Food Safety Risk Posed to Consumers of Table Eggs from Layer Farms in Gauteng Province, South Africa: Prevalence of Enteropathogens, Antimicrobial Residues and Antimicrobial Resistant Bacteria

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ABSTRACT

The cross-sectional study was conducted on 34 large and 5 small layer farms operating in Gauteng province, South Africa to determine the prevalence of selected enteropathogens, resistant pathogens and antimicrobial residues in table eggs collected from the farms. Eggs were collected from all farms based on the daily egg production per farm and the egg shells and contents were tested for the presence of *Salmonella* spp., *Escherichia coli*, *E. coli* O17 and *Campylobacter* spp. using standard methods. The resistance of the bacterial isolates to eight antimicrobial agents was determined using the disc diffusion method. Antimicrobial residues were detected in table eggs using the Microbiological Inhibition Test, Enzyme-linked Immunosorbent Assay (ELISA) and High-performance Liquid Chromatography (HPLC). A questionnaire was administered on each farm to determine the occurrence of risk factors for egg contamination by bacteria and antimicrobial residues. The farm prevalence of *Salmonella* spp., *E. coli*, *E. coli* O157 and *Campylobacter* spp. in table eggs was 7.7%, 48.7%, 0.0% and 0.0% respectively. *S*. Enteritidis and *S*. Ivory were recovered from egg shells on only large farms. Nineteen (48.7%) and 2 (5.1%) of egg shells and egg contents respectively were positive for *E. coli*. Overall, 71.4% of 49 *E. coli* isolates exhibited resistance to one or more antimicrobial agents. The prevalence of resistance was high to doxycycline (53.1%) and oxy-tetracycline (51.0%). The farm prevalence and egg content prevalence of antimicrobial residues was 2.6% (1/39) and 0.5% (1/196) respectively. The residue-positive sample contained Sulfonamides at 79 ppb, and Oxytetracycline at 106 ppb which is lower than the set MRL of 200 ppb for total Tetracyclines. The antimicrobial resistance exhibited by *E. coli* isolates, the isolation of *Salmonella* spp. from eggs and the occurrence of antimicrobial residues in egg content pose food safety and therapeutic threats to consumers.

Keywords Layer farms, Table eggs, Enteropathogens, Antimicrobial residues, Resistance, South Africa

Table eggs are used in the preparation of numerous commercial and home-made products and several egg-borne epidemics of salmonellosis have been reported in humans *(10, 14).* Since eggs could be contaminated or infected horizontally by pathogens such as *Salmonella* in the environment where they are laid or vertically through trans-ovarian transfer they are an important potential source of pathogens *(10, 13, 52).* To date, of all bacterial pathogens, egg-borne *Salmonella*, particularly *S*. Enteritidis, has been the most important cause of outbreaks of food-borne diseases *(10, 27).* Other enteric pathogens, such as *Campylobacter* spp., particularly *C. jejuni*, *Listeria* spp. and *Escherichia coli* have been isolated from eggs, egg products or egg washing and processing facilities *(3, 53).* Infection of parent stock of laying birds and hatching eggs with bacterial pathogens, as well as bacterial contamination of areas where eggs are laid, are therefore important sources of contaminating egg shells and contents. Therefore, the food safety concerns raised by the consumption of contaminated table eggs cannot be ignored.

In the livestock industry, antimicrobial agents are used as feed additives to promote growth, in prophylaxis and in therapy *(19, 38, 50).* Repeated and uncontrolled use of antimicrobial agents, particularly in therapy, has the potential to lead to the development of resistance amongst pathogens *(21, 41).* This has implications for therapeutic failures in poultry with associated economic losses due to mortalities. Furthermore, the use of antimicrobial agents in food animals leads to the excretion of their metabolites in body fluids as well as their accumulation in body tissues or products such as eggs *(29, 42).* Antimicrobial residues are known to contaminate meats, milk and eggs because of the livestock farmers' failure to observe withdrawal periods stipulated for their antimicrobial agents used on the animals prior to slaughter or allowing the

84 products such as milk or eggs to be sold to the unsuspecting public or consumer. It has been
85 established that antimicrobial residues in foods may cause side effects such as direct toxicity,
86 elicit allergic response in consumers, and may also lead to the development of antimicrobial
87 resistance among bacterial pathogens *(11, 57*).

88 In several developing countries, there were reports of unrestricted access of livestock
89 farmers to antimicrobial agents and failure to adhere to stipulated withdrawal periods following
90 administration of these agents to food producing animals (*42, 43, 50*). The occurrence of
91 antimicrobial resistance (AMR) in bacterial pathogens associated with foods has food safety and
92 public health significance. This is because the resistant pathogen could be transmitted, to
93 humans through handling and consumption of such food products, such as table eggs,
94 particularly if consumed raw or under-cooked. There is therefore the risk of therapeutic failures
95 if humans are infected with antimicrobial resistant bacteria (*54, 56).* Antimicrobial residues have
96 been detected in table eggs sampled on layer farms or at retail outlets in Sudan *(51),* Nigeria *(20,*
97 *39),* Tanzania *(43),* Uganda *(48),* and Trinidad and Tobago *(1).*

98 Considering the public health risk posed to consumers by antimicrobial residues in eggs, to
99 reduce the risk of contamination of eggs by antimicrobial residues, maximum residue levels have
100 been established for several antimicrobial agents in foods *(16, 57).* However, the maximum
101 residue levels (MRLs) stipulated for table egg contents destined for human consumption may
102 depend on the regulations enforced in different countries *(26).*

103 In South Africa, information on the microbiology and characteristics of pathogens from
104 poultry farms is scarce. To date there are no available published reports on the microbial quality
105 (microbial and antimicrobial residues) of table eggs produced by layers farms in Gauteng
106 province, South Africa sold to the consumers. The study therefore determined the prevalence

and characteristics of *Salmonella* spp., *E. coli*, *E. coli* O157, *Campylobacter* spp. in table eggs from layer farms across Gauteng province. The study also determined the prevalence of antimicrobial residues in egg contents. Finally, the prevalence of enteropathogens, antimicrobial resistance and antimicrobial residues were related to risk factors on the farms.

