 13'05.49"	LARGE	R 7.00	WOHLFAHRT GRAIN FED EGGS	FRUIT AND VEG STORE GEZINA
197	2008/11/20	SM 11	S25 40'33.25" E028 10'22.48"	LARGE	R 6.60	GROMER EGGS	SHOPRITE SUPERMARKET PRETORIA NORTH
198	2008/11/20	SM 12	S25 40'33.25" E028 10'22.48"	LARGE	R 9.70	GROMER FREE RANGE EGGS	SHOPRITE SUPERMARKET PRETORIA NORTH
199	2008/11/20	SM 13	S25 40'33.25" E028 10'22.48"	LARGE	R 6.59	FARM HOUSE EGGS	SHOPRITE SUPERMARKET PRETORIA NORTH
200	2008/11/20	SM 14	S25 40'43.25" E028 14'24.56"	LARGE	R 7.30	FAIR ACRES EGGS	PICK ' PAY MONTANA CROSSING ZAMBEZI ROAD
201	2008/11/20	SM 15	S25 40'43.25" E028 14'24.56"	MIXED	R 11.00	P 'N P OMEGA-3 EGGS	PICK ' PAY MONTANA CROSSING ZAMBEZI ROAD
202	2008/11/20	SM 16	S25 40'43.25" E028 14'24.56"	LARGE	R 10.00	P 'N P ALL GRAIN FED EGGS	PICK ' PAY MONTANA CROSSING ZAMBEZI ROAD
203	2008/11/20	SM 17	S25 41'55.87" E028 11'00.63"	LARGE	R 10.00	CHECKERS CHOICE FREE RANGE	HYPER CHECKERS MAYVILLE
204	2008/11/20	SM 18	S25 41'55.87" E028 11'00.63"	LARGE	R 7.40	PASTEURISED SAFE EGGS	HYPER CHECKERS MAYVILLE
205	2008/11/20	SM 19	S25 41'55.87" E028 11'00.63"	LARGE	R 7.40	FARM HOUSE GRAIN FED EGGS	HYPER CHECKERS MAYVILLE
206	2008/11/20	SM 20	S25 43'57.71" E028 17'44.15"	LARGE	R 7.60	SPAR EGGS	SILVERTON SPAR SUPERMARKET PRETORIA STREET
207	2008/11/21	SM 21	S25 43'57.71" E028 17'44.15"	LARGE	R 7.00	WOHLFAHRT GRAIN FED EGGS	SILVERTON SPAR SUPERMARKET PRETORIA STREET
208	2008/11/21	SM 22	S25 43'57.71" E028 17'44.15"	LARGE	R 7.40	RIETFONTEIN EGGS	SILVERTON SPAR SUPERMARKET PRETORIA STREET
209	2008/11/21	SM 23	S25 44'39.94" E028 14'45.18"	LARGE	R 8.00	SPAR EGGS	HATFIELD QUICK SPAR PRETORIOUS STREET HATFIELD
210	2008/11/21	SM24	S25 46'18.07" E028 14'04.62"	LARGE	R 8.00	NULAID GRAIN FED EGGS	PICK 'N PAY SUPERMARKET, BROOKLYN SHOPPING CENTRE
211	2008/11/21	SM25	S25 46'18.07" E028 14'04.62"	LARGE	R 7.00	PICK ' PAY NO NAME	PICK 'N PAY SUPERMARKET, BROOKLYN SHOPPING CENTRE
212	2008/11/21	SM26	S25 47'04.52" E028 16'35.50"	LARGE	R 10.45	WOOLWORTHS FREE RANGE EGGS	WOOL WORTHS SUPERMARKET MENLYN SHOPPING MALL
213	2008/11/21	SM27	S25 47'04.52" E028 16'35.50"	LARGE	R 17.55	WOOLWORTHS ORGANIC EGGS	WOOL WORTHS SUPERMARKET MENLYN SHOPPING MALL
214	2008/11/21	SM28	S25 44'45.40" E028 11'35.21"	LARGE	R 10.45	WOOLWORTHS FREE RANGE EGGS	WOOLWORTHS SUPERMARKET SAMMY MARK SQUARE
215	2008/11/21	SM 29	S25 45'13.28" E028 12'30.34"	LARGE	R 7.00	GROMER EGGS	SHOPRITE SUPERMARKET SUNNY SIDE
216	2008/11/21	SM 30	S25 45'04.64" E028 12'11.72"	X-LARGE	R 14.00	WOOLWORTHS FREE RANGE EGGS	WOOLWORTHS SUPERMARKET SUNNY PARK, SUNNYSIDE
217	2008/11/21	SM 31	S25 45'55.98" E028 17'55.05"	LARGE	R 13.00	WOOLWORTHS FREE RANGE EGGS	WOOLWORTHS SUPERMARKET GIFTACRES SHOPPING CENTER
218	2008/11/21	SM 32	S25 45'55.98" E028 17'55.05"	LARGE	R 18.00	WOOLWORTHS ORGANIC EGGS	WOOLWORTHS SUPERMARKET GIFTACRES SHOPPING CENTER
219	2008/11/21	SM 33	S25 40'18.04" E028 06'41.37"	LARGE	R 8.00	NULAID GRAIN FED EGGS	PICK 'N PAY HYPERMARKET WONDERPARK
220	2008/11/21	SM 34	S25 40'18.04" E028 06'41.37"	LARGE	R 8.00	ALZU FREE RANGE EGGS	PICK 'N PAY HYPERMARKET WONDERPARK

221	2008/11/21	SM 35	S25 40'18.04" E028 06'41.37"	LARGE	R 10.00	NULAID FREE RANGE EGGS	PICK 'N PAY HYPERMARKET WONDERPARK
