A pilot study on post-evisceration contamination of broiler carcasses and ready-to-sell livers and intestines (mala) with *Campylobacter jejuni* and *Campylobacter coli* in a high-throughput South African poultry abattoir

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ABSTRACT

To assess post-evisceration contamination of broiler carcasses, 300 samples were randomly selected during routine slaughter in the winter of 2004. The samples originated from 50 chicken carcasses, taken directly after evisceration, as well as 25 samples from ready-to-sell packages of fresh intestines (mala) and livers. The samples were taken in batches over a period of 4 weeks to allow randomised sampling from different farms of origin. Conventional culture-based detection methods of *Campylobacter* spp. usually need 4–6 days to produce a result. The polymerase chain reaction (PCR) used for this study took less than 32 hours. The average contamination rates with *Campylobacter* in both the skin and liver samples were 24 %, and 28 % for intestines. Chicken and chicken products, especially livers and intestines, form an integral part of the traditional diet of many Black South Africans, as they are cheap and readily available in bulk and un-chilled for direct distribution, mainly through street vending and other informal retail outlets. This sudy showed that *Campylobacter* spp. are prevalent in poultry in South Africa. The handling of poultry meat and products contaminated with this organism in households and the potential for cross-contamination of other foods presents a high risk of infection to consumers in South Africa. The study also emphasised the need for further research in this field.

Key words: *Campylobacter jejuni/coli,* PCR, poultry products, South Africa.

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INTRODUCTION

*Campylobacter*iosis in humans is the leading cause of acute bacterial diarrhoea in many countries[1]. Most infections are sporadic and self-limiting and spectacular large outbreaks, severe illness and death are rare[5,15,38,39]. However, due to the direct and indirect costs of the disease, its impact on society can be enormous[10,11,17,42,43].

Most cases of human campylobacteriosis are caused by *Campylobacter jejuni. Campylobacter coli, C. lari* and *C. uppsaliensis* are also recognised as a cause of human gastroenteritis, but less frequently. *C. jejuni* is implicated in approximately 85–99 % of the cases of human campylobacteriosis in developed and developing countries, but the majority of the remaining cases are caused by *E. coli* in developed countries[1,2,30,38,43,51]. Strains like *C. uppsaliensis* and *C. lari* cause infections in humans to a larger extent in developing countries than in developed countries[2].

Enteric campylobacteriosis is a typical zoonosis, which can be transmitted by direct contact with contaminated animals or animal carcasses, or indirectly by ingestion of contaminated food or water. *Campylobacter* are enteric commensals or occasional pathogens in a wide range of animals, which thus form the source of infection for humans. *Campylobacter* can often be isolated from the faeces of dogs and cats, with isolation rates higher in young than in mature animals. Infected pets form a reservoir of infection, especially for children[9,42,43]. Carrier-animals, such as poultry, cattle, sheep and pigs, are sources for food-borne illness rather than for contact infections[9,38,42,43,50]. Faecal contamination of carcasses from the intestinal contents during the slaughtering process and contamination of milk are incriminated as the main routes for food-borne infection of consumers[9,25,40,46]. Poultry meat is cited as the most important source of human campylobacteriosis because most commercially raised poultry harbour *Campylobacter* spp. in their intestinal flora and contamination of carcasses and products is common during slaughtering and processing[4,11,38,49,50].

Contamination of poultry is thought to be nearly universal and colonisation of birds in a flock can be detected from the 2nd and 3rd weeks of age. *Campylobacter* are usually introduced into a flock by single birds and horizontal transmission throughout the remainder of the flock is rapid. The usual infection rate in a flock is 100 %, but a seasonal pattern in reported cases of poultry infections as well as cases of human campylobacteriosis, with a definite peak in warmer months, has been described. Generally, the highest contamination of poultry flocks and poultry meat is reported in summer and early autumn, while contamination rates are low in winter and early spring[2,3,5,8,9,23,24,43]. The large numbers of intestinal *Campylobacter* that are brought into the processing plant with the birds result in massive contamination of birds, processing lines, equipment, hands of workers and finally the end-products of the dressing process. Contamination of carcasses and meat is mainly superficial or subcutaneous, and the incidence of bacteria in muscles is very low[7,45]. The surface parts that are mainly contaminated are the peritoneal cavity, breasts, thighs and drums. Numbers of organisms can exceed 10^6/g[45].