MATERIALS AND METHODS

Layer farms and destination of eggs in Gauteng province. In Gauteng province, South Africa, production systems for laying hens are based primarily on the battery cage system which constitutes most of the farms operating on large commercial scale and small farms under the Developing Poultry Farmers Organization (DPFO). A few farms used a combination of battery cage, free range and with deep litter systems. Eggs from the layer farms are sold to supermarkets through the packing stations or to the roadside informal markets.

Study target population and area. The study was conducted in all layer farms in Gauteng Province, South Africa, with an estimated total population of 6 million-layer birds in 2014 *(47)*.

Study design, sources of samples and sample size. Table eggs from layer farms (large and small) in Gauteng Province in operation during the study period were sampled. Eggs from these farms reached the consumers directly through their sale to the small retailers (formal and informal) or indirectly through the packing stations. The identification and locations of the layer farms were obtained from the database supplied by the South African Poultry Association (SAPA) Statistical Office.

All layer farmers were initially apprised of the study to solicit their support and participation. Approximately 48 h prior to farm visits, the farmers were notified by the technical staff members of the Veterinary Public Health unit of the Department of Agriculture, Forestry and Fisheries (DAFF) who were responsible for sample collection for the study.

On selected layer farms, a standardized questionnaire was used to elicit information on housing types, management practices, use of antimicrobial agents, egg production, and other risk factors for contamination of eggs by bacterial pathogens and antimicrobial residues, was administered to each farmer on the 34 large (20,001 to >300,000 hens) and 5 small (1 to 20,000 hens) farms visited.

Sample size and collection of samples. For sample size determination, the study design used a convenience sampling approach of collecting egg samples from all layer farms in operation during the study period, using a farm house as a unit and collecting 10 eggs per house. This constituted a composite sample. A maximum of 10 houses were sampled from each farm using randomly generated numbers to select the houses on farms that had more than 10 houses. To ensure uniformity in sample collection, a standard operating procedure (SOP) was established for detailed administration of the questionnaires, observations to be made in the poultry houses (for example, the presence of feral birds, rodent droppings), random collection of eggs from houses but representative of the lay-out of cages in the house, aseptic collection (using sterile gloves for each house) of eggs into sterile crates. Eggs were transported to the laboratory within 2-4 h of collection and processed within 24 h of arrival and storage at room temperature. Overall, a total of 39 layer farms operational in Gauteng province were sampled for the study.

Processing of eggs in the laboratory. The isolation, identification and enumeration of bacteria were determined in egg shells and egg contents.

For the isolation of the three microorganisms (*Campylobacter* spp., *Salmonella* spp. and *E. coli*) from egg shell, a crate of 10 eggs from each poultry house constituted a composite sample. A moist sterile swab, dipped in buffered peptone water (BPW) (Oxoid Ltd., U.K.) was applied on the shell surface of each egg. The 10 swabs were dipped in 9 ml of BPW and mixed thoroughly using a vortex mixer and then incubated at 37^{o}C overnight as non-selective enrichment**.**

For the isolation of *E. coli,* the enriched culture growth was used to inoculate MacConkey agar (Oxoid Ltd., U.K.) plates which were incubated at 37^{o}C for 24 h after which isolates resembling *E. coli* were sub-cultured on Columbia blood agar plates and incubated for another 24 h at 37 ^{o} C. Identification was then made by using standard methods (*7*).

To isolate *Salmonella* spp**.,** 100 µl of enriched BPW was inoculated into 10 ml of Rappaport-Vassiliadis soy (RVS) broth (Oxoid Ltd., U.K.) for selective enrichment and incubated overnight at 41.5^{o}C for 24 h *(7).* After 24 h, xylose lysine deoxycholate (XLD) (Oxoid Ltd., U.K.) was used as selective agar plates and were inoculated with the selective enrichment broth and streak for isolation. The agar plates were incubated at 37^{o}C for 24 h. Isolates that showed phenotypic characteristics suggestive of *Salmonella* spp. were identified using the API 10 S identification system.

For the isolation of *Campylobacter* spp., the non-selective enriched BPW growth was inoculated onto *Campylobacter* blood-free containing Charcoal cefoperazone deoxycholate agar (CCDA) supplement (Oxoid Ltd., U.K.) and incubated at 42^{0}C for 3-5 days under micro-aerophilic conditions using an anaerobic jar (Oxoid Ltd., U.K.) filled with a gas mixture of 10 %

carbon dioxide, 6% oxygen and 84% nitrogen (*3, 7, 28*). Suspect *Campylobacter* isolates were then sub-cultured on Columbia blood agar plates and the above process repeated. A Gram stain and oxidase test were done on the suspect *Campylobacter* isolates. Identification of *Campylobacter* spp. was conducted using standard methods *(7, 37).*

To process egg contents, the pointed end of each egg in the pool was sterilized with 70% ethanol and left to air dry. Using sterile a pair of forceps the shell was broken open and contents poured into a 1-litre Schott bottle and thoroughly mixed to obtain homogenous egg content for each composite sample of 10 eggs.

To isolate *E. coli,* 100 µl of the egg mixture was spread on the MacConkey agar using a hockey stick, then incubated for 24 h at 37^{o}C and processed as described above for egg shell samples. To quantify the number of *E. coli* in each composite sample of egg content, 1 ml of egg content was added to 9 ml of sterile saline (10-fold dilution) and further serial 10-fold dilutions were made and plated in duplicate on MacConkey agar. The plates were incubated overnight at 37^{o}C after which the colonies were counted and expressed as colony forming units of *E. coli* per egg content.

To recover *Salmonella* spp., 10 ml of egg content mixture was used to inoculate 90 ml of BPW which was incubated aerobically overnight at 37^{0}C. One hundred microliter (100 µl) of egg content was inoculated into 10 ml Rappaport-Vassiliadis soy (RVS) peptone and the same procedure described earlier for the isolation of *Salmonella* spp. from egg shells was used.