222	2008/11/21	SM 36	S25 28'42.39" E028 06'00.76"	LARGE	R 7.50	SPAR EGGS	SPAR SUPERMARKET BLOCK FF, SOSHANGUVE
223	2008/11/21	SM 37	S25 28'42.39" E028 06'00.76"	LARGE	R 5.00	SPAR EGGS	SPAR SUPERMARKET BLOCK FF, SOSHANGUVE
224	2008/11/21	SM 38	S25 42'33.26" E028 11'06.67"	LARGE	R 7.00	FARM HOUSE GRAIN FED EGGS	SPAR SUPERMARKET PARKTOWN SHOPPING CENTRE
225	2008/11/21	SM 39	S25 42'33.26" E028 11'06.67"	LARGE	R 7.50	SPAR EGGS	SPAR SUPERMARKET PARKTOWN SHOPPING CENTRE
226	2008/11/21	SM 40	S25 40'49.31" E028 11'36.93"	LARGE	R 9.90	FAIR ACRES EGGS	MAKRO MASSTORE WONDERBOOM JUNCTION
227	2009/05/04	S 1	S25 44'27.01" E028 11'24.45"	MIXED	R 7.00	MAGNUM EGGS	BROTHERS SHOP FRUITS AND VEG ANDRIES STREET
228	2009/05/04	S 2	S25 44'56.70" E028 11'27.40"	LARGE	R 6.00	WOHLFAHRT GRAINFED EGGS	L.M SUPERMARKET, CNR ANDIRES AND V.WALT STREET
229	2009/05/04	S 3	S25 45'34.75" E028 11'32.31"	LARGE	R 7.00	ZEEKOEGAT EGGS	4.U SUPERMARKET, ANDRIES STREET
230	2009/05/04	S 4	S25 45'25.61" E028 11'29.77"	LARGE	R 8.50	MORIA EGGS	CALTEX GARAGE, ANDRIES STREET
231	2009/05/04	S 5	S25 45'24.12" E028 11'16.46"	LARGE	R 7.00	QUANTUM EGGS	NO NAME
232	2009/05/04	S 6	S25 45'24.32" E028 11'15.18"	LARGE	R 7.00	WOHLFAHRT GRAIN FED EGGS	NO NAME
233	2009/05/04	S 7	S25 45'24.20" E028 11'19.20"	LARGE	R 7.00	WOHLFAHRT GRAIN FED EGGS	L0W SDE SUPERMARKET, OPPOSITE BOSMAN TRAIN STATION
234	2009/05/04	S 8	S25 45'24.20" E028 11'19.20"	LARGE	R 7.00	ZEEKOEGAT EGGS	BLUE BUTCHERY OPPOSITE BOSMAN TRAIN STATION
235	2009/05/04	S 9	S25 45'23.80" E028 11'23.80"	LARGE	R 7.00	NO BRAND	STATION STORE OPPOSITE BOSMAN TRAIN STATION
236	2009/05/04	S 10	S25 44'53.70" E028 11'56.00"	LARGE	R 7.00	FAIR ACRES EGGS	STAR BUTCHERY, 78, VAN DER WALT STREET
237	2009/05/04	S 11	S25 45'05.57" E028 11'37.37"	LARGE	R 7.00	FAIR ACRES GRAIN FED EGGS	PRIME STAR BUTCHERY, 70, VAN DER WALT STREET
238	2009/05/04	S 12	S25 44'24.56" E028 11'32.47"	LARGE	R 8.00	FAIR ACRES GRAIN FED EGGS	ASTOR SUPERMARKET, CNR BLOED VAN DER WALT STREET
239	2009/05/04	S 13	S25 44'34.86" E028 11'34.15"	LARGE	R 8.00	THORN TREE GRAIN FED EGGS	UNION MEAT MARKET BUTCHERY SHOP
240	2009/05/04	S 14	S25 44'68.20" E028 11'58.00"	MIXED	R 7.00	FARM FRESH EGGS	CENTRAL SUPERMARKET
241	2009/05/04	S 15	S25 44'33.58" E028 11'34.08"	LARGE	R 7.00	KIEPERSOL EGGS	ROYAL HYPER BUTCHERY
242	2009/05/04	S 16	S25 44'36.72" E028 11'33.26"	LARGE	R 7.00	KIEPERSOL EGGS	ROYAL EXPRESS BUTCHERY
243	2009/05/04	S 17	S25 44'54.77" E028 11'36.62"	LARGE	R 8.00	NULAID GRAIN FED EGGS	P 'N P TRAMSHED, VAN DER WALT
244	2009/05/04	S 18	S25 44'54.77" E028 11'36.62"	X-LARGE	R 7.50	P 'N P NO NAME	P 'N P TRAMSHED, VAN DER WALT
245	2009/05/04	S 19	S25 44'45.41" E028 11'35.21"	X-LARGE	R 13.00	WOOLWORTHS FREE RANGE EGGS	WOOLWORTHS SUPERMARKET SAMMY MARKS SQUARE
246	2009/05/04	S 20	S25 44'25.33" E028 11'33.33"	LARGE	R 5.00	NO BRAND	SUPER SAVE BUTCHER VAN DER WALT STREET
247	2009/05/05	N 1	S25 44'24.44" E028 11'37.64"	MEDIUM	R 8.00	WELDHAGEN EGGS	CHERRY SHOP BLOED STREET
248	2009/05/05	N 2	S25 44'24.29" E028 11'36.74"	LARGE	R 7.00	NO BRAND	RATANAMA SUPERMARKET BLOED STREET

249	2009/05/05	N 3	S25 44'24.91" E028 11'31.20"	MEDIUM	R 7.00	WELDHAGEN EGGS	TOP SCORE MEAT SHOP BLOED STREET
