

**Veterinary public health aspects related with food-  
producing wildlife species in the domestic animal,  
human and environment interface**

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## **Declaration**

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## ABSTRACT

The wildlife industry in Namibia continues to grow as the production and consumption of game meat increases. However, the health risks posed by the trade in wildlife and related by-products to livestock and humans have not been fully assessed. The main objective of this study was to investigate the potential health risks related to the increased consumption of game meat and relevant by-products by assessing the quality of game meat, as well as determine the role of game meat species in the transmission of zoonoses.

The microbiological quality and safety of export game meat was assessed. No differences in the aerobic plate count (APC) were observed between the years (2009 and 2010), but the mean *Enterobacteriaceae* count was  $1.33 \pm 0.69 \log_{10}$  cfu/cm<sup>2</sup> compared to  $2.93 \pm 1.50 \log_{10}$  cfu/cm<sup>2</sup> between the years. Insignificant heterotrophic plate count (HPC) levels were detected in 9/23 field water samples, while faecal bacteria (coliforms, *Clostridium perfringens* and enterococci) were not isolated in all samples. Seven serogroups, with the exception of O26, were detected in exotic species. A white tailed deer sample had a serotype belonging to O45 which confirmed positive for *stx1* gene. In springbok, 5/15 pools of faecal samples tested positive for the intimin gene. No *Salmonella* spp were isolated, and all *E. coli* isolates from the meat samples were negative for STEC virulence genes (i.e. *stx1*, *stx2*, *eae* and *hlyA*).

A linear regression analysis was conducted on selected variables to identify the main predictors and their interactions affecting pH of meat 4 hours post-slaughter. In an increasing order of magnitude during winter time, the pH reached at 16-36hr post slaughter in springbok heart, liver, spleen, kidney and lungs was significantly higher than pH 6.0, while no significant differences were observed from the regulatory reference (pH 6.0) in the heart. There was a positive association between the pH of game meat 4hr post-slaughter, and liver congestion. The pH of game meat 4hr post slaughter, increased by 0.11 units per mL increase in liver congestion, and decreased by 0.04 units per minute increase in the shooting to bleeding interval, irrespective of the species.

Worm eggs of strongylids, *Strongyloides papillosus*, *Toxocara* spp, *Trichuris* spp and coccidia were found in variable numbers in both springbok and gemsbok faeces, indicating a potential risk of transmission to other species in the ecosystem. On examination of

carcasses, a novel parasite, *Skjabinodera kuelzii*, was identified and noted to be associated with inguinal fascia and renal fat, but the public health significance remains unclear. Nevertheless, *S. kuelzii* should be considered as of potential significance during routine game meat inspection.

A total of 12 310 springbok were harvested from 26 commercial farms over a period of two years. Tissue samples (i.e. 60 livers, 41 kidneys and 52 hindquarter muscles) were collected from randomly selected healthy animals. The mean values (i.e. above the detection limit) of cadmium (Cd) and lead (Pb) were  $0.10 \pm 0.05 \text{ mg/kg}$  and  $1.04 \pm 0.21 \text{ mg/kg}$  in the liver, respectively; and  $0.33 \pm 0.22 \text{ mg/kg}$  and  $0.905 \pm 0.51 \text{ mg/kg}$  in the kidney of springbok, respectively. The levels of cadmium and lead in the hindquarter muscles were below the detection limit.

Serum samples (n=1692) collected from sheep, goats and cattle from four presumably at-risk farms, and 900 springbok (*Antidorcas marsupialis*) serum samples collected from 29 mixed farming units, were screened for *Brucella* antibodies by using the Rose-Bengal test (RBT). Positive cases were confirmed by complement fixation test (CFT). To assess the prevalence of human brucellosis, 137 abattoir employees were tested for *Brucella* antibodies using the standard tube agglutination test (STAT), and the enzyme linked immunosorbent assay (ELISA). Cattle and sheep from all four farms were negative by RBT and CFT, but two of the four farms carried 26/42 and 12/285 seropositive goats, respectively. *Post mortem* examination of seropositive goats revealed no gross pathological lesions. Culture for brucellae from organs of seropositive animals was negative. None of the wildlife sera tested positive by either RBT or CFT. Occurrence of confirmed brucellosis in humans was linked to the consumption of unpasteurized goat milk, home-made goat cheese and coffee with raw milk and prior contact with goats. All abattoir employees (n=137) tested negative by STAT, but 3 were positive by ELISA. The three abattoir workers were clinically normal, and lacked historical connections with clinical cases.

This study illustrates the importance of microbiological, parasitic and residue monitoring as critical components of a hazard analysis and critical control point based system for game meat. The study also provides the basis for increased integrated health research, surveillance and meat safety risk analysis.

## OPSOMMING

Die Namibiese wilddedryf raak toenemend groter soos die produksie en verbruik van wildsvleis toeneem. Die verwante gesondheidsrisiko's wat die gebruik van wildsvleis en verwante produkte vir mens en dier inhou, is nog nie volledig geassesseer nie. Die doelwit van die studie was om ondersoek in te stel na die potensiële gesondheidsrisiko's wat wildsvleis en verwante neweprodukte vir mens en dier inhou deur middel van die assessering van vleisgehalte en die bepaling van die rol van die wildsvleis spesies in die oordrag van soönoses.

Die mikrobiologiese gehalte en veiligheid van uitvoer wildsvleis was geassesseer. Geen verskille in die aerobiese plaat telling (APC) vir monsters versamel tydens 2009 en 2010 is aangeteken nie. Die gemiddelde *Enterobacteriaceae* telling was  $1.33 \pm 0.69 \log_{10}$  cfu/cm<sup>2</sup> in vergelyking met  $2.93 \pm 1.50 \log_{10}$  cfu/cm<sup>2</sup> tussen die jare. Onbeduidende heterotrofe plaattelling (HPC) vlakke is waargeneem in 9/23 water monsters, terwyl fekale bakterieë (d.i. kolivorme, *Clostridium perfringens* en enterokokke) nie in enige van die monsters geïsoleer is nie. Sewe serogroepe, met die uitsondering van O26, is aangeteken vir die eksotiese spesies. Monsters verky van 'n *white tailed deer* is as positief vir 'n serotipe van O45 getoets, en die teenwoordigheid van die *stx1* geen is bevestig. In springbok het 5/15 poele van fekale monsters positief getoets vir die *intimien* geen. Geen *Salmonella* spp is geïsoleer nie en alle *E. coli* geïsoleer in die vleismonsters was negatief vir die *Stec* virulensie geen (d.i. *stx1*, *stx2*, *EAE* en *hlyA*).

'n Liniêre regressie-analise is op geselekteerde veranderlikes wat as die belangrikste indikatore kan dien, en enige moontlike interaksie wat die pH van wildsvleis 4 uur na-slag kan beïnvloed, uitgevoer. In 'n toenemende orde van grootte gedurende die winter tyd, die pH teen 16-36hr na slagting in springbok hart, lewer, milt, niere en longe was aansienlik hoër as die pH 6.0, terwyl geen beduidende verskille waargeneem is wanneer dit met die regulasie verwysingswaarde van die hart (pH 6.0) vergelyk is nie. Daar was 'n positiewe assosiasie tussen die pH van wildsvleis 4 uur na-slag en mate van aansameling in die lewer. Die pH van wildsvleis 4 uur na-slag, het toegeneem met 0.11 eenhede per mL toename in

lewer aansameling en afgeneem met 0.04 eenhede per minuut toename in die skiet tot uitbloei interval, ongeag die spesie.

Wurmeiers van rondewurms, *Strongyloides papillosus*, *Toxocara* spp, *Trichuris* spp en koksidia het in verskillende ladings in die mis van beide springbok en gemsbok ontlasting, voorgekom. Dit dui op 'n potensiële risiko van oordrag na ander spesies in die ekosisteem. Die voorkoms van 'n nuwe parasiet, *Skjabinodera kuelzii*, in wildskarkasse is aangeteken en was geassosieer met inguinale fascia en renale vet, maar die openbare gesondheidsrisiko bly onduidelik. Daar word aanbeveel dat dié parasiet as 'n potensiële risiko faktor tydens roetine vleisinspeksies beskou moet word.

'n Totaal van 12 310 springbokke is oor 'n tydperk van twee jaar van 26 kommersiële plase geoes. Weefselmonsters (d.i. 60 lewers, 41 niere en 52 agterkwart spiere) is ewekansig versamel van gesonde diere. Die gemiddelde waardes (d.i. hoër as die opsporingslimiet) van kadmium (Cd) en lood (Pb) was  $0.10 \pm 0.05\text{mg/kg}$  en  $1.04 \pm 0.21\text{mg/kg}$  in die lewer onderskeidelik en  $0.33 \pm 0.22\text{mg/kg}$  en  $0.905 \pm 0.51\text{mg/kg}$  in die niere van springbok, onderskeidelik. Die vlakke van kadmium en lood in die agterkwart spiere was laer as die opsporingslimiet.