Campylobacter spp. do not multiply in food, but as the infectious dose for humans is low, relatively few bacteria (400–500) can resuly in an infection. Campylobacteriosis often results from a lack of kitchen hygiene when handling raw chicken or chicken products, from cross-contamination of ready-to-eat foods and from eating undercooked chicken[6,9,17,22,25,29,31,36].

Incidence of human campylobacteriosis

The number of reported cases of *Campylobacter* enteritis is estimated to have risen dramatically over the last

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20–30 years[1,2,48]. In almost all developed countries, campylobacteriosis is now the leading cause of human gastrointestinal infections[2,14,18,48]. Since the mid 1970s, increasing research has been carried out on the role of *Campylobacter* spp. in causing illness in humans, as well as on the development of effective sampling and isolation methods. The rise in reported human cases of *Campylobacter* enteritis is therefore not only a real increase in the incidence of cases, but rather a sign of more concern about the organism as a human pathogen and also as a result of better methods for isolation and detection of *Campylobacter* spp.[15]

Limited information is available regarding the prevalence of human campylobacteriosis in developing countries due to a lack of national surveillance programmes. However, it is likely that the incidence is especially high amongst infants and the young, with the vast majority of infections occurring in children in the first 5 years of life[2,13,14,17]. In children between 0 and 24 months the incidence of infection and the severity of the resulting illness is the highest[2]. A survey performed in South Africa established an infection rate with *C. jejuni* of 14.4 % in black children and 4.9 % in Caucasian children below 2 years of age[13]. Doyle[13] cites Bokkenheuser *et al.* who performed a survey in Soweto where 34 % of all children with diarrhoea were positive for *C. jejuni* while the organism could be detected in 12.5 % of asymptomatic children.

The main causes for the high infection rates in infants in developing countries are a strong environmental exposure together with poverty, overcrowding, malnutrition, poor hygiene and dangerous bottle-feeding habits. Like other diarrhoeal diseases caused by bacteria, *Campylobacter* infections in children result in high mortality rates[21]. Surveys since the 1980s conducted at the Red Cross Children's Hospital in Cape Town revealed that the isolation of *Campylobacter* spp. has risen dramatically. At the same hospital a survey was carried out between October 1990 and September 1997 to determine the distribution of *Campylobacter* spp. from stools obtained from children admitted with diarrhoea[32]. It revealed that thermophilic *Campylobacter* such as *C. jejuni/coli* were present in nearly 50 % of all samples. This rate of *Campylobacter* isolation described by Le Roux and Lastovica in the 'Cape Town Protocol' is unequalled anywhere[13,24,32].

The lower rate of infections in adults in developing countries may be due to a good immunity gained in early childhood, which is thought to be the result of early exposure to the organism[43]. The incidence of infection in developing countries is much higher in rural than in urban populations. Nutritional factors are seen as the major cause for infections in the urban areas, while in rural living conditions the exposure to *Campylobacter* spp. due to environmental contamination from domestic animals is probably the most important factor in transmission[2]. In developing regions where hygienic conditions are poor, the prevalence of *Campylobacter* infections among children is higher than in areas with good hygienic conditions[13].