250	2009/05/05	N 4	S25 44'25.33" E028 11'29.78"	MIXED	R 7.50	FARM FRESH EGGS	FLAMINGO SUPERMARKET BLOED STREET
251	2009/05/05	N 5	S25 44'25.20" E028 11'30.60"	LARGE	R 7.00	ALZU GRAIN FED EGGS	SCORE SUPERMARKET BLOED STREET
252	2009/05/05	N 6	S25 44'25.50" E028 11'28.83"	MEDIUM	R 7.00	RIETFONTEIN EGGS	OBC CHICKENS
253	2009/05/05	N 7	S25 44'25.52" E028 11'30.80"	LARGE	R 8.00	TOP LAY GRAIN FED EGGS	SUPER SPAR SUPERMARKET BLOED STREET
254	2009/05/05	N 8	S25 43'42.69" E028 11'20.39"	LARGE	R 8.00	BUFFELSDRIFT EGGS	MACEDONIA CAFÉ' CAPITAL PARK
255	2009/05/05	N 9	S25 43'27.87" E028 11'14.82"	MEDIUM	R 9.00	WELDHAGEN EGGS	CALTEX GARAGE CAPITAL PARK
256	2009/05/05	N 10	S25 42'48.53" E028 11'05.65"	LARGE	R 10.00	BUFFELSDRIFT EGGS	MATCHRASS SHOP PAUL KRUGER STREET
257	2009/05/05	N 11	S25 42'36.53" E028 11'04.83"	MEDIUM	R 9.00	WELDHAGEN EGGS	CALTEX GARAGE, PAUL KRUGER STREET
258	2009/05/05	N 12	S25 42'33.17" E028 11'06.68"	LARGE	R 7.00	FARM HOUSE GRAIN FED EGGS	SPAR SUPERMARKET, PAUL KRUGER STREET
259	2009/05/05	N 13	S25 42'33.17" E028 11'06.68"	LARGE	R 7.50	SPAR EGGS	SPAR SUPERMARKET, PAUL KRUGER STREET
260	2009/05/05	N 14	S25 42'27.88" E028 11'05.92"	MEDIUM	R 7.00	WELDHAGEN EGGS	MR R5 SHOP, PAUL KRUGER STREET
261	2009/05/05	N 15	S25 42'01.30" E028 11'10.21"	LARGE	R 8.00	NO BRAND	MAYVILLE CAFÉ' PAUL KRUGER STREET
262	2009/05/05	N 16	S25 39'58.87" E028 11'64.60"	LARGE	R 6.50	NO BRAND	TEX FARM, ONDERSTEPOORT, NEAR WONDERBOOM TAXI RANK
263	2009/05/05	N 17	S25 39'14.82" E028 10'54.04"	LARGE	R 28.00	NULAID EGGS (30 EGGS)	ROAD-SIDE SELLER OPP FAC. VET. SCIENCE ONDERSTEPOORT
264	2009/05/05	N 18	S25 36'52.96" E028 10'27.48"	LARGE	R 7.00	GROOT EGGS	ONDERSTEPOORT SUPERMARKET, SOUTPAN ROAD
265	2009/05/05	N 19	S25 36'22.18" E028 10'05.96"	LARGE	R 9.00	FAMILY FAVOURITE EGGS	ELLIS SUPERMARKET BY CALTEX GARAGE, SOUTPAN ROAD
266	2009/05/05	N 20	S25 31'23.36" E028 06'01.16"	X-LARGE	R 7.00	J.C PIENER EGGS	CHILLIES ROAD-HOUSE SHOP SHOSHANGUVE
267	2009/05/05	N 21	S25 31'17.59" E028 05'33.13"	MIXED	R 9.00	MAGNUM EGGS	B.P EXPRESS GARAGE SOSHANGUVE
268	2009/05/05	N 22	S25 31'17.63" E028 05'32.01"	LARGE	R 8.00	NO BRAND	ROAD-SIDE SELLER OPP B.P GARAGE SOSHANGUVE
269	2009/05/05	N 23	S25 30'12.34" E028 04'38.90"	MEDIUM	R 9.00	TOP LAY EGGS	TEMBISA CAFÉ' SOSHANGUVE
270	2009/05/05	N 24	S25 28'57.57" E028 04'01.00"	LARGE	R 7.00	FARMERS FAVOURITE EGGS	U-SAVE SHOPRITE SUPERMARKET SOSHANGUVE
271	2009/05/05	N 25	S25 28'42.28" E028 06'00.79"	LARGE	R 7.50	EGGBERT EGGS	SPAR SUPERMARKET SOSHANGUVE BLOCK FF
272	2009/05/05	N 26	S25 28'42.28" E028 06'00.79"	LARGE	R 6.00	SPAR EGGS	SPAR SUPERMARKET SOSHANGUVE BLOCK FF
273	2009/05/05	N 27	S25 28'42.28" E028 06'00.79"	LARGE	R 8.00	NO BRAND	ROAD-SIDE SELLER, BLOCK FF SOSHANGUVE
274	2009/05/06	N 28	S25 30'23.10" E028 06'26.17"	LARGE	R 6.50	QUANTUM EGGS	PHAAHLA SUPERMARKET, SOSHANGUVE
275	2009/05/06	N 29	S25 30'23.10" E028 06'26.17"	MIXED	R 9.00	MAGNUM EGGS	ROSINAH TUCK SHOP,
276	2009/05/06	N 30	S25 29'49.89" E028 05'37.64"	MIXED	R 6.50	MAGNUM EGGS	OBC CHICKENS SOSHANGUVE PLAZA

277	2009/05/06	N 31	S25 29'49.89" E028 05'37.64"	LARGE	R 7.00	GROMER EGGS	SHOPRITE SUPERMARKET SOSHANGUVE PLAZA