For the isolation of *Campylobacter* spp., the egg contents were used to inoculate *Campylobacter* blood-free agar and the same procedure described above for the isolation of *Campylobacter* spp. from egg shell samples was used.

Characterization of the *E. coli* and *Salmonella* isolates. The resistance of the isolates to selected antimicrobial agents and their serotypes were determined using phenotypic methods.

To determine the resistance of bacteria to antimicrobial agents, the selection of antimicrobial agents used in the current study was based of those commonly available and used in the poultry industry in South Africa. The disc diffusion method according to the CLSI *(14)* guidelines was used to determine the resistance of isolates of *Salmonella* and *E. coli* to the following antimicrobial agents: Amoxicillin (30 mcg), Enrofloxacin (5 mcg), Fosfomycin (50 mcg), Fosfomycin plus T (40 mcg), Norfloxacin (10 mcg), Sulphamethoxazole/Trimethoprim (25 mcg), Doxycycline (30 mcg) and Oxytetracycline (30 mcg). Interpretation of test results was as stipulated by the CLSI *(15).*

Serotyping of pathogens. All isolates biochemically identified as *Salmonella* spp. were confirmed and serotyped at Onderstepoort Veterinary Research (OVR), the national centre for serotyping *Salmonella* spp.

The presence of O157 *E. coli* strains amongst the *E. coli* isolates was determined using the latex agglutination test (Oxoid Ltd., UK).

Assay for antibiotic residue. Reagent kits for ELISA kits (R-Biopharm, AG, Darmstadt, Germany), and the Four Plate Microbiological Inhibition Test were used in the study and the four-plate test media was prepared as described by Bogaerts and Wolf (*9).*

Screening for antimicrobial residues. For each pool of ten egg contents, 3 g was homogenized and centrifuged for 10 minutes and 100 µl each of the sample supernatant and

220 negative control, poured onto the four-plate media. The plate was incubated for 20 minutes at
221 room temperature, then incubated for 3 h at 65^{o}C.

222 Screening for antimicrobial residues in egg contents by enzyme-linked immunosorbent
223 assay (ELISA), 1 g of each pool was weighed into a centrifuge tube, and 2 ml methanol was
224 added, and vortexed for 30 seconds. The samples were centrifuged for 10 minutes at 4000 rpm,
225 1.5 ml of the supernatant was transferred into clean tubes and evaporated to dryness under a
226 gentle stream of nitrogen gas. The residue was dissolved in 0.5 ml sample dilution buffer, to
227 which 1 ml of n-hexane was added and vortexed for 10 seconds followed by centrifugation for
228 10 minutes at 4000 rpm. The lower phase of the centrifuged sample, 50 µl, was used in the
229 ELISA test. To perform the ELISA, 50 µl each of the standard or sample were added to
230 duplicate wells followed by the addition of 50 µl of enzyme conjugate solution to each well. The
231 antibody, 50 µl, of antibody solution was added to each well and mixed gently. The plate was
232 incubated for 1 h at room temperature. The reagents were decanted, and the plate was washed 3
233 times with 250 µl washing buffer. Thereafter, 100 µl of the substrate chromogen was added to
234 each well, mixed gently and incubated for 15 minutes at room temperature in the dark. The
235 reaction was stopped with 100 µl of the stop solution to each well. The absorbance was measured
236 at 450 nm within 30 minutes after addition of stop solution and the samples were qualitatively
237 classified as positive or negative for residues as recommended by the kit manufacturer. The
238 ELISA was used to quantify the concentration of only sulfonamides in the sample positive for
239 the residue by MIT.

240

241 **Quantitative antimicrobial residue analysis.** The chromatographic system used was an
242 HP 1200 Series (Agilent Technologies, USA) which consisted solvent degasser, auto-sampler

243 with 100 µl loop, quaternary pump, column thermostat, fluorescence detector (FLD) and diode
244 array detector (DAD) system. The chromatographic column used was a C18 (150 mm x 4.6 mm,
245 5 µm). Two SPE cartridges, Oasis HLB (60 mg, 3 ml) of Waters (Milford, MA, USA) and
246 BondElut C18 (500 mg, 3 ml) from Agilent Technologies (USA) were used, mounted on a SPE
247 manifold (J.T. Baker, USA) and a vacuum pump was used. The HPLC was used to quantify the
248 concentration of tetracycline only, being one of the most commonly used antimicrobial agents in
249 livestock in South Africa.
250
251 **Statistical analysis.** The prevalence of microorganisms on egg shells and/or in egg
252 contents was compared for the different types of layer farms (large and small), the management
253 practices and other risk factors were related to the frequency of isolation of selected pathogens
254 by the Chi-square tests using SPSS version 10 (SPSS Inc., Chicago, IL, USA). All statistical
255 analyses were two-tailed and interpreted at the 5% level of significance. A similar analysis was
256 done for the prevalence of resistance to antimicrobial agents amongst the bacterial isolates and
257 the prevalence of antimicrobial residues in egg contents. For data, other than frequency
258 comparison, the Analysis of Variance (ANOVA) was used to determine the existence of
259 significant differences amongst values.
260 **RESULTS**
261
262 **Questionnaire survey findings.** The risk factors (farm size, housing type, pest infestation
263 and use of antimicrobial agents) for contamination of table eggs by enteropathogens and
264 antimicrobial residues, and the number of table eggs collected are shown in Table 1. A total of
265 all 39 operating layer farms in Gauteng province, comprising 34 large and 5 small (DPFO) were
266 sampled with median (range) of hens-in-lay for the 39 farms was 47,149 (964 – 538,656). The

predominant housing type was the battery cage system, 84.6% (33/39); Rodent and feral bird infestations were experienced by 21 (53.8%) and 20 (51.3%) farms respectively. For the 39 layer farms, 2 (5.1%), 3 (7.7%), 7 (17.9%) and 7 (17.9%) used antimicrobial agents as growth promoters, for prophylaxis, for treatment and observed withdrawal periods after use respectively. Antimicrobial agents are used as growth promoters and for treatment only on the large farms. For the study, a total of 196 crates of pooled eggs, comprising 10 eggs per crate i.e. 1960 eggs were processed consisting of 1860 and 100 eggs from the large and small farms respectively.