Serum monsters (n=1692) is van skape, bokke en beeste van vier vermoedelik hoë risiko plase en springbok (*Antidorcas marsupialis*, n=900) van 29 gemengde boerdery sisteme versamel en getoets vir die teenwoordigheid van *Brucella* teenliggaampies deur middel van die Rose-Bengal-toets (RBT). Positiewe gevalle is bevestig deur die komplement binding toets (CFT). Die voorkoms van menslike brusellose is bepaal deur 137 abattoir werknemers te toets vir *Brucella* teenliggaampies deur gebruik te maak van die standaard buis agglutinasie toets (STAT) en die ensiembinding immunosorberende toets (ELISA). Beeste en skape van die vier hoë risiko plase het negatief getoets met die RBT en CFT metodes, maar bokke van twee van die vier plase het seropositief getoets (26/42 en 12/285 onderskeidelik). Nadoodse ondersoek van seropositief bokke het geen patologiese letsels aangedui nie. Die kultuur van orgaanmonsters van seropositief diere vir *Brucellae* was negatief. Die monsters versamel van wild het negatief getoets deur middel van die RBT en CFT toets metodes. Die voorkoms van brusellose in mense in die studie was geassosieer met die gebruik van ongepasteuriseerde melk, tuisgemaakte bokmelkkaas en koffie met

ongepasteuriseerde melk, asook direkte kontak met bokke. Alle abattoir werknemers (n=137) het negatief getoets met die STAT metode, maar drie werknemers het positief getoets met die ELISA metode. Die drie abattoir werkers was klinies normaal en het nie vorige kontak met bevestigde kliniese gevalle gehad nie.

Hierdie studie bevestig die belang van mikrobiologiese, parasitiese en residu monitering as kritieke komponente van 'n gevaar-analise en kritiese kontrolepunt gebaseerde stelsel vir die produksie en verbruik van wildsvleis. Die studie verskaf 'n basis vir toekomstige navorsing gefokus op 'n geïntegreerde benadering van mens- en diergesondheid, monitering en vleis veiligheid risiko-analises.

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**LIST OF PUBLICATIONS AND OR ARTICLES SUBMITTED FOR PUBLICATION THAT HAVE EMANATED FROM THIS STUDY**

**Magwedere K, Bishi A, Tjipura-Zaire G, Eberle G, Hemberger Y, Hoffman LC, Dziva F** (2011) Brucellae through the food chain: the role of sheep, goats and springbok (*Antidorcus marsupialis*) as sources of human infections in Namibia. *Journal of the South African Veterinary Association* 82(4):205-212.

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**Magwedere K, Dang HA, Mills E, Cutter CN, Roberts E, DebRoy C** (2012) Incidence of the presence of shiga toxin-producing *Escherichia coli* (STEC) strains in beef, pork, chicken, deer, boar, bison and rabbit retail meat. *Journal of Veterinary Diagnostic Investigation*. In press.

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The dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has been unavoidable.

## CHAPTER 1

### GENERAL INTRODUCTION

Veterinary Public Health (VPH) is defined by the Food and Agriculture Organisation (FAO) as the contributions to the physical, mental and social well-being of humans through an understanding and application of veterinary science and this generally relates to the understanding, prevention and control of zoonotic diseases and food safety issues (WHO/FAO/OIE, 1999). Irrespective of the existence of animal-related hazards that can potentially affect human health and global economies, food of animal origin is among the most commonly consumed nutrients by human communities around the world (Daniel *et al.*, 2011; Drury, 2011). Across the African continent and the world at large, the growing importance of promoting a coordinated national and regional approach to ensuring veterinary public health has gained recognition (AU-IBAR, undated; CTA, 2012; Magalhães, 2010). Comprehensive strategies on the application of integrated risk-based quality assurance systems centred around good hygiene practice (GHP), good manufacturing practice (GMP) and hazard analysis and critical control point (HACCP) procedures at production system, field game harvesting and slaughterhouse levels have played crucial roles in controlling potential hazards arising from game meat production (Govender & Katsande, 2011; Van der Merwe *et al.*, 2011; Van Schalkwyk, 2011). Although controversially considered to be among the greatest threats to biodiversity in some developing countries, the harvest of wildlife for human consumption is valued at several billion dollars annually and provides an essential source of meat for millions of rural people living in poverty (Brashares *et al.*, 2011).

The preference of organically-produced and highly nutritious game meat by today's population increases the risk of microbiological and non-microbial hazards (Smith-Spangler *et al.*, 2012; Van Loo *et al.*, 2012). With a small population of 2.1 million and a land mass of 824,116 km<sup>2</sup>, game farming has become a thriving business in Namibia's semi-arid and arid regions, ranging from small-holder conservancies in rural areas to large commercial game ranches which slaughter for the export market (Hoffman *et al.*, 2010;

Hoffman, 2010; Lindsey, 2011). Access to export markets is considered as one of the most influential factors in developing; national strategies, road maps and plans including setting priorities on specific meat safety and zoonotic diseases in Namibia (Bishi & Kamwi, 2008). Illnesses from food-borne hazards are a significant global health concern but population level incidence estimates are often uncertain due to underreporting and in some cases, the difficulty in attributing illness to meat consumption (Rocourt *et al.*, 2003; WHO, 2002). Although the movement of micro-organisms within animal populations and from animals to humans could successfully be controlled (if detected early), 56 zoonoses have recently been reported to be responsible for 2.5 billion cases of human illness and 2.7 million deaths annually in the developing world (Cima, 2012; Grace *et al.*, 2012).

It is well known that a variety of hazards exist in livestock, however, the impact of zoonotic diseases and/or unsafe meat causing the majority of human illnesses and/or death in the Namibian population, Africa and beyond has remained an unsolved mystery. With increasing economic emphasis placed on wildlife, the sanitary aspects of game meat are therefore of cardinal importance to Namibia (Aguirre *et al.*, 2006; EU, undated; Lindeque *et al.*, 1996; Turnbull *et al.*, 1992). Food hygiene issues have become a critical issue in modern day meat production practices, particularly with the emergence of the wildlife meat industry. Whilst extensive work has been undertaken on guidelines for the production of safe game meat for export and parameters required for a sustainable game meat industry in Namibia (Van Schalkwk & Hoffman, 2010; Van Schalkwk, 2011), private standards requirements often go beyond official standards thereby calling the need to address some knowledge gaps on public health aspects along the game meat value chain. The present studies were thus designed in order to get to grips with veterinary public health challenges primarily in Namibia by generating information on some of the identified knowledge gaps.

### **Justification of the present studies**

With shortcomings in food safety compliance estimated to be costing African agricultural and food products' exporters over US\$1 billion per annum in lost exports, sanitary

requirements has been identified as one of the most important non-tariff barriers (CTA, 2009). Stringent standards which generally vary from country to country are increasingly imposed on the international food trade by both public institutions and private companies as consumers demand that the food they eat must be safe (CTA, 2009). Minimum production and harvesting process' hygiene parameters requirements for a sustainable game production system in Namibia have recently been established (Paulsen *et al.*, 2011; Van Schalkwyk, 2011). Coupled with this, guidelines for harvesting game meat for export have also been prescribed (Hoffman & Van Schalkwyk, 2010). As outlined above, legislation and associated regulatory requirements are in place; governing the slaughter, local sale and export of game meat. The nutritive value, wholesomeness and chemical composition of game meat have been extensively studied in Namibia and elsewhere.

The demand for game meat exists and is anticipated to increase due to the rising preference of organically-produced food. Although measures have been put in place to guarantee the safe production of game meat, there are still some gaps in knowledge on the public health aspects which exert huge implications on the development of effective control strategies (Bekker, 2011; Van Schalkwyk, 2011; Paulsen *et al.*, 2011; Govender & Katsande, 2011; Thiermann & Hutter, 2009). Furthermore, the impact of the current risk management strategies on meat hygiene at the livestock-wildlife-environmental interface has not been thoroughly studied. About 41% of farmers practising mixed game farming in South Africa lack effective control strategies to prevent the interaction between game and domestic animals (Bekker, 2011). Not surprising, this promotes uncontrolled spillover of infections in either direction. The quality assurance system in existence in Namibia is based on domesticated livestock species, so it is unclear whether the system works well for food-producing wildlife species where no vaccination, no ante-mortem inspection and no individual identification of the animal entering the food chain takes place. Foodborne pathogens pose a greater threat to human health than other types of hazards linked to the consumption of food and this scenario puts public protection from these hazards as a top priority (Food safety, 2006; Olson, 2011). In Africa and other developing countries, zoonotic gastrointestinal diseases and brucellosis have been listed as two of the most