278	2009/05/06	N 32	S25 29'44.60" E028 05'27.89"	LARGE	R 7.50	KIEPERSOL GRAIN FED EGGS	MABOPANE TAXI RANK
279	2009/05/06	N 33	S25 29'44.60" E028 05'27.89"	MIXED	R 6.50	MAGNUM EGGS	B.P EXPRESS GARAGE MABOPANE
280	2009/05/06	N 34	S25 29'43.73" E028 05'07.31"	MIXED	R 7.00	MAGNUM EGGS	OBC CHICKENS MABOPANE
281	2009/05/06	N 35	S25 29'43.73" E028 05'07.31"	LARGE	R 8.00	WOHLFAHRT GRAIN FED EGGS	FRUITS AND VEG MABOPANE
282	2009/05/06	N 36	S25 29'43.73" E028 05'07.31"	LARGE	R 7.00	GROMER EGGS	SHOPRITE SUPERMARKET MABOPANE
283	2009/05/06	N 37	S25 29'43.73" E028 05'07.31"	LARGE	R 6.50	TOP LAY GRAIN FED EGGS	P 'N P MABOPANE
284	2009/05/06	N 38	S25 31'08.03" E028 05'32.99"	LARGE	R 7.00	WOHLFAHRT GRAIN FED EGGS	CHIPHILE SUPER SAVE SUPER MARKET, S'GUVE SHOPPING CTR
285	2009/05/06	N 39	S25 32'50.20" E028 06'01.05"	LARGE	R 6.50	NO BRAND	VIVA TAKALANI SUPERMARKET, SOSHANGUVE
286	2009/05/06	N 40	S25 31'24.09" E028 02'08.34"	LARGE	R 7.00	GROMER EGGS	SHOPRITE SUPERMARKET MORULA
287	2009/05/06	N 41	S25 30'46.38" E028 02'40.88"	LARGE	R 6.10	KIEPERSOL GRAIN FED EGGS	O.K GROCER MABOPANE
288	2009/05/07	N 42	S25 28'59.28" E028 02'57.63"	LARGE	R 7.00	THORN TREE GRAIN FED EGGS	MAGGIES SUPERMARKET SOSHANGUVE
289	2009/05/07	N 43	S25 29'42.95" E028 03'20.02"	LARGE	R 7.60	NULAID GRAIN FED EGGS	SCORE SUPERMARKET NUMBAWANE STREET SOSHANGUVE
290	2009/05/07	N 44	S25 29'42.95" E028 03'20.02"	MEDIUM	R 8.00	KIEPERSOL EGGS	ENGENE GARAGE NUMBAWANE
291	2009/05/07	N 45	S25 30'35.47" E028 03'33.12"	MIXED	R 7.50	MAGNUM EGGS	MARYS CONVENIENT STORE NUMBAWANE
292	2009/05/07	N 46	S25 30'09.21" E028 03'47.43"	LARGE	R 8.00	QUANTUM EGGS	TAWABA SUPERMARKET
293	2009/05/07	N 47	S25 30'09.21" E028 03'47.43"	X-LARGE	R 9.00	WELDHAGEN GRAIN FED EGGS	NO NAME
294	2009/05/07	N 48	S25 30'09.21" E028 03'47.43"	LARGE	R 7.00	J.C PIENER EGGS	IKA SUPERMARKET SOSHANGUVE
295	2009/05/07	N 49	S25 31'22.31" E028 04'06.34"	LARGE	R 8.00	FAMILY FAVOURITE EGGS	TSHENOLO CONVENIENT STORE
296	2009/05/07	N 50	S25 31'14.39" E028 03'42.27"	MEDIUM	R 8.50	TOP LAY EGGS	TSHIAMO SUPERMARKET SOSHANGUVE
297	2009/05/07	E 1	S25 44'46.74" E028 11'30.54"	LARGE	R 7.95	NULAID GRAIN FED EGGS	JET MART CHURCH STREET CBD
298	2009/05/07	E 2	S25 44'46.03" E028 11'31.67"	X-LARGE	R 9.00	NULAID GRAIN FED EGGS	SHOPRITE SUPERMARKET CHURCH STREET CBD
299	2009/05/07	E 3	S25 44'45.64" E028 11'47.57"	MIXED	R 8.00	MAGNUM EGGS	EXPRESS SUPERMARKET OPPOSTE RESERVE BANK CHURCH STR
300	2009/05/07	E 4	S25 44'42.75" E028 12'17.76"	LARGE	R 7.00	NULAID GRAIN FED EGGS	SHOPRITE ARCADIA
301	2009/05/07	E 5	S25 44'40.80" E028 13'54.62"	LARGE	R 8.00	WELDHAGEN EGGS	NADA MINI MARKET CHURCH STREET
302	2009/05/07	E 6	S25 44'40.56" E028 13'02.11"	LARGE	R 8.60	QUANTUM EGGS	ETING SUPERMARKET CHURCH STREET
303	2009/05/07	E 7	S25 44'34.40" E028 14'29.13"	LARGE	R 8.50	WELDHAGEN EGGS	ENGENE GARAGE CHURCH STREET BY HATFIELD
304	2009/05/07	E 8	S25 44'32.11" E028 14'47.66"	MEDIUM	R 8.50	WELDHAGEN EGGS	STAR MART BY HATFIELD

305	2009/05/07	E 9	S25 43'56.73" E028 17'03.02"	LARGE	R 8.50	FAIR ACRES	ENGEN GARAGE OPP. SILVERTON VILLAGE SHOPPING CENTRE
306	2009/05/07	E 10	S25 43'58.39" E028 17'02.49"	LARGE	R 8.50	NO BRAND	TIP TOP MEAT SILVERTON VILLAGE SHOPPING CENTRE