Prevalence of enteropathogens on egg shells and contents and antimicrobial residues in egg contents. The prevalence of enteropathogens in table eggs is displayed in Table 2. The overall farm prevalence of enteropathogens in table eggs (shells and contents) was 7.7% (3 of 39) and 48.7% (19 of 39) for *Salmonella* spp. and *E. coli* respectively with all positive samples originating from large farms only. Two (5.1%) of the 39 farms had egg contents positive for *E. coli*. All the samples were negative for *E. coli* O157 and *Campylobacter* spp.

The egg prevalence for *Salmonella* spp. was 2.0% (4 of 196 pooled eggs), all originated from egg shells and from the large farms. The serotypes detected were *S*. Enteritidis 9,12:g,m:- (2 farms) and *S*. Ivory (1 farm).

The prevalence of *E. coli* in pooled egg shells and egg content was 49.9% (96 of 196) and 2.0% (2/196) respectively. The two content-positive eggs were also shell-positive. Of the 2 egg-content positive samples, the total aerobic plate counts exceeded 3,000 colony forming units per ml.

289 **Odds ratio for contamination of egg shells by *E. coli* and *Salmonella* spp.** The odds
290 ratio, i.e. the risk factors or probability of egg shells from farms being contaminated by *E. coli*
291 were as follows: farms that used antimicrobial agents for treatment (3.04), experienced pest
292 (insects, flies, wasps, etc.) infestation (3.00), encountered rodent problem (2.10), experienced
293 feral bird problem (1.57), used antimicrobial agents as growth promoters (1.06) and used
294 antimicrobial agents for prophylaxis (1.00).

295 The odds ratio for egg shells from farms being contaminated by *Salmonella* spp. was as
296 follows: farms that used antimicrobial agents for treatment (2.42) and experienced rodent
297 problem (1.79).

298

299 **Resistance of *E. coli* and *Salmonella* spp. isolates to antimicrobial agents.** Overall, the
300 farm prevalence for resistant *E. coli* was 47.1% (16/34) and 0.0% (0/5) for large and small farms
301 respectively (Table 3). Of the 49 isolates of *E. coli* recovered from table eggs (shells and
302 contents), 35 (71.4%) exhibited resistance to one or more antimicrobial agents comprising a
303 frequency of resistance for egg shells and egg contents being 77.8% (35/45) and 0.0% (0/4)
304 respectively. Of the 49 isolates of *E. coli* tested, the frequency of resistance was 53.1%, 51.0%,
305 38.8%, 24.5%, 6.1% and 6.1% to doxycycline, oxy-tetracycline, sulphamethoxazole-
306 trimethoprim (SXT), amoxicillin, enrofloxacin and norfloxacin respectively. The differences
307 were statistically significant ($P<0.0001$). All (100.0%) the *E. coli* isolates were susceptible to
308 fosfomycin and fosfomycin plus Tylosin. For the 35 *E. coli* resistant isolates, 28 (80.0%) and 24
309 (68.8%) exhibited resistance to oxy-tetracycline and doxycycline respectively.

The four isolates of *E. coli* recovered from egg contents were sensitive to the eight antimicrobial agents tested.

Of the four isolates of *Salmonella* spp. recovered, 2 (50.0%) exhibited resistance to doxycycline only, both being *S.* Enteritidis isolates.

A total of 12 resistance patterns were exhibited by the 35 resistant isolates of *E. coli* (Table 4). The predominant patterns were sulphamethoxazole/trimethoprim-doxycycline-oxytetracycline, 9 (25.7%); doxycycline–oxytetracycline, 6 (17.1%); amoxicillin-sulphamethoxazole/trimethoprim-doxycycline-oxytetracycline, 5 (14.3%); amoxicillin, 4 (11.4%) and oxytetracycline, 4 (11.4%). Overall, 27 (77.1%) of the 35 resistant isolates were multi-drug resistant with 17 (48.6%) and 10 (28.6%) resistant to 3 or more and 2 or more antimicrobial agents respectively.

Odds ratio for contamination of egg shells and contents by resistant *E. coli.* The odds ratios for the contamination of egg shells from farms by resistant *E. coli* strains were as follows: used antimicrobial agents for treatment (4.55), used antimicrobial agents as growth promoters (1.50) and used antimicrobial agents for prophylaxis (1.43).

For egg contents, the odds ratio for the isolation of resistant strains of *E. coli* was 5.0 for farms that used antimicrobial agents for treatment.

Detection of antimicrobial residues in table egg contents. The farm prevalence for antimicrobial residues in table eggs was 2.6% (1/39) while the egg prevalence was 0.5% (1 of

196 crates), with the pooled egg contents being positive for 5 antimicrobial residues, namely quinolone, macrolide, aminoglycoside, tetracycline and beta lactam based on the broad-spectrum veterinary drug residue microbiological inhibition test (MIT). The sample that was positive for Tetracyclines on the MIT was confirmed by the HPLC Tetracycline Method to have a concentration of 106 ppb for Oxytetracycline. However, Sulfonamide concentration (79 ppb) in the positive sample by ELISA was used as the final result. On the antimicrobial residue-positive farm, the composite egg content originated from 1 (14.3%) pool of 7 pools of egg contents (i.e. 1 pool of 10 eggs from a poultry house out of 7 pools of 70 egg contents). The antimicrobial residue-positive farm was a large farm with a daily egg production of 81,450, used a battery cage system, reported frequent infestation by feral bird, indicated the use of antimicrobial agents for treatment only under the supervision of a veterinarian and observed withdrawal periods after treatment.

DISCUSSION

The farm prevalence for *Salmonella* spp. in table eggs in the current study was 7.7% with only egg shells contaminated by the pathogen. This is considerably lower than the 100.0% reported by Indar et al. *(30)* and the 40% reported for 35 farms in three Caribbean countries with a range of 26.1% for small farms to 77.8% for large farms *(4)*. Chemaly et al. *(12)* has also reported a farm prevalence of 39.3% for *Salmonella* spp. on table eggs. Similarly, Adesiyun et al. *(3)* reported that 13% of layer farms studies had table eggs positive for *Salmonella* spp.