important hazards in terms of their impact on human deaths, livestock sector and severity in people, along with their amenability to agriculture-based control (Grace *et al.*, 2012). However, it was noted that there could be some variations on the prevalence and risk ranking of the common neglected tropical zoonotic diseases taking into consideration the demographic, cultural, social, economical, political and scientific peculiarities between Namibia and other African countries (Borremans & Belmain, 2012; Noden & Van der Colf, 2012; AU-IBAR, undated). Noteworthy, a new generic risk-based sanitary certification for TVC, *Enterobacteriaceae*, *Salmonella* and *E.coli* for meat export has been adopted by default, and there is an urgent need to examine its suitability for game meat (EU, 2010). The effect of lowering carcass pH on certain pathogenic micro-organisms such as aphovirus, arbovirus, and *Brucella* is well-established, and this provides data required in the risk management and assessment of some previously reported pathogens. Such pH data is lacking for some game carcasses and their offals. Furthermore, this data has implications on the overall microbiological quality of meat, hence the need to investigate factors influencing post-mortem pH changes. Cases of lead contamination in cattle offal have been reported in Namibia in the past, but intriguingly, the spatial pattern and source of contamination has remained elusive (Midzi, 2012). Considering that some bullets used for harvesting game meat are lead-based, and the potential environmental contamination during the hunting process, it is necessary to assess whether this has effect on the quality of meat. In addition, lead and cadmium are some of the heavy metals contained in industrial waste which potentially contaminate the grazing pastures and the environment, in general. The highest concentrations of these chemical hazards occur in the liver and kidneys, organs involved in detoxification and excretion respectively. Therefore, it was imperative to examine the sanitary aspects of wildlife meat, potential contamination with food-borne microorganisms, zoonotic parasites and heavy metals in order to inform on control measures along the meat production chain requiring improvements. Although a greater percentage of the population may come into contact with game meat as opposed to live animals, this does not reduce the threat of live animals to public health, thus we selected to investigate primarily the food-producing springbok as a potential source of infection to humans.

## Objectives of current work

- i) Assess the microbiological quality of game meat and factors influencing offal pH
- ii) Assess meat safety by screening for selected food-borne bacterial pathogens
- iii) Evaluate the incidence of parasitic contamination
- iv) Examine contamination levels of selected chemical hazards
- v) Investigate whether rearing game at livestock-wildlife impacts on transmission of disease to humans.

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## CHAPTER 2

### LITERATURE REVIEW<sup>1</sup>

#### 1. The livestock industry and regulation of livestock by-products in Namibia

Namibia has a human population of approximately 2.1 million people of which 70% are directly or indirectly engaged in agriculture (Bishi & Kamwi, 2008; Mushendami *et al.*, 2008). The greater percentage of Namibian land falls under unfavourable arid and semi arid conditions, therefore, livestock farming is the predominant agricultural activity which accounts for about 90% of the total agricultural production (Mushendami *et al.*, 2008; Mwinga *et al.*, 2010). The commercial sector comprises 4 200 farmers on 6 337 holdings totalling 28.7 million hectares (Ha), thus an average of 6 800 Ha per holding which contributes to about 80% of the agricultural output (Bishi & Kamwi, 2008). The communal areas cover 30.8 million Ha and are utilized by some 150 000 households with user rights on cropping lands and communal rights to grazing land. Communal agriculture provides livelihood to 41% of all households in the country (Bishi & Kamwi, 2008; Sweet *et al.*, 2006). In communal areas, livestock plays multiple purposes in the sustainance of the people's livelihoods and wellbeing, among these, the provision of nutrition, labour, draught power, manure, milk, meat, household cash income from sales, and also acts as a form of storing wealth, socio-cultural support and food security. Income is generated mainly from the sale of livestock and their products within the community. Recent years have seen a greater economic emphasis placed on wildlife and the tourism sector by the government of Namibia and in some parts of the world. Consequently, small conservancies have mushroomed in rural areas (Van Schalkwyk *et al.*, 2010). To the rural community's advantage, this is bringing in additional income for some households especially where wildlife harvesting, photographic safaris, trophies and tourism take place (Lühl, 2010).

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**Magwedere K, Hemberger MY, Hoffman LC, Dziva F** (2012) Zoonoses: a potential obstacle to the growing wildlife industry of Namibia. *Infection Ecology & Epidemiology* 2. doi: 10.3402/iee.v2i0.18365. Epub 2012 Oct 15.

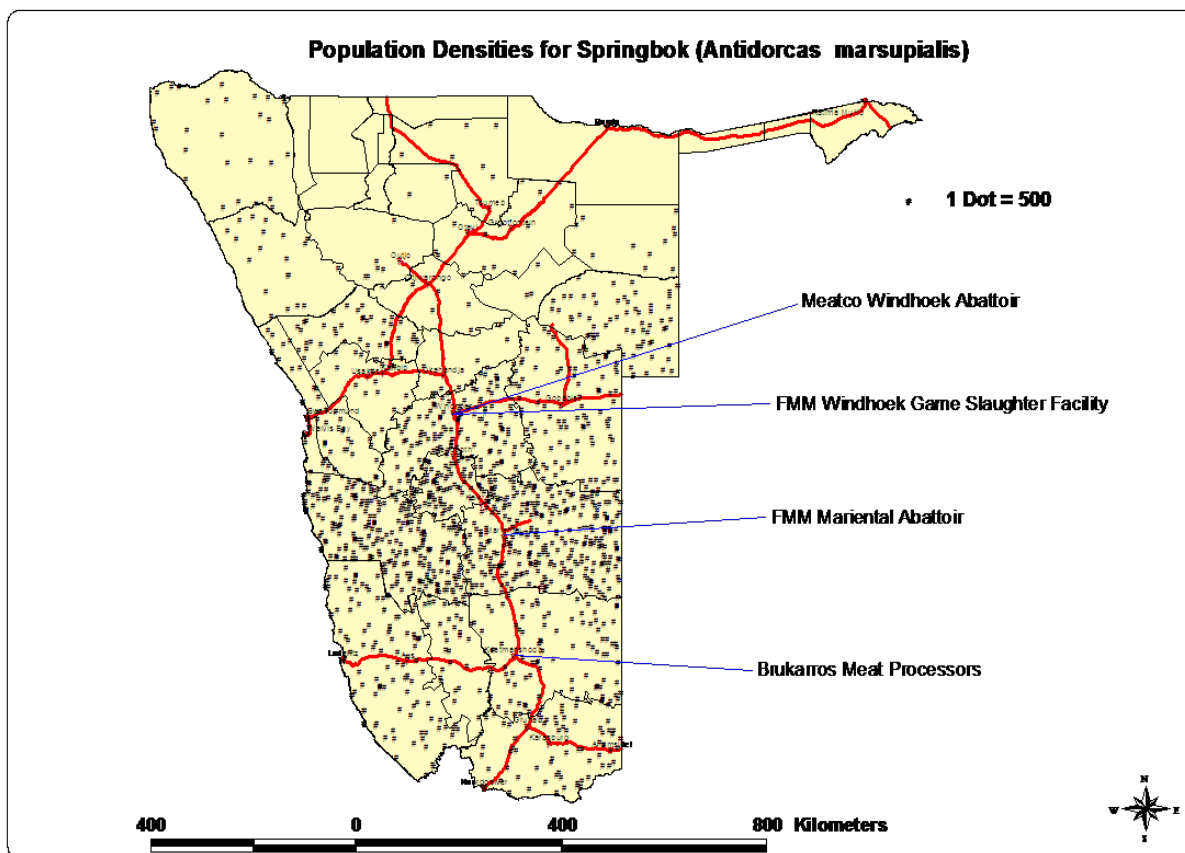
## **2. The wildlife industry of Namibia**

The overall national objectives, goals and aspirations are included in the national vision 2030 policy where the National Agricultural Policy places emphasis on the development of the livestock sector by providing expanded animal health, extension, research, training and advisory services, supporting the establishment of conservancies in communal areas and commercial rights over wildlife to freehold land owners in order to enhance productivity in the sector (Bishi and Kamwi, 2008). Maintenance of ecosystems, biological diversity and utilization of living natural resources on a sustainable basis is a requirement in the Namibian Constitution Act No. 34 of 1998 Article 95 and Nature Conservation Ordinance, Number 4 of 1975 as amended (MET, 2010; Schalkwyk *et al.*, 2010). The biodiversity initiatives are expected to contribute to national development and provides for an economically based system of sustainable management and utilization of game in communal areas (Richardson, 1998).