307	2009/05/07	E 11	S25 43'57.37" E028 17'44.22"	LARGE	R 7.50	RIETFONTEIN EGGS	SPAR SILVERTON
308	2009/05/07	E 12	S25 43'57.37" E028 17'44.22"	LARGE	R 7.00	WOHLFAHRT GRAIN FED EGGS	SPAR SILVERTON
309	2009/05/07	E 13	S25 43'57.37" E028 17'44.22"	LARGE	R 7.60	SPAR EGGS	SPAR SILVERTON
310	2009/05/07	E 14	S25 43'59.58" E028 17'44.22"	MIXED	R 8.50	MAGNUM EGGS	SHELL GARAGE SILVERTON
311	2009/05/08	E 15	S25 43'59.47" E028 17'58.68"	LARGE	R 8.00	NULAID GRAIN FED EGGS	JET SUPERMARKET SILVERTON
312	2009/05/08	E 16	S25 43'59.73" E028 18'06.30"	LARGE	R 7.00	NULAID GRAIN FED EGGS	SHOPRITE SILVERTON
313	2009/05/08	E 17	S25 44'00.76" E028 18'55.28"	LARGE	R 9.00	VLEIVIEW FARM GRAIN FED EGGS	COUNTRY FRESH VIVA SUPER SAVE
314	2009/05/08	E 18	S25 43'51.80" E028 19'02.74"	LARGE	R 8.70	ZEEKOEGAT EGGS	TOTAL GARAGE SILVERTON
315	2009/05/08	E 19	S25 43'13.94" E028 19'54.26"	LARGE	R 7.00	TOP LAY EGGS	WALTLOO MEAT AND CHICKEN ZASM STREET MAMELODI
316	2009/05/08	E 20	S25 43'08.37" E028 19'53.41"	LARGE	R 7.50	WOHLFAHRT GRAIN FED EGGS	SUPER SEVEN SUPER MARKET ZASM STREET
317	2009/05/08	E 21	S25 43'03.73" E028 20'14.51"	LARGE	R 7.60	NULAID GRAIN FED EGGS	SCORE SUPERMARKET DENNEBOOM, MAMELODI
318	2009/05/08	E 22	S25 43'03.73" E028 20'14.51"	LARGE	R 6.00	WELDHAGEN GRAIN FED EGGS	SEGWAGWA SUPERMARKET, DENNEBOOM, MAMELODI
319	2009/05/08	E 23	S25 43'03.73" E028 20'14.51"	LARGE	R 7.00	NO BRAND	JUICE AND DAIRY DENNEBOOM, MAMELODI
320	2009/05/08	E 24	S25 43'03.73" E028 20'14.51"	LARGE	R 6.50	WELDHAGEN GRAIN FED EGGS	PROBITUM SUPERMARKET, DENNEBOOM, MAMELODI
321	2009/05/08	E 25	S25 43'03.01" E028 22'02.92"	LARGE	R 7.00	ALZU GRAIN FED EGGS	SCORE SUPERMARKET MAMELODI
322	2009/05/08	E 26	S25 43'03.01" E028 22'02.92"	LARGE	R 7.30	TOP LAY GRAIN FED EGGS	SCORE SUPERMARKET MAMELODI
323	2009/05/08	E 27	S25 42'48.60" E028 22'31.38"	MEDIUM	R 6.00	TOP LAY EGGS	KHULANI MEAT AND CHICKEN TSAMAYA AVENUE MAMELODI
324	2009/05/08	E 28	S25 42'49.58" E028 22'29.39"	LARGE	R 7.65	FARM EGGS	TOTAL GARAGE MAMELODI
325	2009/05/08	E 29	S25 42'43.32" E028 22'40.74"	MIXED	R 7.45	FARM FRESH EGGS	LE BAMBA SUPERMARKET MAMELODI
326	2009/05/08	E 30	S25 41'55.64" E028 25'21.24"	LARGE	R 7.50	TOP LAY EGGS	P 'N P MAHUBE CITY MAMELODI
327	2009/05/08	E 31	S25 41'55.64" E028 25'21.24"	LARGE	R 6.50	WELDHAGEN GRAIN FED EGGS	P 'N P MAHUBE CITY MAMELODI
328	2009/05/08	E 32	S25 43'18.16" E028 24'05.57"	LARGE	R 8.00	TOP LAY EGGS	ROAD SIDE SELLER OPPOSITE UNIV. PRET. MAMELODI CAMPUS
329	2009/05/08	E 33	S25 43'19.29" E028 23'21.59"	LARGE	R 6.40	TOP LAY EGGS	B.P GARAGE MAMELODI
330	2009/05/08	E 34	S25 43'16.70" E028 23'07.62"	LARGE	R 7.00	TOP LAY EGGS	ROAD SIDE SELLER MAMELODI
331	2009/05/08	E 35	S25 42'40.96" E028 22'25.51"	LARGE	R 7.50	NO BRAND	OSIZWANI BUTCHERY MAMELODI
332	2009/05/08	E 36	S25 42'40.96" E028 22'25.51"	LARGE	R 7.50	NO BRAND	OSIZWANI SUPERMARKET

333	2009/05/08	E 37	S25 42'53.37" E028 19'58.23"	LARGE	R 7.00	TOP LAY EGGS	SHOPRITE MAMELODI
334	2009/05/08	E 38	S25 42'53.37" E028 19'58.23"	LARGE	R 8.00	TOP LAY GRAIN FED EGGS	SHOPRITE MAMELODI
335	2009/05/08	E 39	S25 42'49.17" E028 20'06.81"	LARGE	R 9.00	NULAID GRAIN FED EGGS	WALTLOO MEAT AND CHICKEN MAMELODI CROSSING