Clinical signs of human campylobacteriosis

Symptomatic *Campylobacter* infections are marked by gastrointestinal illness, which is often clinically indistinguishable from those caused by other enteric pathogens[13]. Generally, *Campylobacter* enteritis is self-limiting and treatment is not necessary; but infections can lead to potentially dangerous long-term consequences like bacteraemia, meningitis, pneumonia, miscarriage, reactive arthritis (RA) and an acute flaccid paralytic disease (Guillain-Barré syndrome: GBS)[20,23,43,45]. *Campylobacter jejuni* is the inducent antecedent infection in approximately 30 % of all cases of GBS, while reactive arthritis, which leads to the impaired movement of various joints, occurs in approximately 2 % of all *C. jejuni* enteritis cases[36,44].

Evidence shows that immunocompromised individuals are at increased risk for *Campylobacter* infections. Patients with HIV/AIDS were found to be 39 times as likely as immunocompetent individuals to have campylobacteriosis. Patients with HIV/AIDS and campylobacteriosis also showed an increased incidence of bacteraemia and hospitalisation compared with non-infected AIDS patients[44]. Bacteraemia is uncommon and transient in immunocompetent people while immunocompromised individuals are predisposed to *Campylobacter jejuni*-induced bacteraemia and a higher mortality caused by the infection. Surveillance performed over 10 years in England and Wales revealed an incidence of 25.8 % in immunocompromised patients. Although pregnant women and elderly people are usually considered as immunocompromised, no evidence exists to show predisposition of these population groups to *Campylobacter* infection. However, *Campylobacter jejuni/coli* can have deleterious effects on the foetus, including stillbirth, abortion, meningitis or bacteraemia in the newborn[44]. While much research has been carried out on the importance of *Campylobacter jejuni/coli* as a food-borne zoonosis in many parts of the world, including European and Asian countries, North America and Australia, information on the current situation in southern Africa is limited.

The main objectives of this study were to determine the extent of contamination of poultry carcasses and products in a large-scale South African chicken processing plant and to develop a convenient and practical method for identifying *Campylobacter jejuni* and *Campylobacter coli* in samples.

MATERIALS AND METHODS

Pilot study

A pilot study was first performed to determine the specific conditions of the PCR methodology. No differentiation between the detection of *C. jejuni* and *C. coli* by PCR used was made in this study. A pure culture of *C. jejuni* obtained from a dog was used in the preparation of the control culture in the pilot study as well as the positive control in the field study[39]. The *C. jejuni* strain used was cultivated anaerobically at 42 °C and diluted in sterile phosphate-buffered saline (PBS) to a concentration of approximately 10^7 bacteria per mℓ. The optical density of the solution was determined to be about 0.226 at a wavelength of 535 nm by using a LKB Biochrome Ultrospec II spectrophotometer. Of this undiluted (pure) culture solution, 10-fold dilutions in phosphate-buffered saline (PBS) up to 10^{-6} were made. The undiluted solution of *C. jejuni* contained 5.84×10^7 bacteria/mℓ and the highest dilution of 10^{-6} contained 5.84×10^1 bacteria/mℓ. From each solution, 200 μℓ was used for direct bacterial DNA extraction and a further 200 μℓ of each dilution was used to spike poultry samples.

In this study, liver tissue, intestines and skin of poultry were used. Each skin sample consisted of a pool of 5 samples removed aseptically from different sites on the carcass (neck, both thighs and both sides of the breast). Of each sample, 25 mg was removed and cut into small pieces before the DNA was extracted. The QIAamp DNA Mini Kit (QUIAGEN GmbH, Hilden, Germany) was used for DNA extraction and as the tissue samples were solid, the incubation period with Proteinase K at 56 °C was prolonged to approximately 18 hours to achieve complete lysis.

For the spiking of samples and for use as negative tissue controls in the PCR, samples of liver, intestines and skin were obtained aseptically from a specific pathogen-free (SPF) chicken and prepared for extraction as described above. To the tissue samples, 200 μℓ of each bacterial

dilution ranging from undiluted to a dilution of 10^{-6} was added. The extraction was then performed and each spiked sample and the pure bacterial dilutions were examined by PCR.