Regarding the frequency of isolation of *Salmonella* spp. from egg shells in the current study, 2.0% of the pooled egg shells (4 of 196), is slightly higher than the frequency of 1.05%

354 reported by Chemaly et al. *(12)* for layer farms in France where a high holding capacity (>30,000
355 laying hens) was identified as a factor, the 0.07 to 0.4% in Canada reported by Poppe et al. *(45)*
356 and the failure (0.0%) to isolate the pathogen from egg shells in Australia *(13)*, St. Lucia and
357 Grenada *(4)*, Ethiopia *(31)* and Brazil *(35)*. Our finding in the current study, however, is low
358 compared with the isolation rate of 3.5% for *Salmonella* spp. in egg shells in Thailand *(46*), 3.6%
359 in the USA *(33)* and reports of three studies in Trinidad which documented isolation rates for
360 *Salmonella* spp. in pooled egg shells of 3.8% *(3),* 4.7% *(30)* and 12.5% *(4)* and the 34% reported
361 in a study conducted in Spain *(23).*

362 In this study, no *Salmonella* spp. was isolated from 196 pooled egg contents studied. The
363 finding agrees with other reports where the pathogen was not recovered from egg contents, in
364 Australia *(13),* St. Lucia and Grenada in the Caribbean *(4),* Spain *(23)* and in the USA *(33).*
365 However, *Salmonella* spp. have been isolated from the egg contents of table eggs by others at
366 different rates such as 0.67% in China *(24),* 1.2% in Thailand *(46)* and 1.2%, 7.6% and 12.5% in
367 Trinidad *(3, 4, 30).*

368 It was not a surprise to have detected in the current study that the risk factors, assessed by
369 the odds ratio, most important for the contamination of egg shells with *Salmonella* spp. were the
370 use of antimicrobial agents for treatment and infestation by rodent. Rodent infestations have been
371 associated with contamination of eggs by *Salmonella* spp. *(22, 55)* and it was significant that all
372 *Salmonella*-positive eggs in our study originated from the large farms. Denagamage et al. (*17),*
373 in a systematic review of risk factors associated with *Salmonella* in laying hen farms, reported
374 that risk factors associated with *S.* Enteritidis infection in laying hens were flock size, housing
375 system, and farms with hens of different ages. As a summary, this systematic review

demonstrated that *Salmonella* contamination of laying hen flocks and shell eggs in layer production systems is multifactorial.

Although only 4 isolates of *Salmonella* spp. were recovered from eggs, all from shells in the current study and it is important to have detected that 75% of the isolates were *S.* Enteritidis. Other studies have reported similar predominance of the serotypes being associated with chicken eggs sampled on layer farms. In several studies on farm eggs in Trinidad, *S.* Enteritidis was the predominant serotype where Indar et al. *(30)* reported that 0.8% of egg contents were positive for *S*. Enteritidis and suggested trans-ovarial transmission. Adesiyun et al. *(3)* also found that *S.* Enteritidis constituted 58.3% (14/24) isolates of *Salmonella* spp. recovered from table eggs. Lestari et al. *(36)* also reported the predominance of serovars Enteritidis, Kentucky and Hadar in their study. Of food safety importance is the fact that *S.* Enteritidis has been reported to be the predominant *Salmonella* serotype in egg-associated human salmonellosis except for the countries of Oceania where *S.* Typhimurium is most prevalent *(14).* Similarly, the predominance of other serovars of *Salmonella* from table eggs have been reported by others. Saitanu et al. *(46)* reported that of the 134 strains of *Salmonella* from table eggs tested, 24 serotypes were confirmed and *S.* Cerro (4.8%), *S.* Amsterdam (4.3%) and *S.* Typhimurium (1.3%) were predominantly encountered while only two samples were contaminated with *S.* Enteritidis. Also, in a study conducted in the Caribbean region, Adesiyun et al. *(4)* reported that three different serotypes of *Salmonella* (S. Mbandaka 6,7: z10:e,n,z15, Polyvalent A-negative *Salmonella* and *S.* Montevideo 6,7:g,m,s:) were mostly isolated from freshly laid eggs on layer farms and that *S.* Enteritidis represented only 2.9% of the *Salmonella* serotypes isolated. The authors suggested that there may have been a changing pattern in the contamination of table eggs by *Salmonella* serovars.

Based on the rather low frequency of isolation of *Salmonella* spp. from egg shells (2.0%) and failure to detect the pathogen in the egg contents of table eggs in Gauteng province the risk of egg-borne salmonellosis in human consumers is therefore extremely low.

In our study, the farm prevalence of *E. coli* in table eggs (shells and/contents) was 48.7% and *E. coli* was isolated from 23.0% of the 196 pooled egg shells. This is lower than the prevalence of 37.0% reported by Adesiyun et al. *(3)* for table eggs in Trinidad but higher than the 11.9% which was reported in the USA *(33).*

The important risk factors for contamination of table egg shells with *E. coli* in the current study, as also found for *Salmonella* spp., included pest (rodents, free-flying birds, insects, flies, wasps) infestation, which have also been associated with contamination of egg shells *(25*). The use of antimicrobial agents (therapy, prophylaxis and growth promoters) was also determined to be important risk factors for isolation of *E. coli* and may reflect unsuccessful use of the antimicrobial agents to control collibacillosis and other infections on these farms.

It was significant that 5.1% of the pooled egg contents were positive for *E. coli.* This is comparable with the report of a study in the USA where 5.2% of table egg contents were positive for *E. coli (33).* This prevalence is however higher than the 3.8% reported for egg contents in Trinidad *(3)* and the 0.33% prevalence for egg contents in China *(24).*

It is known that egg shells are contaminated horizontally by *E. coli* and other microorganisms from the environments where the eggs are laid while egg contents (albumen and yolk) are contaminated trans-ovarially and through shell penetration by microorganisms *(10, 13, 52).* It is imperative to mention that the two egg contents positive for *E. coli* in the current study were also shell-positive for microorganism. The study was unable to confirm whether the isolates

of *E. coli* recovered from the shells and contents were similar or related since molecular techniques such as the pulse-field gel electrophoresis (PFGE) *(34)* which could establish their relatedness was not used. The possibility of *E. coli* penetrating the shells, through cracks in the shells not visible to the naked eye, to contaminate the contents can however not be ignored.