336	2009/05/08	E 40	S25 42'48.69" E028 20'04.83"	LARGE	R 7.00	FAMILY FAVOURITE EGGS	SSS SUPERSTORE MAMELODI CROSSING
337	2009/05/12	W 1	S25 44'47.85" E028 11'13.47"	LARGE	R 8.00	ZEEKOEGAT EGGS	AFRICAN PRIDE SHOP BY CHURCH SQUARE
338	2009/05/12	W 2	S25 44'49.56" E028 10'49.69"	LARGE	R 9.00	QUANTUM EGGS	SMALL SHOP BY PRESIDENTS BUILDING CHUCH STREET
339	2009/05/12	W 3	S25 44'51.85" E028 10'04.21"	MIXED	R 8.00	MAGNUM EGGS	STEVES CAFÉ' ATTERIDGEVILLE ROAD, CHURCH STREET
340	2009/05/12	W 4	S25 44'53.91" E028 09'43.22"	X-LARGE	R 9.00	MORIA EGGS	DELI QUEEN FRUITS AND VEG ATTERIGDEVILLE ROAD
341	2009/05/12	W 5	S25 44'53.93" E028 09'43.19"	MEDIUM	R 8.50	WELDHAGEN EGGS	DELI QUEEN FRUITS AND VEG ATTERIGDEVILLE ROAD
342	2009/05/12	W 6	S25 44'53.93" E028 09'43.19"	X-LARGE	R 9.50	WELDHAGEN EGGS	ATHINA FRUITS AND VEG ATTERIDGEVILLE ROAD
343	2009/05/12	W 7	S25 44'54.90" E028 09'28.08"	MIXED	R 9.95	FARM FRESH EGGS	NAPOLI CAFÉ' ATTERIDGEVILLE ROAD
344	2009/05/12	W 8	S25 44'54.90" E028 09'28.08"	LARGE	R 7.00	WELDHAGEN EGGS	MODERN TRADING CENTER ATTERIDGEVILLE ROAD
345	2009/05/12	W 9	S25 44'56.17" E028 09'09.52"	LARGE	R 9.95	WELDHAGEN EGGS	M.K SUPERMARKET AND FAST FOOD ATTERIDGEVILLE ROAD
346	2009/05/12	W 10	S25 44'56.17" E028 09'09.52"	LARGE	R 9.00	NO BRAND	KHANS SUPERMARKET BY B.P GARAGE ATTERIDGEVILLE ROAD
347	2009/05/12	W 11	S25 44'53.20" E028 08'53.73"	LARGE	R 7.00	GROMER EGGS	SHOPRITE SUPER MARKET QUAGGA SHOPPING CENTER
348	2009/05/12	W 12	S25 44'53.20" E028 08'53.73"	LARGE	R 9.95	WOOLWORTHS FREE RANGE EGGS	WOOLWORTHS SUPERMARKET QUAGGA SHOPPING CENTER
349	2009/05/12	W 13	S25 44'53.20" E028 08'53.73"	LARGE	R 8.89	P 'N P FREE RANGE EGGS	P 'N P QUAGGA SHOPPING CENTER
350	2009/05/12	W 14	S25 44'53.20" E028 08'53.73"	LARGE	R 6.50	P 'N P NO NAME	P 'N P QUAGGA SHOPPING CENTER
351	2009/05/12	W 15	S25 44'53.20" E028 08'53.73"	LARGE	R 8.89	ALZU FREE RANGE EGGS	P 'N P QUAGGA SHOPPING CENTER
352	2009/05/12	W 16	S25 45'01.69" E028 07'56.74"	LARGE	R 8.50	QUANTUM EGGS	SHELL GARAGE ATTERIDGEVILLE ROAD
353	2009/05/12	W 17	S25 45'41.85" E028 05'07.02"	LARGE	R 7.70	QUANTUM EGGS	SHELL GARAGE ATTERIDGEVILLE
354	2009/05/12	W 18	S25 45'54.99" E028 04'54.86"	LARGE	R 8.00	QUANTUM EGGS	AFRICAN SUPPLY SHOP ATTERIDGEVILLE
355	2009/05/12	W 19	S25 45'54.99" E028 04'54.86"	LARGE	R 8.00	J.C PIENAAR EGGS	AFRICAN SUPPLY SHOP ATTERIDGEVILLE
356	2009/05/12	W 20	S25 46'06.29" E028 04'40.14"	LARGE	R 7.00	THORN TREE GRAIN FED EGGS	LOBAY GAP SUPERMARKET ATTERIDGEVILLE
357	2009/05/12	W 21	S25 46'06.29" E028 04'40.14"	LARGE	R 7.00	WELDHAGEN EGGS	LOBAY GAP SUPERMARKET ATTERIDGEVILLE
358	2009/05/12	W 22	S25 46'13.83" E028 05'23.98"	X-LARGE	R 7.70	GROMER EGGS	SHOPRITE SUPER MARKET ATTERIDGEVILLE
359	2009/05/12	W 23	S25 46'13.83" E028 05'23.98"	LARGE	R 8.00	KIEPERSOL EGGS	ROYAL BUTCHERY ATTLYN SHOPPING CENTER ATTERIDGEVILLE
360	2009/05/12	W 24	S25 46'13.83" E028 05'23.98"	LARGE	R 7.50	RIETFONTEIN EGGS	NIZAM SUPERMARKET ATTERIDGEVILLE

361	2009/05/12	W 25	S25 46'17.84" E028 04'18.50"	LARGE	R 7.50	QUANTUM EGGS	NEW SUPERMARKET ATTERIDGEVILLE
362	2009/05/12	W 26	S25 46'17.84" E028 04'18.50"	LARGE	R 7.50	THORN TREE GRAIN FED EGGS	NEW SUPERMARKET ATTERIDGEVILLE
363	2009/05/12	W 27	S25 46'17.84" E028 04'18.50"	LARGE	R 8.00	QUANTUM EGGS	N.V SUPERMARKET ATTERIDGEVILLE