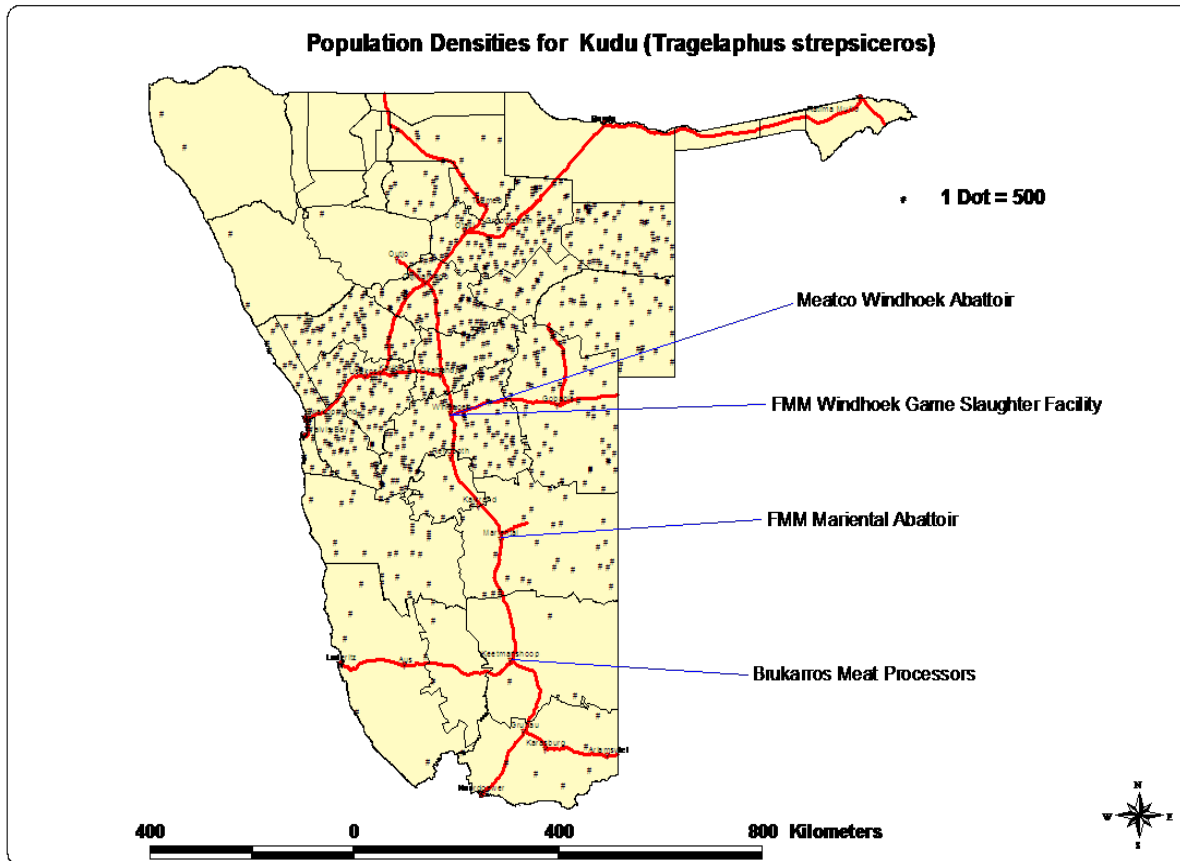
### **2.1. Distribution of food-producing wildlife species**

About 15-25% of private farmland is used for commercial game rearing primarily for: auctioning, hunting, harvesting, live game capture and wildlife viewing (Turpie *et al.*, 2010). There are approximately 400 registered commercial hunting farms, ranging from 3 000 to 10 000 ha (Turpie *et al.*, 2010). Over 90% of Namibia's large mammals occur outside protected areas with some 80% in privately owned commercial agricultural lands (Barnes 1995b; Richardson, 1998). In year 2009, gemsbok and springbok population estimates stood at 388 411 and 731 563 respectively (Van Schalkwyk, 2011). When land owners were granted rights to the wildlife, the numbers of harvestable wildlife mammals were estimated to have increased by some 70% and species diversity by 44% over a period of 45 years (Smith *et al.*, 2012; Van Schalkwyk, 2011). The economic contribution of wildlife is estimated to have increased from 5% to 11% of the total economic value of privately owned rangelands (Barnes & De Jager 1995; Turpie *et al.*, 2010). As a result, greater economic weight is currently placed on the wildlife industry and the tourism sector compared to livestock farming in Namibia and this has led to the establishment of numerous managed wildlife conservancies and to the spiralling of game farming units on

many private farmlands including some rural areas. Population of food producing wildlife mammalian species has been estimated at a minimum of two million (Van Schalkwyk *et al.*, 2012). In terms of numbers, the major wildlife species under current and future consideration for commercial game meat export are; springbok (*Antidorcas marsupialis*), gemsbok (*Oryx gazella*), kudu (*Tragelaphus strepsiceros*), mountain zebra (*Equus zebra hartmannae*) and red hartebeest (*Alcelaphus buselaphus*) (Van Schalwyk, 2011). The widespread distribution of springbok and kudu respectively in Namibia is shown in Figure 1 & 2.



**Figure 2.1:** The population densities and distribution of springbok in Namibia (MET, 2007; Van Schalkwyk, 2011).



**Figure 2.2:** The population densities and distribution of kudu in Namibia (MET, 2007; Van Schalkwyk, 2011).

## 2.2. Regulatory instruments for export of game meat

Safety of products of animal origin, trade and export of animal commodities and control of animal disease are increasingly governed by international, national and private standards (AU-IBAR, undated). To enact and enforce benchmarks for international harmonization and equivalents guaranteeing the trade of safe meat, the Republic of Namibia acceded to various international instruments since independence in 1990. Under Article 144 of the Namibian Constitution, such international agreements form part of the laws of Namibia (Mosoti *et al.*, 2006). These international instruments include the World Trade Organisation (WTO) Agreement on the Application of Sanitary and Phytosanitary (SPS) measures, the various World Organisation for Animal Health (OIE) and Codex Alimentarius

Commission standards and Article 16 of the Southern African Development Community (SADC) Protocol on trade relating to SPS measures (Mosoti *et al.*, 2006).

Sanitary regulatory requirements vary depending on whether the meat is for the local market only or both export and local markets. Meat for the local market is covered by the Public Health Act of 1919 and also by the revised draft Public and Environmental Health Bill of 2012 and Food Safety Bill of 2012 (Mosoti *et al.*, 2006). The Public and Environmental Health Bill and its draft regulations aim to provide a framework for a structured uniform health system within the Republic of Namibia, taking into account the obligations imposed by the Constitution and other laws on international, national, regional, district and local government levels. All these regulatory instruments are equally applicable to the wildlife meat industry.

For the export market, Namibia fulfils the basic animal health, public health and welfare requirements for the production of meat products as laid down in the relevant EU legislation and regularly undergoes inspections by the Commission's Food and Veterinary Office (FVO). The Namibian game harvesting standards circulars/guidance for the harvesting of game for meat export purposes incorporates all the requirements (Harmonization and equivalence) of international standards and main trading partners. Harmonization ensures that the same export requirements for the introduction of fresh meat are applied while equivalence recognizes the exporting country's measures as acceptable even if they are different from those of the importing country, so long as an equivalent level of protection is provided (WTO, 2006). Before meat and products can be introduced into the EU territory, certain rules are respected. Council Directive 2002/99/EC forms the legal basis for all animal health rules while the food hygiene laws are covered under Regulation (EC) No 852/2004, Regulation (EC) No 178/2002, Regulation (EC) No 854/2004 and Regulation (EC) No 853/2004, Commission implementing regulation (EU) No 739/2011, Commission regulation (EU) No 16/2012 and Commission Regulation (EU) No 150/2011, Commission regulation 206/2010; Council directive 2003/99/EC, South Africa Meat Safety Act of 2000 and its regulations and veterinary procedural notices (DAFF, undated; EU, undated). The key institutions with specific departments or agencies that share the mandate and competency on issues of SPS measures in Namibia are the Ministry

of Agriculture, Water and Forestry (MAWF), the Ministry of Health and Social Services (MOHSS), the Ministry of Trade and Industry (MTI), Ministry of Fisheries and Marine Resources (MFMR) and Ministry of Education (ME). Other organizations involved in SPS indirectly are the Namibian Agronomic Board, the Meat Board of Namibia, local municipalities and the Namibian Standards Institute (NSI). The key regulations covering livestock and livestock products are the Animal Health Act, 2011, Animal Diseases and Parasites Act, 1956, Medicines and Related Substances Control Act, 2003 (Act No. 13 of 2003) as amended, the Undesirable Residues in Meat Act, 1991 as amended, the Abattoir Industry Act, 1976 and the Registration of Fertiliser, Farm Feed, Stock Remedies and Agricultural Remedies Act, 1947 (Anonymous, 2010; Bekker *et al.*, 2012). Animal Health in Namibia is regulated and implemented primarily by the Directorate of veterinary services (DVS) and to some extent, by MOHSS, MFMR, MET and municipalities at secondary level. This legislation has broadly been adopted for wildlife meat despite the variance in the rearing, antemortem inspection and slaughtering practices.