The failure to detect *E. coli* O157 strains in either egg shell or egg contents agreeswith the findings of Adesiyun et al. *(2)* where all the egg samples tested were also negative for *E. coli* O157. Dipineto et al. *(18)* had however isolated Shiga-toxin *E. coli* (STEC) from 26 (3.6%) of the 720 cloacal swabs of layers samples. Therefore, it appears that table eggs may not be important in the transmission of *E. coli* O157 strains.

The failure to detect *Campylobacte*r spp. in either egg shell or contents in the current study agrees with the findings of Ge et al. *(24)* in China where for the internal contents of eggs, none (0.0%) was positive for *Campylobacter* and with the very low isolation rate of 1.1% reported by Adesiyun et al. *(3)* where only 2 of 184 pooled egg shell, egg contents or both were positive. Similarly, Sulonen et al. *(53)* reported examining a total of 360 table eggs from Finnish organic laying hens for the presence of *Campylobacter* spp. and detected the organism in only 1 (0.28%) egg shell sample. It therefore appears that consumption of table eggs poses a low risk for human campylobacteriosis in South Africa or elsewhere.

It is well established in the livestock industry that antimicrobial agents are used for treatment, prophylaxis and as growth promoters *(19, 38, 50)* and with inappropriate use, lead to side effects such as the occurrence of antimicrobial residues in animal products such as meat, milk and eggs resulting in allergic reactions, development of resistant bacterial strains *(21, 41)*. In our study, on the five small farms, 20% used antimicrobial agents for prophylaxis only while

442 of the 34 large farms 20.6% used antimicrobial agents for therapy and 5.9% each used them as
443 growth promoters and prophylaxis. It was equally significant that all the large farms that used
444 antimicrobial agents for treatment also claimed adherence to withdrawal periods following
445 treatment of layers. This is contrary to the report of a study in Uganda by Sasanya et al. *(48)*
446 who found 95% of the 60 farmers never observed withdrawal periods although 80% of them
447 knew the importance of withdrawal periods. Similarly, in Tanzania, Nonga et al. (*43)* reported
448 that 80% of the farmers had knowledge of antimicrobial withdrawal period to be observed before
449 eggs from treated hens are sold for human consumption and almost 85% were unaware of
450 possible effects of antimicrobial residues in humans.

451 The infrequent use of antimicrobial agents as growth promotes in the current study is
452 comparable to the findings in Sudan where a questionnaire survey of layer farmers reported that
453 only 5% stated using antimicrobial agents for growth promotion with quinolones, reported to
454 constitute one-third *(19).* Only 30% of the farmers had heard of antibiotic resistance; poor
455 knowledge of farmers on antibiotic use, antibiotic resistance and zoonotic infections was found.

456 In a study conducted in Sudan, Sirdar et al. *(50)* reported that 49% of the layer farms were
457 on antibiotic treatment during a survey and that 59% of the farms had used antibiotics within the
458 last 3 months and concluded that farmers and producers had a lack of knowledge about
459 antimicrobial residues, their withdrawal periods and the risk posed by the consumption of these
460 residues.

461 In the current study, of a total of 49 isolates of *E. coli* recovered, 35 (71.4%) exhibited
462 resistance to one or more of the eight antimicrobial agents tested. It cannot be over-emphasized
463 that such a high prevalence of resistance to antimicrobial agents could cause adverse effects on
464 therapeutic interventions on the layer farms and consumers of contaminated eggs. The

prevalence of resistance detected in the current study is however considerably lower than the findings reported by Adesiyun et al. *(2)* in a study conducted on *E. coli* isolates from table eggs (shells and contents) where 88.1% were resistant to one or more of the seven antimicrobial agents tested. Lower prevalence of resistance to antimicrobial agents by *E. coli* isolates from table eggs have been reported by others such as the 58.33% reported for eggs in Northwest Spain *(6)* and the 64.7% reported for isolates in Grenada *(8).* Unlike our study where all the 4 isolates of *E. coli* from egg contents were sensitive to the eight antimicrobial agents, Arathy et al. *(8)* reported that 52.4% of *E. coli* isolates recovered from egg yolks exhibited resistance to antimicrobial agents.

In addition to the rather high prevalence (71.4%) of resistance to antimicrobial agents amongst table egg isolates of *E. coli*, it is equally of concern that multi-drug resistance was prevalent (77.1%) amongst the 35 resistant *E. coli* isolates in the current study. Variable prevalence of multi-drug resistance in *E. coli* isolates have been documented by others, 10.9% in Grenada *(8),* 46.6% in Trinidad *(2)* and 100.0% in Nigeria *(44).*

It has been established that the prevalence on resistance to antimicrobial agents reflects their use or overuse in the livestock industry *(19, 38, 50).* It is also of therapeutic significance that amongst the eight antimicrobial agents used in the poultry (broilers and layers) farms in Gauteng province, resistance was relatively high (24.5% to 53.1%) to doxycycline, oxy-tetracycline, sulphamethoxazole-trimethoprim (SXT) and amoxicillin. This finding may affect their effectiveness in treating infections in layer farms in the province. Compared to published reports on the prevalence of resistance of *E. coli* to these antimicrobial agents, considerable variable prevalence rates have been documented for table eggs isolates of *E. coli*, such as the 9.4% to SXT *(2),* 29.9% to tetracycline *(33),* 57% to doxycycline and 81.0% to amoxicillin *(44).*

It was important to have detected a very low prevalence of resistance to enrofloxacin and norfloxacin (6.1%) and to fosfomycin and fosfomycin plus T (0.0%). This agrees with the report of Arathy et al. *(8)* that none (0.0%) of *E. coli* isolates from table eggs was resistant to enrofloxacin. However, considerably higher prevalence of resistance to norfloxacin have been reported for isolates of *E. coli* from chickens by others, 36.9% *(58)* and 96% *(40).*