364	2009/05/13	W 28	S25 46'40.82" E028 04'00.53"	LARGE	R 8.00	RIETFONTEIN EGGS	FREEDOM STORE ATTERIDGEVILLE
365	2009/05/13	W 29	S25 46'58.23" E028 03'01.89"	LARGE	R 8.00	FARM FRESH EGGS	TRINCO STORE ATTERIDGEVILLE
366	2009/05/13	W 30	S25 47'08.51" E028 02'36.84"	X-LARGE	R 7.50	J.C PIENAAR EGGS	BAFANA BAFANA SUPERMARKET ATTERIDGEVILLE
367	2009/05/13	W 31	S25 47'08.88" E028 02'33.89"	LARGE	R 7.80	TOP LAY EGGS	MASHIGO SUPERMARKET ATTERIDGEVILLE
368	2009/05/13	W 32	S25 47'14.27" E028 02'18.86"	LARGE	R 7.00	QUANTUM EGGS	TRPICA SUPERMARKET ATTERIDGEVILLE
369	2009/05/13	W 33	S25 47'11.95" E028 02'05.51"	MEDIUM	R 7.50	TOP LAY EGGS	MASUNDA SUPERMARKET ATTERIDGEVILLE
370	2009/05/13	W 34	S25 47'10.98" E028 02'29.27"	LARGE	R 7.00	NO BRAND	ROAD-SIDE SHOP ATTERIDGEVILLE
371	2009/05/13	W 35	S25 47'10.98" E028 02'29.27"	LARGE	R 7.50	QUANTUM EGGS	ROAD-SIDE SHOP ATTERIDGEVILLE
372	2009/05/13	W 36	S25 47'10.98" E028 02'29.27"	LARGE	R 7.50	WELDHAGEN EGGS	ROAD-SIDE SHOP ATTERIDGEVILLE
373	2009/05/13	W 37	S25 47'10.98" E028 02'29.27"	MEDIUM	R 6.50	WELDHAGEN EGGS	ROAD-SIDE SHOP ATTERIDGEVILLE
374	2009/05/13	W 38	S25 47'10.98" E028 02'29.27"	LARGE	R 7.50	RIETFONTEIN EGGS	ROAD-SIDE SHOP ATTERIDGEVILLE
375	2009/05/13	W 39	S25 47'10.98" E028 02'29.27"	LARGE	R 7.00	FAIR ACRES EGGS	ROAD-SIDE SHOP ATTERIDGEVILLE
376	2009/05/13	W 40	S25 47'10.98" E028 02'29.27"	LARGE	R 9.00	ZEEKOEGAT EGGS	ROAD-SIDE SHOP ATTERIDGEVILLE
377	2009/05/13	W 41	S25 47'10.98" E028 02'29.27"	X-LARGE	R 9.00	J.C PIENAAR EGGS	ROAD-SIDE SHOP ATTERIDGEVILLE
378	2009/05/13	W 42	S25 47'10.98" E028 02'29.27"	LARGE	R 8.50	RIETFONTEIN EGGS	ROAD-SIDE SHOP ATTERIDGEVILLE
379	2009/05/13	W 43	S25 47'10.98" E028 02'29.27"	LARGE	R 7.00	QUANTUM EGGS	ROAD-SIDE SHOP ATTERIDGEVILLE
380	2009/05/13	W 44	S25 47'10.98" E028 02'29.27"	LARGE	R 8.00	WELDHAGEN EGGS	ROAD-SIDE SHOP ATTERIDGEVILLE
381	2009/05/13	W 45	S25 47'10.98" E028 02'29.27"	LARGE	R 7.50	NO BRAND	ROAD-SIDE SHOP ATTERIDGEVILLE
382	2009/05/13	SS 1	S25 43'05.26" E028 13'05.49"	LARGE	R 6.50	WELDHAGEN GRAIN FED EGGS	P 'N P GEZINA
383	2009/05/13	SS 2	S25 43'05.26" E028 13'05.49"	LARGE	R 6.50	P 'N P NO NAME	P 'N P GEZINA
384	2009/05/13	SS 3	S25 43'05.26" E028 13'05.49"	LARGE	R 8.99	WELDHAGEN FREE RANGE EGGS	P 'N P GEZINA
385	2009/05/13	SS 4	S25 43'05.26" E028 13'05.49"	LARGE	R 7.00	TOP LAY GRAIN FED EGGS	P 'N P GEZINA
386	2009/05/13	SS 5	S25 43'05.26" E028 13'05.49"	LARGE	R 8.00	ALZU FREE RANGE EGGS	P 'N P GEZINA
387	2009/05/13	SS 6	S25 43'05.26" E028 13'05.49"	LARGE	R 7.00	WOHLFAHRT GRAIN FED EGGS	FRUITS AND VEG GEZINA
388	2009/05/13	SS 7	S25 42'33.26" E028 11'06.67"	LARGE	R 7.00	FARM HOUSE GRAIN FED EGGS	SPAR SUPERMARKET PARKTOWN

389	2009/05/13	SS 8	S25 42'33.26" E028 11'06.67"	LARGE	R 7.50	SPAR EGGS	SPAR SUPERMARKET PARKTOWN
390	2009/05/13	SS 9	S25 41'55.87" E028 11'00.63"	LARGE	R 10.00	CHECKERS CHOICE FREE RANGE	CHECKERS SUPERMARKET MAYVILLE
391	2009/05/13	SS 10	S25 41'55.87" E028 11'00.63"	LARGE	R 7.40	PASTEURISED SAFE EGGS	CHECKERS SUPERMARKET MAYVILLE
392	2009/05/13	SS 11	S25 41'55.87" E028 11'00.63"	LARGE	R 7.40	FARM HOUSE GRAIN FED EGGS	CHECKERS SUPERMARKET MAYVILLE