### **3. Game meat production in Namibia**

#### **3.1. Demand and marketing**

The demand for game meat in developed and developing countries continues to grow as the consumption of such meat increases with available income and people's preferences (Daniel *et al.*, 2011). Namibia has a thriving wildlife population that is reared and slaughtered for both domestic and export markets, thus placing wildlife on a different platform from its traditional past. Because rearing and slaughter practices tend to differ from those of livestock species, concerns on meat safety have arisen and these have inevitably presented a challenge to the existing regulatory framework of the meat industry. About 75% of Namibian game farmers hunt wildlife for own consumption, and 15-25% of private farmland is used for commercial game production for ranching, hunting, live game capture and wildlife viewing (Turpie *et al.*, 2010). Approximately 275 tonnes of game meat were exported to South Africa and European Union between 2009 and 2011 through registered game export establishments (Van Schalkwyk, 2011). It is estimated that 4300



tonnes of game meat were produced annually in Namibia during the period between 2001 and 2005 (Laubscher *et al.*, 2007). Lindsey (2011) estimates that between 16 to 26 000 tonnes of game meat is produced annually on Namibian farmlands. Of this, oryx (*Oryx gazelle*), greater kudu (*Tragelaphus strepsiceros*) and springbok (*Antidorcas marsupialis*) contribute approximately two thirds of the total game meat produced on freehold farms in the form of trophy hunting, followed by own use with a relatively small proportion produced through harvest specifically for meat to sell e.g. “shoot-and-sell” and wildlife export harvesting (Lindsey, 2011). Wildlife products (meat, biltong and offal) are common food items for many communities worldwide despite national regulations in some countries prohibiting such consumption (Chaber *et al.*, 2010). Like Namibia, major markets for New Zealand venison are Europe and USA with 80% of the exports imported into Europe (Wild and Hunt, 2011). In recent years, wildlife meat exports to Europe from both South Africa and Namibia have increased steadily as a result of game meat producers satisfying the preference of discerning first world consumers of meat produced in a sustainable and eco-friendly manner. Moreso, the production meets ethical and safe harvesting methods, ensuring safety, wholesomeness and nutritive value (Hoffman *et al.*, 2010; Hoffman, 2010). Concurrent with expansion of wildlife in Namibia is a decline in free range domestic animal farming, particularly sheep and cattle resulting in some export abattoirs processing game meat species (Figure 3) during the April to August months where the seasonal supply of beef and sheep is low to make use of their under-utilized processing facilities (Van Schalkwyk, 2011). Clearly, this highlights a high demand for the organically-produced game meat.



maintainance of consumer confidence and market position (Ehiri *et al.*, 2001; Food safety, 2006). Any person with intent of harvesting or processing a game animal, game animal carcasses and game animal meat for sale is required to do so after receiving approval upon application. An application for registration is approved in accordance with the provisions stipulated by the Chief Veterinary Officer. Upon receiving the application for registration, the state veterinarian or his deligate will assess the documents, establishment, equipment, premises or facilities and then make a determination on whether regulatory requirements or set standard(s) are met (Van Schalkwyk & Hoffman, 2010).

Abattoir state veterinarian requires animal health decleration records for the offloading of the harvested game. It is therefore imperative that any game animal producer or game harvester request field ante-mortem or game animal health inspection service of a farm or area and receives approval from the local state veterinarian or the designee prior to the commencement of game harvesting (Anonymous, 2000; Van Schalkwyk, 2011). Initial application for field game animal farm inspection service will be made by an official harvesting team leader to the state veterinarian in a prescribed form. The applicant requesting inspection furnishes the certifying veterinary official such production and health information as may be required on forms provided by the field state veterinarian (OIE, 2002). Upon receipt of the completed application or request, the state veterinarian schedules a farm/area inspection for approval (Codex, 2005; OIE, 2002). After the inspection, an inspection report is forwarded to the harvester or an official responsible for the certification of the game meat (Van Schalkwyk & Hoffman, 2010).

An approved harvester will submit a harvesting program (comprising of the following information: date of intended harvest; name and contact details of harvesting team leader; name and registration number of the farm(s)/area, game meat inspector's name, name of receiving export establishment/processing plants with contact details, species and numbers to be harvested) to the nearest state veterinarian office before harvesting commences. It is a voluntary good practice requirement for all immediate farm owners are to be notified at least seven days before the intended harvest (Codex, 2005; Van Schalkwyk,

2011; Van Schalkwyk & Hoffman, 2010). Export approved field mobile harvesting depots have standard operating procedure manuals based on HACCP principles verified at the harvesting field depot. All employees should be trained on aspects of general hygiene and specific work instructions upon recruitment and thereafter at least once on a yearly basis. Standardized process hygiene items are monitored by the harvesters during harvesting and processing in compliance to the requirement in the harvester standard operating procedure (SOP) manual (Van Schalkwyk & Hoffman, 2010).

To limit the impact of temperature and flies, game harvesting is done at night and during the winter season (April to August) to a greater extend. During this period, night environmental temperature is always low (below 12°C) thereby limiting the influence of temperature and pests on the quality and sanitary condition of the carcasses. Harvesting vehicles registered by the competent authorities are allowed to harvest for export (Van Schalkwyk & Hoffman, 2010). On the day of official hunting, each participating harvesting truck is inspected to confirm the availability of chemical sterilizer and cleanliness, running warm and cold water, working lights, clean protective clothing for hunting assistants and disinfectant among other requirements. Field water is chlorinated using liquid sodium hypochlorite or other approved food grade disinfectants. During commercial harvesting, the wild animals are flushed out of the bush by harvesting vehicles in freehold camps on dark moonless nights, followed and shot in the head or cranial part of the neck at a close range after being blinded with a strong light. Light caliber rifles with telescopes are used to secure minimal meat loss from bullet wounds.

In the field, shot animals are picked and bled while on the truck rails within 10 minutes. Hygienic evisceration of some rough offal (stomach, intestines and rectum) is done in the field within 20-60min after bleeding time and the killed animals are brought to the field harvesting depot within 2-3 hours of bleeding for further red offal (lungs, heart, liver and spleen) evisceration and storage in a temperature controlled refrigerated truck. Alternatively, the harvested animals are directly transported to the abattoir for further evisceration and dressing without passing through the harvesting depot. Animals with

head or neck shorts are generally accepted as suitable for possible further processing for export. Meat samples collected at the harvested game abattoir are from traceable carcasses and while skin on, had been tagged and kept in the field mobile abattoir refrigerated truck and permanent abattoir for 16-72 hours at cooling unit temperatures above 2°C for 24 hours and carcasses temperatures below 7°C from 24 hours onwards before being processed and deboned. Carcasses which had passed the post-mortem inspection and without visible contamination qualify for export status and further microbiological sampling.

### **3.3 Good hygiene practices and meat safety assurance system approach in reducing hazards of harvestable wildlife in Namibia**

Namibia has an integrated approach to food safety in the livestock sector that aims to assure a high level of food safety, animal health, animal welfare, farm management, feed and licks, identification and traceability, environmental protection, documentation and conformity assessment through coherent farm-to-table measures (Hoffman & Lühl, 2012; Anonymous, 2010; OIE, 2002). The cornerstone of this approach is a Public-Private partnership between the government and the meat industry which drafted the Farm assured Namibian meat scheme (FANMeat) standards for livestock producers. The FANMeat is a quality assurance system for animal production food safety and aims to assist farmers to meet their responsibilities to produce safe food of animal origin. All producers who are members of the FANMeat are required to be in possession of a copy of the FANMeat standards and must be able to show it. The guide provides the guidelines and requirements that the producer needs to follow to implement the FANMeat program on their farm while the conformity assessment provides the set of procedures and/or criteria which are used by a producer or auditor to assess the conformance of the individual producer to the requirements of the FANMeat (Anonymous, 2010). The mandate of the FANMeat scheme is with the meat industry and the verification competence is with the government regulatory services where the later audit every farm at least once per year and the auditing results are forwarded to the farmer and meat industry for issuing corrective action.

### **3.3.1 Chilling of partially dressed game carcasses**

Chilling is used successfully as a critical control point based on the efficacy of the chilling process to reduce the number of bacteria on meat irrespective of variations in the capabilities of chillers and chill-carcass cycles (Lenahan *et al.*, 2009; Bekker *et al.*, 2011). The physiology of the meat product influences the likelihood of pathogens to be able to adhere and survive over time on the product (Paulsen *et al.*, 2011; Laury *et al.*, 2009). Partially dressed carcasses and offal must be chilled within 4 hours of harvesting and without maturation, a carcass core temperature of 7°C should be accomplished within 24 hours after chilling commences (Anonymous, 2000; Codex, 2005).

### **3.3.2 Transport of partially dressed game carcasses**

The chilling unit of a vehicle used for the transport of partially dressed carcasses should have the potential to chill such carcass to a temperature of less than 7°C within 24 hours of having been loaded (Anonymous, 2000). The refrigerator unit's setting should preferably be set above 2.0°C and below 3°C for the first 24 hours and thereafter at 0°C to achieve the desired carcass temperature. However, for carcasses and offal destined for the domestic market, to achieve temperatures below 7°C within 24 hours, some harvesters set their cooling vehicle temperatures initially between 0 to -5°C for the 1<sup>st</sup> few hours until after loading of harvested game is completed and thereafter at -1°C to 2°C depending on the environmental temperature and season (Ehiri *et al.*, 2001).

### **3.3.3 Receiving of partially dressed game carcasses at an export game abattoir**

All partially dressed game carcasses received at a game abattoir are accompanied by an inspection report from the registered inspector at the harvesting depot, except if an abattoir is situated on the game farm where harvesting is done. Partially dressed game carcasses received at a game abattoir are offloaded and moved to the holding chilling unit without delay (Anonymous, 2000; EU, 2004a).