A semi-nested PCR assay was used to detect the *Campylobacter* spp.[47] Oligonucleotide primers from the *C. jejuni flaA* and *C. coli flaB* sequences with the following sequences were used: CF03-JT (5′-GCT CAA AGT GGT TCT TAT GC-3′), CF04-JT (5′-GCT GCG GAG TTC ATT CTA AGA CC-3′) and CF02-JT (5′-AAG CAA GAA GTG TTC CAA GTT T-3′). The concentrations of the primers were 76 pmol/$\mu\ell$ for primer CF04-JT, 69 pmol/$\mu\ell$ for primer CF02-JT and 79 pmol/$\mu\ell$ for primer CF03-JT. The primers were obtained from Inqaba Biotech (Pretoria, South Africa).

The 1st PCR step was performed with primers CF03-JT and CF04-JT and resulted in the amplification of a fragment of 340 to 380 base-pairs (bp) as described by Waage *et al.*[47]. A total volume of 25 $\mu\ell$ was used which contained Red Taq Ready Mix PCR reaction mix (12.50 $\mu\ell$) (Sigma-Aldrich), 0.25 $\mu\ell$ of each original primer, distilled water (9.50 $\mu\ell$) and the extracted DNA sample (2.50 $\mu\ell$). The 2nd PCR step was performed with the primers CF03-JT and CF02-JT and the resulting amplification was a fragment of 180– 220 bp as described by Waage *et al.*[47]. A total volume of 25 $\mu\ell$ was used and contained UDG Invitrogen (12.50 $\mu\ell$), 0.25 $\mu\ell$ of each primer, water (11.50 $\mu\ell$) and 0.50 $\mu\ell$ of PCR product from the 1st step.

The same PCR programme was used for both steps of the PCR: a pre-PCR step at 42 °C for 2 min, heat denaturation at 94 °C for 10 min, followed by 40 cycles consisting of heat denaturation at 94 °C for 5 sec, primer annealing at 53 °C for 30 sec and DNA extension at 72 °C for 40 sec per cycle. After the last cycle, the samples were held at 72 °C for 10 min to complete synthesis of all strands and were kept at 4 °C until analysed. The PCR products were analysed on a 2 % agarose gel.

Field study

To determine the status of *Campylobacter* in commercially available chickens, samples were taken at a high-throughput South African commercial poultry abattoir over a period of 3 weeks in late winter. The dates of sampling were chosen in a way to ensure that each batch of samples originated from a different farm supplying broilers to the abattoir. Fresh chicken carcasses were obtained at the evisceration stage prior to chilling using the principle of systematic probability sampling – the basis of random sampling. Livers and intestines (mala) were obtained at the packaging stage at the abattoir in ready-to-sell packages after vacuum pumping from the harvesting area in chilled water at 4–7 °C, but prior to freezing (Fig. 1).