393	2009/05/13	SS 12	S25 40'33.25" E028 10'22.48"	LARGE	R 6.60	GROMER EGGS	SHOPRITE PRETORIA NORTH
394	2009/05/13	SS 13	S25 40'33.25" E028 10'22.48"	LARGE	R 9.70	GROMER FREE RANGE EGGS	SHOPRITE PRETORIA NORTH
395	2009/05/13	SS 14	S25 28'42.39" E028 06'00.76"	LARGE	R 7.50	SPAR EGGS	SPAR SUPERMARKET BLOCK FF SOSHANGUVE
396	2009/05/13	SS 15	S25 40'18.04" E028 06'41.37"	LARGE	R 8.00	NULAID GRAIN FED EGGS	P 'N P WONER PARK
397	2009/05/14	SS 16	S25 40'18.04" E028 06'41.37"	LARGE	R 8.00	ALZU FREE RANGE EGGS	P 'N P WONER PARK
398	2009/05/14	SS 17	S25 40'18.04" E028 06'41.37"	LARGE	R 10.00	NULAID FREE RANGE EGGS	P 'N P WONER PARK
399	2009/05/14	SS 18	S25 40'18.04" E028 06'41.37"	LARGE	R 9.00	P 'N P FREE RANGE EGGS	P 'N P WONER PARK
400	2009/05/14	SS 19	S25 45'13.28" E028 12'30.34"	LARGE	R 7.00	GROMER EGGS	SHOPRITE SUNNYSIDE
401	2009/05/14	SS 20	S25 45'04.64" E028 12'11.72"	X-LARGE	R 14.00	WOOLWORTHS FREE RANGE EGGS	WOOLWORTHS SUPERMARKET SUNNY PARK
402	2009/05/14	SS 21	S25 47'04.52" E028 16'35.50"	LARGE	R 10.00	WOOLWORTHS FREE RANGE EGGS	WOOLWORTHS SUPERMARKET MENLYN PARK
403	2009/05/14	SS 22	S25 47'04.52" E028 16'35.50"	MIXED	R 18.00	WOOLWORTHS ORGANIC EGGS	WOOLWORTHS SUPERMARKET MENLYN PARK
404	2009/05/14	SS 23	S25 46'18.07" E028 14'04.62"	LARGE	R 7.50	EGGBERT EGGS	CHECKERS SUPERMARKET BROOKLYN
405	2009/05/14	SS 24	S25 46'18.07" E028 14'04.62"	LARGE	R 10.00	CHECKERS CHOICE FREE RANGE	CHECKERS SUPERMARKET BROOKLYN
406	2009/05/14	SS 25	S25 46'18.07" E028 14'04.62"	LARGE	R 7.50	PASTEURISED SAFE EGGS	CHECKERS SUPERMARKET BROOKLYN
407	2009/05/14	SS 26	S25 43'57.71" E028 17'44.15"	LARGE	R 7.60	SPAR EGGS	SPAR SILVERTON
408	2009/05/14	SS 27	S25 43'57.71" E028 17'44.15"	LARGE	R 7.00	WOHLFAHRT GRAIN FED EGGS	SPAR SILVERTON
409	2009/05/14	SS 28	S25 43'57.71" E028 17'44.15"	LARGE	R 7.40	RIETFONTEIN EGGS	SPAR SILVERTON
410	2009/05/14	SS 29	S25 40'43.25" E028 14'24.56"	LARGE	R 7.30	FAIR ACRES EGGS	P 'N P MONTANA
411	2009/05/14	SS 30	S25 40'43.25" E028 14'24.56"	MIXED	R 11.00	P 'N P OMEGA-3 EGGS	P 'N P MONTANA
412	2009/05/14	SS 31	S25 40'43.25" E028 14'24.56"	LARGE	R 10.00	P 'N P ALL GRAIN FED EGGS	P 'N P MONTANA
413	2009/05/14	SS 32	S25 40'46.03" E028 14'24.38"	LARGE	R 8.00	FARM HOUSE GRAIN FED EGGS	SPAR MONTANA
414	2009/05/14	SS 33	S25 40'46.03" E028 14'24.38"	LARGE	R 10.00	BOSCHVELD FREE RANGE EGGS	SPAR MONTANA
415	2009/05/14	SS 34	S25 40'46.03" E028 14'24.38"	LARGE	R 7.00	THORN TREE GRAIN FED EGGS	SPAR MONTANA
416	2009/05/14	SS 35	S25 40'46.03" E028 14'24.38"	LARGE	R 9.40	THORN TREE FREE RANGE EGGS	SPAR MONTANA

417	2009/05/14	SS 36	S25 40'46.03" E028 14'24.38"	LARGE	R 8.00	SPAR EGGS	SPAR MONTANA
418	2009/05/14	SS 37	S25 40'46.03" E028 14'24.38"	X-LARGE	R 8.00	FARM HOUSE FREE RANGE EGGS	SPAR MONTANA
419	2009/05/14	SS 38	S25 44'39.94" E028 14'45.18"	LARGE	R 8.00	SPAR EGGS	SPAR HATFIELD
420	2009/05/14	SS 39	S25 45'55.98" E028 17'55.05"	LARGE	R 13.00	WOOLWORTHS FREE RANGE EGGS	WOOLWORTHS SUPERMARKET GIFT ACRES
421	2009/05/14	SS 40	S25 45'55.98" E028 17'55.05"	LARGE	R 18.00	WOOLWORTHS ORGANIC EGGS	WOOLWORTHS SUPERMARKET GIFT ACRES
422	2009/05/14	SS 41	S25 40'49.31" E028 11'36.93"	LARGE	R 9.90	FAIR ACRES EGGS	MAKRO SUPER STORE WONDERBOOM JUNCTION