### **3.3.4 Identification and traceability**

Livestock must be identified so as to trace their products from them individually or as a group back to the farm of origin (Anonymous, 2010 & 2011). A registered game harvesting team submits a harvesting program to the competent authority prior to commencement of game harvesting. On the day of harvesting on approved farms, each game animal harvested is marked with a tag bearing the hunter's name and order number of arrival at the field harvesting depot. The tagged carcasses and its red offal are loaded into the refrigerated truck by the harvesting team. A game harvesting control document is completed per harvest and or every farm and every delivery to the processing export abattoir. The harvesting control document at minimum indicates the: name of the hunting team, hunting location, nature conservation harvesting permit number, competent authority field inspector, name of meat examiner, date of hunting, name of farm owner, number of game booked, tag numbers of harvested game and truck seal number. Offloading and dressing at the game export abattoir is done according to game carcass owner and tag numbers. After dressing, the carcass is weighed and marked by a computer generated tag indicating abattoir estimation number, carcass number, slaughter date, product type, batch/lot number and weight. Carcasses are placed on the market with the computer generated tag. For carcasses destined for further processing (deboning), the tag is scanned on the deboning computer for every carcass that enters the meat cutting room and all the tracing information on the tag is stored in the deboning computer. The serial number and slaughter date is then reflected on the traceability record printed from the deboning computer when carcass scanning took place. After deboning, the carcass serial number is recorded on the production sheet, which also displays the traceability code which also includes freezing/production date and expiry date. From the position of the serial number concerned on the boning list, the time which the carcass is packed can be calculated based on the number of carcasses processed per unit time of which the calculated time and traceability code on the production sheet will indicate the carton that contains the carcass in question (Commission regulation, 2011; EU, 2004a; Commission regulation, 2012). The meat cartons, displays the traceability code as indicated by the production sheet and the production time. Although it is a requirement that every livestock owner must have his/her

registered stock branded in Namibia, this does not apply to game animals. Game owners tend to fence their farms to an extent that prevents escape of their animals.

### **3.3.5 Water quality**

Each field harvesting depot should have an adequate supply of drinking quality water for cleaning and sanitation purposes. In order to receive an inspection, all harvesting teams must provide competent authority personnel with documentation certifying that the supply of water complies with the drinking water standards. The harvesting team may operate at a location where it can directly utilize either a municipal water supply or well/borehole water. Alternatively, it is permissible to transport water in a water holding tank to the harvesting location as long as there is a water report certifying the potability of the water source. This documentation is made available for review at all operational locations before initiating game harvesting activities at the specific site. For a private well/borehole, this documentation is to be renewed annually for any recurring game harvesting location (Council Directive 98, 1998; Van Schalkwyk, 2010; Anonymous, 2005).

### **3.3.6 Cleaning and sanitation requirements for game harvesting teams**

Game harvesting establishments are required to develop, implement, and maintain written sanitation standard operating procedures (SSOP) (Anonymous, 2000; EU, 2004 & 2004a; Kamwi, 2007). The SSOP describes all procedures an official harvesting team will conduct at every harvest, before and during harvesting, sufficient to prevent direct contamination of the harvested meat (Anonymous, 2000 & 2012; Chukwuocha *et al.*, 2009). Stepwise procedures that are to be conducted prior to operations are identified as such, and address at a minimum; the cleaning of harvested game contact surfaces of facilities, equipment, and utensils (Anonymous, 2000 & 2012; Allwood *et al.*, 2004). The SSOP specify the frequency with which each procedure is to be conducted (Anonymous, 2012). The effectiveness of the SSOP and the procedures therein in preventing direct contamination of harvested game is routinely evaluated.

## **4. Microbiology and potential obstacles to the wildlife meat industry**



Transmissible diseases should be considered as one of the potential obstacles to this emerging industry. Around 60% of all human pathogens are zoonoses that are equally harboured by domestic and wild animals (Allen *et al.*, 2012; Bekker *et al.*, 2012). Of the emerging infectious diseases, 75% of these are zoonoses predominantly associated with wildlife animals, clearly highlighting an increasing threat arising from these animal species (Allen *et al.*, 2012). Because rearing and slaughter practices tend to differ from those of livestock species, the likelihood of encountering these tends to increase since there is no ante-mortem inspection. In Namibia, a wide variety of neglected tropical zoonotic diseases have recently been reviewed (Noden & van der Colf, 2012), and a potential threat of some of these zoonoses to the wildlife meat industry exists. The risks caused by consumption of game meat are primarily associated with lack of hygiene during processing and with unrecognised zoonotic diseases which can be transferred to humans consuming the meat. Important zoonotic risks in wildlife mammalian species capable of infecting the greatest number of genera include gastrointestinal zoonotic pathogens (*Salmonella* spp, STEC, *Yersinia enterocolitica*, *Yersinia pestis*, *Clostridia* spp, *Campylobacter* spp, *Toxoplasma* spp ), *Trichinella* spp, *Staphylococcus aureus*, *Brucella* spp, *Leptospira* spp, *Franciella tularensis*, *Mycobacterium bovis*, prions, Hepatitis E, Phlebovirus, Lyssavirus, Influenza A viruses, *E.granulosus*, *Chlamydia* spp, *Borrelia* sp (Bekker *et al.*, 2012; Borremans & Belmain, 2012; FAO, 2012; Hotez & Kamath, 2009; Katakebwa *et al.*, 2012; Pavlin *et al.*, 2009; Paulsen *et al.*, 2011).

Heavy metals warrant special attention because of their vast global distribution and high potential toxicity coupled with members of the animal kingdom, including humans, that ingest soil either involuntarily or deliberately (the latter practice being known as geophagy or geophagia) (Abrahams, 2011). Indeed soil (geophagia), water and plant material are the main sources of minerals for wildlife (Mincher *et al.*, 2008; Belli *et al.*, 1993; Mahaney *et al.*, 1990). Lead and cadmium are the heavy metals routinely monitored in Namibia livestock industry. In livestock meats (muscle, liver and kidneys), toxic cadmium and lead occurs in organ meats (Ambushe *et al.*, 2012; Dzoma *et al.*, 2010; Falandysz, 1994; Midzi, 2012).

The impact of zoonoses and food safety issues on human and animal health and welfare can not be emphasized enough. A growing world population requires more food, especially safe and wholesome sources of protein (Karesh *et al.*, 2005). As a result, food security and safety issues have taken centre-stage on the global platform—all geared to safeguard human health. Since some zoonoses are notifiable diseases, these consequently impose a huge economic burden on farmers through compulsory slaughter, loss of access to export markets and the local meat industry (Anonymous, 2000; Bekker *et al.*, 2012; EU, 2002; OIE, 2002). With ruminant wildlife species increasingly entering the human food chain, coupled with a thriving managed wildlife for tourism purposes in Namibia, it is prudent to examine the extent to which such selected diseases may affect this emerging industry.

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## CHAPTER 3

### **Microbiological quality of springbok meat and factors contributing to post-mortem pH changes in selected wildlife species edible offal<sup>1,2</sup>**

#### **1. INTRODUCTION**

Meat and edible offal add to a range of foods which are nutritiously attractive. The edible offal is highly prized in South East Asia and Africa, while demand is variable and low in Australia and USA respectively, (Fatma & Mahdey, 2010; Pearson & Dutson, 1988) and in slaughtered animals edible offal contributes approximately 33% of the edible material (Aduku *et al.*, 1990).

It is estimated that about 75% of the emerging human infectious diseases arise from animal reservoirs (Allen *et al.*, 2012) of which rift valley fever (RVF) has been placed at number three on the list of the 17 most dangerous animal threats after Foot and Mouth disease (FMD) and Influenza (Mandell & Flick, 2011). Within the animal-human interface, transmission of zoonotic foodborne pathogens frequently depends on factors such as food consumption habits and level of processing (El-Nesser *et al.*, 2007; Fatma & Mahdey, 2010; Michael *et al.*, 2011; USDA, 2011).

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**Magwedere K, Hemberger HY, Hoffman LC, Dziva F (2012)** Investigation of the contributing factors to post-mortem pH changes in springbok (*Antidorcas marsupialis*), eland (*Taurotragus oryx*), red hartebeest (*Alcelaphus buselaphus*) and kudu (*Tragelaphus strepsiceros*) edible offal. *Journal of the South African Veterinary Association*. Inprint.

pH below 6.0 is commonly used to destroy some dangerous animal pathogens and ensure the safety of livestock products (OIE, 2012). The pH of meat is important for good meat quality and for inactivating viral and bacterial animal microbes, a number of which are endemic in southern Africa (European Commission, 2001; Falenski *et al.*, 2010; Fatma & Mahdey, 2010). However, *invitro* effects of pH on pathogens in the laboratory and on meat under natural conditions have been reported not to be correlated due to possible differences in ideal temperature, water activity, presence of competitive microorganisms under field conditions and a variety of other factors (Bouvier, 1960; Fatma & Mahdey, 2010; IFT/FDA, 2003; Sadler, 1960).