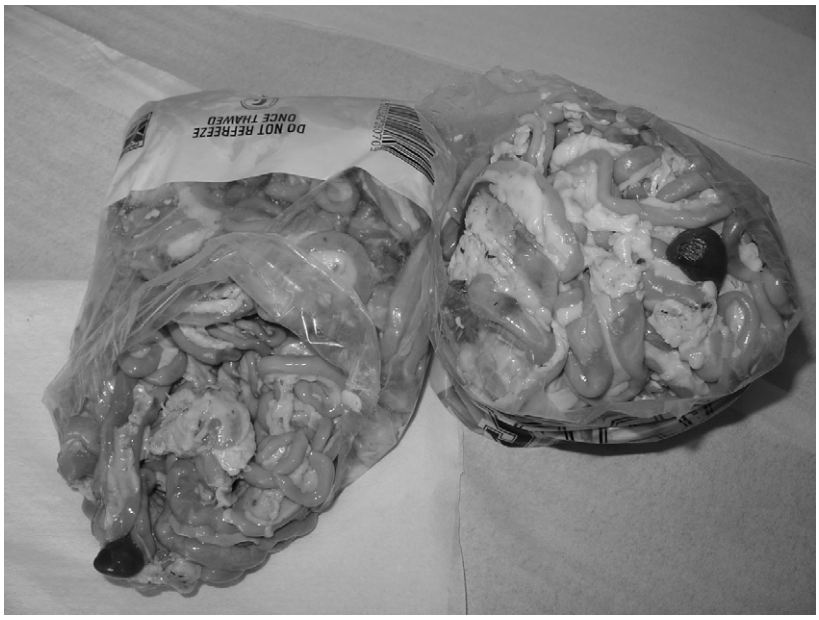

Fig. 1: **Ready-to-sell packages of fresh intestines (mala).**

Samples were taken within a 3-week period in August/September and 50 carcasses and 25 samples of liver and intestines (mala) each were included in the study.

Carcasses were obtained by systematic sampling at the post-evisceration and pre-chilling stages directly from the processing line and 5 skin samples were taken aseptically from the neck, both thighs and both sides of the breast of each carcass. All skin pieces from 1 carcass were transferred into a small plastic bag as 1 pooled sample per carcass and each bag was numbered sequentially. After skin samples were taken, ready-to-sell packages of fresh intestines (mala) and liver were obtained at the packaging stage, prior to freezing. All samples were placed on ice and immediately transported to the laboratory for testing. The DNA extraction process was started within 3 hours of collection. To avoid the risk of cross-contamination, extraction and PCR was performed in batches. Each batch contained 5 liver, 5 intestine (mala) and 10 skin samples.

The samples were processed in the laboratory as described above and incubated at 56 °C for about 16–18 hours (overnight) to ensure complete lysis of tissue and bacterial cells. The extraction process was completed on the following day and the DNA was stored at –20 °C until used for PCR.

RESULTS

The specificity of the primers was determined by performing the PCR on the undiluted bacterial solution and tissue samples (liver, skin, intestines (mala) of a presumed *Campylobacter*-free (SPF) chicken. Sterile water was not processed together with the extraction of samples, but included in the PCR as a negative control. The semi-nested PCR step resulted in a fragment of the expected size of 180–220 bp for the undiluted bacterial culture, while the SPF chicken tissue samples showed no amplification of *Campylobacter* DNA.

The sensitivity of the primers was tested by subjecting all bacterial dilutions as well as all tissue samples spiked with the range of bacterial dilutions to the PCR. Negative (unspiked SPF tissue samples) and positive (pure bacterial culture) controls were included in each PCR batch. All tissue samples taken from the specific pathogen-free (SPF) chicken were negative in the PCR. The undiluted culture solution, as well as all tissue samples spiked therewith, showed DNA bands of the expected size.

The results of the pilot study revealed the high sensitivity of the primers and method, which enables the detection of DNA equivalent to 58 bacterial cells per mℓ or 12 cells per PCR, based on the results for a bacterial dilution of 10^{-6}.

With respect to the correlation of contamination rates of different tissues within the same batch, the following was observed. The 1st batch showed high contamination of skin (70 %) and moderate contamination of liver samples (40 %), while all intestine (mala) samples were negative. The 2nd batch was more homogeneous, with 60 %, 40 % and 30 % for liver, intestines (mala) and skin, respectively. The same applied to batch 3, with 20 % positive samples each for liver and intestines (mala) (Fig. 2), and 10 %

Table 1: **Field samples: PCR results listed according to batches.**

Sample	Liver (5 per batch)		Intestines (5 per batch)		Skin (10 per batch)	
	Positive (Total)	Positive (%)	Positive (Total)	Positive (%)	Positive (Total)	Positive (%)
Batch 1	2	40	0	–	7	70
Batch 2	3	60	2	40	3	30
Batch 3	1	20	1	20	1	10
Batch 4	0	–	0	–	0	–
Batch 5	0	–	4	80	0	–

positive samples for skin. While all tissue samples of the 4th batch were negative, 80 % of all intestine (mala) samples in batch 5 showed positive results, but all liver and skin samples in this batch were negative. In conclusion, the liver and the skin samples showed the same average contamination rate with *Campylobacter* spp. A total of 6 of 25 samples (24 %) of liver tissue was positive and 12 of 50 pooled samples of skin (24 %) gave positive PCR results. The intestine (mala) samples showed a slightly higher rate of *Campylobacter* spp. contamination, namely 7 of 25 samples (28 %). These results are reflected in Table 1.