The potential effectiveness of the use of fosfomycin (Fosbac) and Fosfomycin (Fosbac) plus Tylosin cannot be over-emphasized because all the *E. coli* and *Salmonella* isolates were sensitive to the antimicrobial agents. The superior efficacy of fosfomycin to other antimicrobial agents on *E. coli* has been demonstrated in several studies, particularly on poultry farms *(32, 49).*

For the four *Salmonella* isolates, resistance was exhibited by two to doxycycline only. This is at variance with the findings of Adesiyun et al. *(5)* who reported that all 9 isolates from table eggs in three Caribbean countries were resistant to one or more of the eight antimicrobial agents tested. Also, contrary to our study where no multi-drug resistance was detected, other studies have documented multi-drug resistance amongst *Salmonella* spp. isolated from table eggs, 1% *(2),* 53.4% *(36)* and 100.0% *(31).*

The farm prevalence and egg prevalence for antimicrobial residues was 2.6% and 0.5% respectively for table eggs in the current study. The farm prevalence is considerably lower than the 100% reported for table eggs sampled from 29 smallholder layer farmers in Tanzania *(43),* the 61% to 72% reported over monthly samplings of layer farms in Sudan *(51),* the 36% reported for eggs from 25 commercial layer farms in Enugu, Nigeria *(20)* and the 6.5% for 23 commercial layer farms in Trinidad *(1).* Regarding the egg prevalence of residues in pooled egg contents, only 0.5% (1/196) samples were positive in the current study which is considerably lower than

6.5% (12/184) reported for pooled eggs in Trinidad *(1),* 12.9% (4/31) of farms positive in Abuja, Nigeria *(39)* and the 98.3% for eggs tested in Uganda *(48)* and 100.0% of eggs tested in Tanzania *(43).* It is however pertinent to mention that the reported prevalence of antimicrobial residues in table eggs is affected by the detection systems used which have different sensitivity and specificity *(1, 16, 39, 43, 51, 57).*

The finding of low farm and egg prevalence in our study could be a manifestation of the use on antimicrobial agents and observation of the withdrawal period under the supervision of veterinarians as reflected in the questionnaire survey.

It was alarming that the only farm positive for antimicrobial residues was positive for quinolone, macrolide, aminoglycoside, tetracycline and beta lactam, an indication of gross misuse of the five classes of antimicrobial agents on this farm. This farm, pooled eggs from house #2 of 7 houses sampled was positive, antimicrobial agents were not used for prophylaxis nor as growth promoters but used for treatment on the prescription of the veterinarian and from the questionnaire, the farmer claimed that the withdrawal period was observed following administration of antimicrobial agents. This could be explained, in part, by possible breakdown in communication regarding antimicrobial use and observation in the poultry house on the farm.

Reported prevalence of residues detected in table eggs reflect the types of residues assayed for, the frequency of use of the agent (prophylaxis, treatment and as growth promoters) in the poultry industry, adherence to withdrawal period, and the sensitivity and specificity of assay methods. It is therefore prudent to selectively assay for antimicrobial residues commonly available or used by layer farms in the areas or countries where studies are conducted. In Abuja, Nigeria where it was known that chloramphenicol, although a banned antibiotic, was being used

by layer farmers reported that 7.0% table egg contents were positive *(39).* Adesiyun et al. *(1)* tested pooled eggs for residues and detected sulphonamides (6.5%), macrolides (3.8%), tetracycline (2.7%) and penicillin (0.0%) while Sasanya et al. *(48)* in Uganda, 98.3% of the samples that had detectable sulfonamide residues came from farmers who applied antimicrobials in feeds/ water.

It is important to mention that based on the three types (Microbiological Inhibition Test, ELISA and HPLC), used in the current study, the two antimicrobial agents Sulfonamides (79 ppb) and Oxytetracycline (106 ppb) at relatively low levels. For example, in South Africa Government Notice No. R. 1387 of 19 November 1999 (*25)* set maximum residue level (MRL) of 200 ppb for total tetracyclines which is considerably higher than detected. Information is unavailable for the MRLs for other antimicrobial agents in table eggs.

In conclusion, the frequency of detection of *Salmonella* spp. (2.0%) from pooled egg shell only and *E. coli* (49.9%) from both pooled egg shells and contents, the failure to isolate *Campylobacter* spp. and *E. coli* O17 strains from table eggs, all suggest that table eggs from layer farms in Gauteng province pose minimal health risk of salmonellosis, moderate risk of colibacillosis and virtually no risk of infection by verocytotoxigenic *E. coli* and campylobacteriosis to consumers of table eggs from the layer farms studied. The relatively high resistance of *E. coli* strains (71.4%) isolated from table eggs can however not be ignored because of the potential therapeutic implications while the prevalence of antimicrobial residues in egg content, albeit low, also has food safety implications for consumers.

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Disclosure Statement

No competing financial interests exist.

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TABLE 1. *Occurrence of risk factors for contamination of table eggs by bacterial pathogens*

		Size	Housing		Pests		
	No. of	Median (range) total No. of:	Housing type[b]		No. (%) of farms with infestation by:		
Type of farm[a]	farms	hens in-lay	Battery Cage	Free range	Rodent	Feral birds	Insect[c]
Large	34	49,524 (1,050 to 538,656)	28 (82.4)	4 (11.8)	18 (52.9)	18 (52.9)	9 (26.5)
Small (DPFO)[d]	5	1,500 (964 to 15,800)	5 (100.0)	0 (0.0)	3 (60.0)	2 (40.0)	1 (20.0)
Total	39	47,149 (964 to 538,656)	33 (84.6)	4 (10.3)	21 (53.8)	20 (51.3)	10 (25.6)

[a]Large farms with 100,001 - >300,000 hens and small farma 1-20,000 hens

[b]Other types of housing included: deep litter, 1 (2.6%) and battery cage and and free range, 1 (2.6%)

[c]Included flies, wasps and mites

[d]Developing Poultry Farmers Organization

TABLE 2. *Frequency of use of antimicrobial agents and observation of withdrawal period*