RVF is a serious livestock and public health threat while FMD is a disease of socioeconomic importance (Hyslop, 1970; OIE, 2012). Inactivation of the RVF and FMD viruses in skeletal muscle and heart muscle occurs rapidly after animal death as a result of lactic acid formation which accompanies rigor mortis and causes the pH to drop to levels between 5.5 and 6.0. (AVMA, 2010; Bachrach *et al.*, 1975; Blackwell, 1984; Bengis & Veeary, 1997; Evans *et al.*, 2008; OIE, 2011; Pharo, 2002; Scott, 2003). *Brucella melitensis* and *Brucella abortus* have been detected at varying counts in edible offal (Fatma & Mahdey, 2010). The number of *Brucella* organisms per gram of muscle is normally small and rapidly decreases with the drop in meat pH (European Commission, 2001). The survival period of *B.abortus* in yogurt at pH 3.60 and 3°C is one day and while the optimal pH range of brucella is between pH 6.6 and 7.4 at 37°C, the maximum pH is 8.4 and the minimum is 4.1 (Falenski *et al.*, 2010; ICMSF, 1996; Lerche & Entel, 1959; Zobell & Meyer, 1932).

Several studies have been undertaken to evaluate the microbiological quality of game and their by-products (Atanassova *et al.*, 2008; Gill, 2007; Holds *et al.*, 2008; Membré *et al.*, 2011; Wahlström *et al.*, 2003; Van Schalkwyk *et al.*, 2011; Van der Merwe *et al.*, 2011). The total aerobic counts (APC) for gemsbok, kudu, springbok, zebra, beef and mixed game salami were found in the range of 7.11- 8.12 log<sub>10</sub> colony forming units (cfu) per gram, with 1.50-2.80 log<sub>10</sub> cfu/g representing *E. coli* (Van Schalkwyk *et al.*, 2011). Of particular significance, springbok salami had the highest coliform and *E. coli* counts with peak counts

of  $3.22 \log_{10}$  cfu/g and  $2.80 \log_{10}$  cfu/g respectively (Van Schalkwyk *et al.*, 2011), suggesting a possible breach in the overall level of bacterial contamination compliance during harvesting and or processing stages. *Enterobacteriaceae* organisms are readily inactivated by sanitizers and the count reflects the level of environmental hygiene (Cox *et al.*, 1988; Gabis & Faust, 1988) and hence can be used as an evaluation tool for good manufacturing practices (Van Schothorst & Oosterom, 1984). Whilst APC and *Enterobacteriaceae* counts provide information on quality, safety of meat can be assured by ascertaining the absence of foodborne pathogens and or antimicrobial resistance. Antimicrobial effects of lactates have been investigated in meat and meat products, and their efficiency against many pathogens and spoilage microorganisms without posing any health risk for consumers has been established (Bingol & Bostan, 2007; Koos, 1992; Shelef, 1989; Debevere, 1989; Lemay *et al.*, 2002). There are many factors influencing post harvesting concentration of natural antimicrobial lactic acid (pH) of game meat, among others the slaughter method, stunning, bleeding, the initial microbiological load, deboning, packaging and storage conditions (Hoffman *et al.*, 2007 & 2009). The objective of this study was therefore to investigate the microbial quality and the contributing factors to post-slaughter pH changes in selected wildlife species

## **2. MATERIALS AND METHODS**

Harvesting of wildlife species for pH measurements and microbiological sampling were done independently.

### **2.1 Laboratory analysis**

The samples were analysed for their microbiological (APC, *Enterobacteriaceae* and *E.coli*, coliforms, HPC, *Clostridium perfringens* and enterococci) and physicochemical characteristics (pH).

## **2.2 pH and temperature measurement**

The pH and temperature were measured using a testo model 205 (Testo AG, Germany) temperature and pH meter with automatic temperature compensation. The selection of farms included in this study was based on application by owners for game harvesting for commercial purposes. For eviscerated carcasses with skin on, meat pH was measured 16 to 34 hours post-slaughter by inserting the probe in the middle of the *Longissimus dorsi* and for edible offal organs, the pH meter probe was inserted into the organ. Calibration of the testo meter was done using standard calibration solution at the beginning of the measurements and after every five readings.

## **2.2 Harvesting of wildlife species for pH measurements**

### **2.3.1 Winter night harvesting**

The springbok were harvested between July and August 2010. Average night temperatures ranged from 0 to 17°C. Relative humidity is defined as the amount of water vapor in a sample of air compared to the maximum amount of water vapor the air can hold at any specific temperature in a form of 0 to 100%. Relative humidity was measured using a hygrometer (Hygrocheck, Hanna Africa) and verified by readings from Intellicast the authority in weather (RH, undated). Average maximum and minimum humidity was 50% and 7% respectively. pH measurements were taken from five consignments of springbok harvested on different days and delivered to the abattoir at a minimum of 10 carcasses per group. In total, 55 harvested springbok carcasses and organs (liver, lung, heart, spleen and kidney) were selected from visually inspected and passed carcasses.

### **2.3.2 Summer daylight harvesting**

The harvesting was done on one farm and the random effects of the three harvesting camps on the farm were assumed to be normally distributed. Species under consideration were eland (*Taurotragus oryx*), red hartebeest (*Alcelaphus buselaphus*), springbok (*Antidorcus marsupialis*) and kudu (*Tragelaphus strepsiceros*). All red hartebeest were males except for one female, springbok were females except for two males, kudu were females and eland were males except for one female. All animals were mature. The game harvesting was done

during the day and during the summer month of February. Average day temperatures ranged from 22 to 31°C. A progressive decrease in the relative humidity from morning to evening was observed with a maximum humidity of 84% at approximately 07H20 and a minimum humidity of 23% at approximately 17H00. All harvested game included in this study were healthy. The shooting position was consistently the upper neck and head. In springbok, evisceration of the stomachs and intestines (white offal) was done one hour fifteen minutes after bleeding while in the hartebeest, Oryx and Kudu, it was done at bleeding soon after shooting (Van Schalkwyk *et al.*, 2011).

### 2.3.3 Body Condition Scoring System

All harvested animals were given a visual body condition score as depicted in Table 3.1.

**Table 3.1:** Assessment of body condition criteria at bleeding using the rating system outlined below (Gerhardt, 2003).

Ribs	1	Deep grooves between ribs, even immediately behind shoulder
	2	Ribs fairly well covered immediately behind shoulder (by the <i>Latissimus dorsi</i> ).
	3	Can still feel ribs, but grooves are not deep
	4	Ribs nearly flush with tissue between them.
Hips/spine	1	Hip bones very distinct (no back fat). Spine very distinct.
	2	Some padding over hips. Spine very distinct.
	3	Hips fairly well padded. Spine partly covered along each side.
	4	Hips well padded. Spine is flush with or nearly covered with fat.

The selection of variables was based on previous post mortem observations during game harvesting inspections. The time (in minutes) taken chasing the identified wild animal until shooting as well as the time from shooting to severing blood vessels (carotid and jugular) in the neck below the jaw line by means of a deep cut with a sharp disinfected knife was recorded. The liver and lung temperature and pH were recorded at 4 hours from the point of severing of blood vessels in the neck soon after the red offal (heart, liver, kidney, spleen,

lungs, trachea and esophagus) evisceration. Liver and lung congestion was scored using the rating system of the quantity (ml) of blood collected in a graduated tube from a longitudinal cut across the whole organ followed by organ squeezing for 2 minutes. The cut was made on the gastric surface of the liver and dorsal surface of the lung. Liver and lung organs that had blood on the incision line after 2 minutes which did not flow into the tube were given a score of 0.09ml while organs with no blood were given a score of 0 ml.

## **2.4 Microbiological analysis of harvested springbok carcasses and water**

To exclude the influence of temperature on microbial growth during transit in suitable cooling vehicles with temperatures set at 3°C, carcass temperatures were taken from a minimum of 3 carcasses in each truck location (front, middle and rear) at delivery by placing a Testo penetration thermometer (Testo AG, Lenzkirch, Germany) into the internal rump muscles. A batch was defined as a hunting day's delivery consignment with numbers varying between 73 and 235 springbok carcasses. A total of 52 batches were harvested over the 2 years. Only one batch of harvested game was processed per day before any domestic species were slaughtered at the same abattoir. A total of 2324 out of 6185 springbok harvested in 2009 and 1401/5906 springbok in 2010 that passed the routine meat inspection process (i.e. initial export criteria) were considered in the microbiological analysis.