DISCUSSION

Pilot study

The pilot study of this research project aimed at the determination of specificity and sensitivity of primers and the PCR method for the detection of *Campylobacter.* As the tissue samples used varied in terms of composition and possible bacterial contamination, an extraction method suitable for all 3 types of tissue had to be used. Therefore the tissue protocol as described in the QIAamp DNA Mini Kit Manual was used. However, the prescribed time for incubating the samples with Proteinase K was insufficient and the tissue did not lyse completely. The incubation time was increased to between 16 and 18 hours for complete lysis to occur. The primers were chosen in accordance with the protocol of Waage *et al.,* as they used those primers successfully with a variety of food and water samples[47].

The 1st step PCR failed to show all bands of the expected size. The results were clearly enhanced by the 2nd, semi-nested PCR step, indicating this to be a more accurate and specific method to detect *Campylobacter,* although it can lead to contamination of the PCR products. As little as 58 bacteria per mℓ of the tissue extract or 12 bacteria per PCR could be detected by the semi-nested method. This is agreement with similar assays described by Mandrell and Wachtel[33], with detection rates of 35–120 *Campylobacter* cells per mℓ[35].

Field study

The field study was performed on 300 tissue samples (skin, livers, intestines (mala)). The samples were obtained by systematic sampling at the post-evisceration stage of the processing line of a high-throughput poultry abattoir and as ready-to-sell packages prior to freezing. All samples were tested for the presence of *Campylobacter* spp. by a semi-nested PCR assay. The sampling site at the processing line was chosen because the evisceration stage prior to chilling is regarded as one of the most critical points with regard to the risk of cross-contamination during the processing of poultry[29,34].

The different tissues included in the study were chosen according to the predilection sites of *Campylobacter* in poultry as described in the literature[37,41] and with regard to the nutritional importance of the different products. As an enteric pathogen, *Campylobacter* is commonly found in the intestinal flora of poultry and carcass contamination is common during processing[6,50]. Furthermore, livers and intestines (mala) of poultry form an important part of the traditional diet in the African population[12]. While livers have been the subject of various research papers, intestines (mala) as an important edible poultry product have not been addressed in previous work.

As many authors described the skin of neck, breast and thighs as the predilection sites for *Campylobacter* on the chicken carcass, those sites were sampled for this study[8,9,30]. Thomas and McMeekin described the topography of poultry skin with regard to contamination with microorganisms[45]. According to their study, organisms are partly trapped in feather follicles, channels and folds of skin of carcasses or products and are therefore not readily removable. Consequently, surface swabs and washes might not include all bacteria present on a carcass. Based on these findings we decided that lysed samples of tissue should be used rather than washes or swabs to ensure that all bacteria trapped in tissue folds and attached to the surface are detected. Five skin samples per carcass were obtained from the sites mentioned and processed as a pooled sample.

Most other studies were based on carcass washes or rinses and included an enrichment stage before further processing and examination of the samples. Our approach was different in that we used solid tissue samples and direct processing, thus avoiding the possible influence of substances in the enrichment media on the PCR. As one aim of the study was to detect any *Campylobacter* in the tissue samples, a differentiation between viable and non-viable organisms was not necessary. Furthermore, we wanted to limit the time