Type of farm[a]	No. of farms	Use of antimicrobial agents as:			Observation of withdrawal period	San Median (range) of eggs Crates[b] collected
		Growth promoters	Prophylaxis	Treatment		
Large	34	2 (5.9)	2 (5.9)	7 (20.6)	7 (20.6)	5 (1 to 10)
Small (DPFO)[c]	5	0 (0.0)	1 (20.0)	0 (0.0)	0 (0.0)	2 (1 to 4)
Total	39	2 (5.1)	3 (7.7)	7 (17.9)	7 (17.9)	4 (1 to 10)

[a]Large farms with 100,001 - >300,000 hens and small farma 1-20,000 hens

[b]A crate consisted of 10 table eggs

[c]Developing Poultry Farmers Organization

npling of eggs:

Total No. of crates collected	Total No. of eggs tested
186	1860
10	100
196	1,960

TABLE 3. *Prevalence of enteropathogens and antimicrobial residues in table eggs*

Type of farms	No. of farms	No. (%) of samples positive for: *Salmonella*[a]	*E. coli*[b]	*E. coli* O157	*Campylobacter* spp.	Antimicrobial residue[c]
Large	34	3 (8.8)	19 (55.9)	0 (0.0)	0 (0.0)	1 (2.9)
Small (DPFO)[d]	5	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total	39	3 (7.7)	19 (48.7)	0 (0.0)	0 (0.0)	1 (2.6)

[a]Of the three farms positive for *Salmonella* spp., the frequency of isolation per crate (10 eggs) was as follows:
Farm #33--1 (20.0%) of 5 crates *(S*. Enteritidis 9,12:g,m:-)
Farm #17--1 (25.0%) of 5 crates (S. Ivory) and
Farm #13--2 (28.6%) of 7 crates (both S. Enteritidis 9,12:g,m:-)
All 4 (2.0% of 196 crates) isolates of *Salmonella* originated from egg shells

[b]Overall, 19 (48.7%) of egg shells were positive for *E. coli* compared with 2 (5.1%) of egg content
The prevalence of *E. coli* in pooled eggs (shells and contents) was 23.0% (45 of 196) and 2.0% (4 of 196) for egg shell

[c]Of a total of 196 crates (1960 eggs) screened for residues only 1 (0.5%) was positive (Farm #3)
for 5 antimicrobial agents (Quinolones, Macrolides, Aminoglycosides, Tetracycline and Beta lactam)

[d]Developing Poultry Farmers Organization

TABLE 4. *Prevalence of resistant strains of E. coli table eggs by farm*

Type of sample	Layer farms[a]	Number of *E. coli*[b] isolates tested	No. (%) of isolates resistant[c]	No. (%) resistant to antimicrobi				
				AMOX[d]	ENRO	FOSF	FOSF+ T	NOR
Egg shell	A	1	1					
	B	4	4	3				
	C	3	3					
	D	1	1					
	E	1	1	1				
	F	2	2					
	G	1	1					
	H	1	1					
	I	1	0					
	J	1	1					
	K	2	2	1	1			1
	L	2	2					
	M	8	5	4				
	N	3	3		1			1
	O	3	0					
	P	4	3	1				1
	Q	3	3					
	R	3	2	2	1			1
	S	1	0					
	Subtotal (n =19)	45	35 (77.8)	12 (26.7)	3 (6.7)	0 (0.0)	0 (0.0)	3 (6.7)
Egg content								
	T	3	0					
	U	1	0					
	Subtotal (n = 2)	4	0 (0.0)					

	Total	49	35 (71.4)	12 (24.5)	3 (6.1)	0 (0.0)	0 (0.0)	3 (6.1)
P-value (ANOVA)		P < 0.0001						

[a]All farms positive for *E. coli* were the large commercial farms i.e. all DPFO farms were negative

[b]Of the 4 isolates of *Salmonella* recovered, 2 (50.0%) exibited resistance to antimicrobial agents each resistant to doxycycline

[c]Resistant to one or more antimicrobial agents

[d]Amoxycillin--AMOX (30 mcg), Enrofloxacin--ENRO (5 mcg), Fosfomycin--(FOSF) (50 mcg), Fosfomycin plus T--(FOSF+ T (40 mcg) Sulphamethoxazole/Trimethoprim--SXT (25 mcg), Doxycycline--DOXY (30 mcg) and Oxytetracyclins--OXY (30 mcg)

al agents :		
SXT	DOXY	OXY
1	1	1
2	1	1
2	3	2
1	1	1
1	1	2
1	1	1
1	1	1
1	1	1
		1
	2	1
3	5	5
3	3	3
2	2	1
	2	2
1	2	2
19 (42.2)	26 (57.8)	25 (55.6)

19 (38.8) 26 (53.1) 25 (51.0)

, Norfloxacin nichotinate --NOR (15 mcg),

TABLE 5. *Resistance patterns exhibited by E. coli isolates*

Resistance Pattern[a]	Number (%) of isolates of *E. coli*[b]
SXT-DOXY-OXY[c]	9 (25.7)
DOXY-OXYT	6 (17.1)
AMOX-SXT-DOXY-OXY	5 (14.3)
AMOX	4 (11.4)
OXY	4 (11.4)
AMOX--NOR-SXT-DOXY-OXY	1 (2.6)
NOR-SXT-DOXY-OXY	1 (2.9)
AMOX-DOXY-OXY	1 (2.9)
AMOX-OXY	1 (2.9)
ENRO-NOR	1 (2.9)
SXT-DOXY	1 (2.9)
AMOX-SXT	1 (2.9)

[a]A total of 3 isolates from egg shells and 4 from egg contents were sensitive to all 8 antimicrobial agents tested

[b]Of a total of 35 isolates of *E. coli* (all egg shells) that exhibited resistance to antimirobial agents

[c]AMOX: Amoxacillin, ENRO: Enrofloxacin, FOSF: Fosfomycin, FOSF+: Fosfomycin plus T, NOR: Norfloxacin, SXT: Sulphamethazole/Trimethoprim, DOXY: Doxycycline and OXY: Oxytetracycline