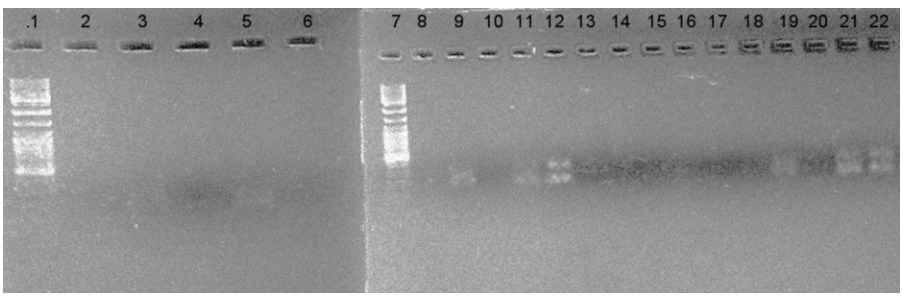

Fig. 2: **PCR results of liver and intestine** (mala) **samples in the third batch. 1**, DNA ladder; **2**, SPF liver (negative control); **3**, H_2O (negative control); **4**, H_2O (2nd step PCR, negative control); **5**, liver sample 11; **6**, liver sample 12; **7**, DNA ladder; **8**, Liver sample 13; **9**, liver sample 14; **10**, liver sample 15; **11**, SPF liver spiked with bacterial culture (positive control); **12**, pure bacterial culture (positive control); **13**, SPF intestines (negative control); **14**, H_2O (negative control); **15**, H_2O (2nd step PCR, negative control); **16**, intestine (mala) sample 11; **17**, intestine sample 12; **18**, intestine sample 13; **19**, intestine sample 14; **20**, intestine sample 15; **21**, SPF intestines spiked with bacterial culture (positive control); **22**, pure bacterial culture (positive control).

necessary to complete the assay. Even with the prolonged time required for tissue lysis during the extraction stage, examination of tissue samples could be completed in about 28 hours, from the time of sampling to the visualisation of the PCR product.

Human campylobacteriosis is an important food-borne zoonosis. The handling of raw chicken products in the household bears high risks of cross-contamination and infection for consumers[21,33]. In the African context, these risk factors for the transmission of *Campylobacter* cannot be overemphasised. Chicken and chicken products form a substantial part of the traditional diet as they are inexpensive and readily available outside of supermarkets and other retail outlets. Intestines (mala) are commonly sold by street vendors and hawkers. The products are usually obtained at abattoirs and butchers and sold in the streets on the same day[12] (Fig. 3).

Safe food storage temperatures (cold chain) are difficult to maintain, and street vendors and hawkers do not have readily accessible hand-washing facilities in the form of running water. Instead, buckets are used, sometimes without soap, and toilet facilities are not available[28]. Hence, potential health risks have been identified in association with an initial contamination of raw food with pathogenic organisms and the handling of it by street vendors[34]. The subsequent handling of such products in households and the potential for cross-contamination of other foods therefore presents a high risk of infection to consumers. Attempts to introduce intervention measures at farm level to reduce the initial bacterial load of poultry entering the processing plants, have met with limited success[9]. However, more emphasis should be placed on the introduction of good management practices on the farm, including the use of an all-in-all-out-system, with proper cleanout and disinfection between flocks [6,16,19,22,26,27,40]. During processing, risk assessment models for the facilities and the introduction of HACCP programmes are essential measures to reduce the risk of cross-contamination with *Campylobacter*[2,27].

In addition, information on measures to avoid cross-contamination, on temperature control of products and on correct core temperatures when cooking, and the education of consumers regarding the importance of hygiene in the kitchen and during food handling, will remain the primary line of defence in attempts to eradicate *Campylobacter* from poultry products and to decrease the incidence of human campylobacteriosis[21,37,49].

In conclusion, this study has shown that

Fig. 3: **Fresh poultry products sold by a street vendor.**

Campylobacter spp. are prevalent in poultry in South Africa and that the contamination of poultry meat and products with this organism is a health hazard. Moreover, detection method developed for this study, which is fast and sensitive, should be routinely applied. As the study was the first of its kind performed in South Africa, it should be considered baseline research which it is hoped will lead to further investigations to determine whether the application of appropriate control measures is needed.

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