### **2.4.1 Carcass sampling and processing for APC and *Enterobacteriaceae***

The sampling frequency in carcasses passing initial export criteria was determined by selecting the 6<sup>th</sup> carcass from each of the 9 batches (2009) and 7 batches (2010) restricted to a total of 5 carcasses per batch. Approximately, 5 x 2 cm<sup>2</sup> was sliced using a sterile knife from each of the 4 sites (flank, lateral rump, brisket and breast) and pooled into a sterile Stomacher® plastic bag, giving a total of 45 pooled meat samples in 2009 and 35 in 2010. The APC and *Enterobacteriaceae* counts were determined using standard procedures described elsewhere (van Schalkwyk *et al.*, 2011). Briefly, 25g (representing 40cm<sup>2</sup> surface area) of sliced meat was homogenized in 225 ml Butterfields' Phosphate buffer (BPB) using a Stomacher® 400 Circulator (Seward Ltd, West Sussex, UK) to give a 1:10 dilution and



kept on ice. Serial dilutions were prepared according to the International Organization for Standardization (ISO) methods for the preparation of dilutions (ISO 6887-1) using 1 ml of the homogenate. The APC was determined by the pour-plating protocol (ISO 4833) and *Enterobacteriaceae* were enumerated according to ISO 21528-2:2004. Results were presented as the mean  $\log_{10}$  cfu/cm<sup>2</sup> of 5 carcasses of each sampled batch. Batches exceeding the *Enterobacteriaceae* (2.50  $\log_{10}$  cfu/cm<sup>2</sup>) level were re-sampled for pathogenic *E. coli*.

#### **2.4.2 Microbiological analysis of field water used in game processing**

To exclude any carcass contamination due to processing water, 23 samples of farm tap water used in harvesting and processing were collected for determining heterotrophic plate count (HPC) and faecal pollutants (Reasoner & Geldreich, 1985). Briefly, 1L volumes were collected in sterile plastic bottles containing 1 ml of 10% sodium thiosulfate (to neutralize residual chlorine) after a 2 min flow to waste. Samples were transported in refrigerated(ice packs) cooler boxes to the laboratory and processed within 24 hours for HPC and selected faecal bacterial pollutants (coliforms, *Clostridium perfringens* and enterococci) following filtration of 100 ml volumes of water through Millipore HA membranes (47 mm, 0.45  $\mu$ m pore size; Merck Millipore, Bedford, Massachusetts, USA). HPCs were obtained after placing filter membranes on R2A agar (0.05% yeast extract, 0.05% Proteose Peptone, 0.05% Casamino acids, 0.05% glucose, 0.05% soluble starch, 0.03% K<sub>2</sub>HPO<sub>4</sub>, 0.005% Mg<sub>2</sub>SO<sub>4</sub>.7H<sub>2</sub>O and 0.03% sodium pyruvate; Reasoner & Geldreich, 1985) and faecal pollutants were detected by placing filter membranes on Lactose TTC agar (Merck) for coliforms; m-CP agar (Oxoid) for *Clostridium perfringens* and Slanetz and Bartley agar (Oxoid) for enterococci. The plates were incubated at 22°C and 37°C under appropriate atmospheric conditions for each bacterial species for up to 7 days as recommended (Reasoner & Geldreich, 1985; Reasoner, 2004). Where required, coliforms were enumerated according to the ISO 9308-1:2004, whilst those of *Clostridium perfringens* and enterococci bacteria were done according to ISO 8199:2005 and ISO 7899-2:2004, respectively.

## **2.5 Statistical Analysis**

For microbiological quality and carcass temperatures, statistical analysis was done by one way analysis of variance (SAS 9.1.3 ©2002-2003, SAS Institute Inc. Cary, NC, USA) and differences were considered significant at  $p < 0.05$ . LSD (Least Significant Difference) and Tukey's Studentized Range (HSD) were used as multiple comparison tests. The effect of incubation temperature on HPCs was tested using a Student's *t*-test where  $p < 0.05$  was considered significant.

For winter night harvesting, student *t*-test and one way analysis of variance (ANOVA) using the Statistical Analysis Systems (SAS) 9.1.3 (SAS Institute Inc. Cary, NC, USA ©2002-2003) was used to evaluate pH measurements between organs and reference pH value with significance at  $p < 0.05$  and  $p < 0.01$  respectively. Data are expressed as mean  $\pm$ SD.

For the summer daylight harvesting, all analysis was done using Stata version 10 (Stata Corp, Stata statistical software, version 10, College Station, Texas, USA). Descriptive statistics describing all variables was used on the data. Linear regression was used to evaluate the association between pH after 4hrs (outcome) and the predictor variables. Univariate analyses between pH after 4 hrs and all the potential predictors (time from chasing to shooting, shooting to bleeding time, offal temperature at four hours post- slaughter, liver congestion, lung congestion, body condition score, environmental temperature, humidity, species, liver and lung pH at four hours post slaughter) were first evaluated to detect those variables that were statistically significant. This was followed by fitting multi-variable models evaluating the association between pH after 4hrs and the identified predictors of interest controlling for potential confounder variables. A  $p < 0.05$  was considered significant. Model diagnostics was conducted to evaluate the assumptions of homoscedasticity and normality of residuals.

## **3 RESULTS**

### **3.1 Microbiological condition of carcasses**

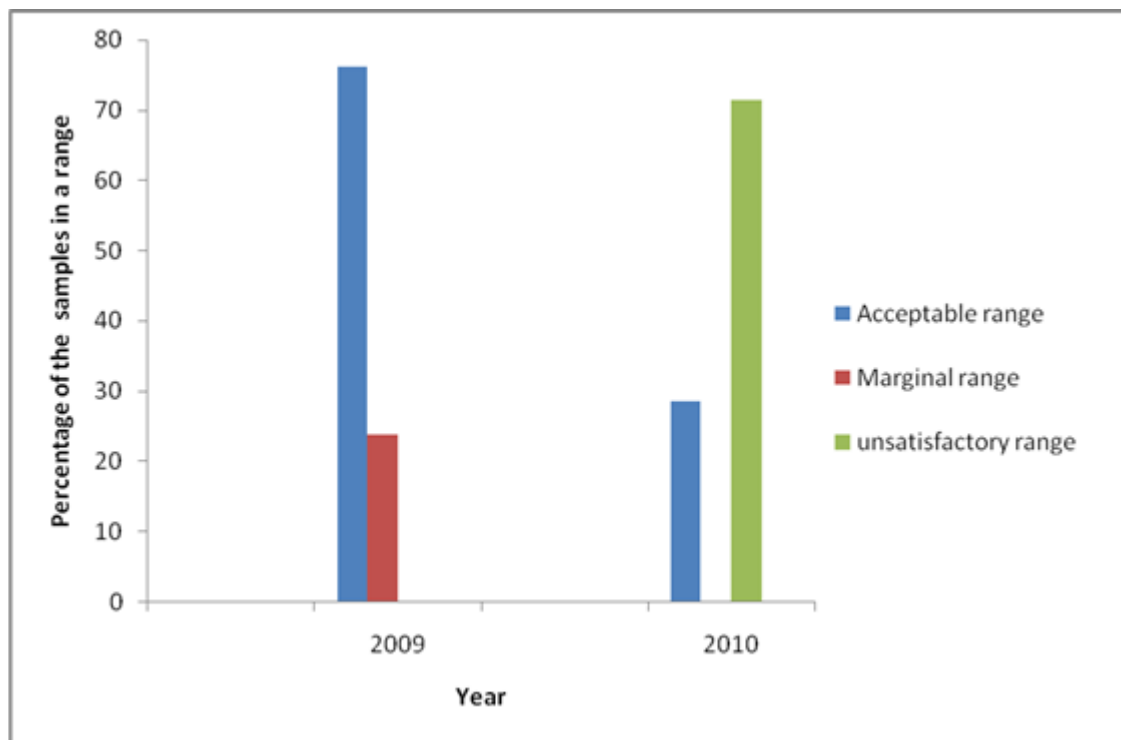
The APC values for 2009 ranged between 1.79 and 3.36  $\log_{10}$  cfu/cm<sup>2</sup> giving a mean of  $2.58 \pm 1.02$  whereas those for 2010 were between 2.23 and 5.26  $\log_{10}$  cfu/cm<sup>2</sup> with a mean of  $3.49 \pm 1.36$  (Table 3.2). However, there was no significant ( $p > 0.05$ ) differences in the mean APC values between the two years. On other hand, the *Enterobacteriaceae* counts ranged between 0.8 and 1.86  $\log_{10}$  cfu/cm<sup>2</sup> for 2009 with a mean of  $1.33 \pm 0.69$ , and for 2010 the counts were between 1.54 and 4.32  $\log_{10}$  cfu/cm<sup>2</sup> giving a mean of  $2.93 \pm 1.50$ . Statistical differences ( $p < 0.05$ ) were obtained between the mean *Enterobacteriaceae* count for 2009 and 2010.

**Table 3.2:** Mean level ( $\pm$ SD) of Aerobic Plate Count (APC) and Enterobacteriaceae in springbok carcasses samples collected in 2009 and 2010 at the abattoir sorting area before chilling.

Year	No. of batches sampled	Mean APC count (cfu/cm <sup>2</sup> )	Mean <i>Enterobacteriaceae</i> count (cfu/cm <sup>2</sup> )	Number of batches exceeding the maximum limit for <i>Enterobacteriaceae</i>	No. of samples from re sampled batches from which <i>E coli</i> was isolated
2009	9	$2.58^a \pm 1.02$ (n=45)	$1.33^c \pm 0.69$ (n=45)	0	-
2010	7	$3.49^b \pm 1.36$ (n=35)	$2.93^c \pm 1.50$ (n=35)	5	18
Total	16	-	-	5	18

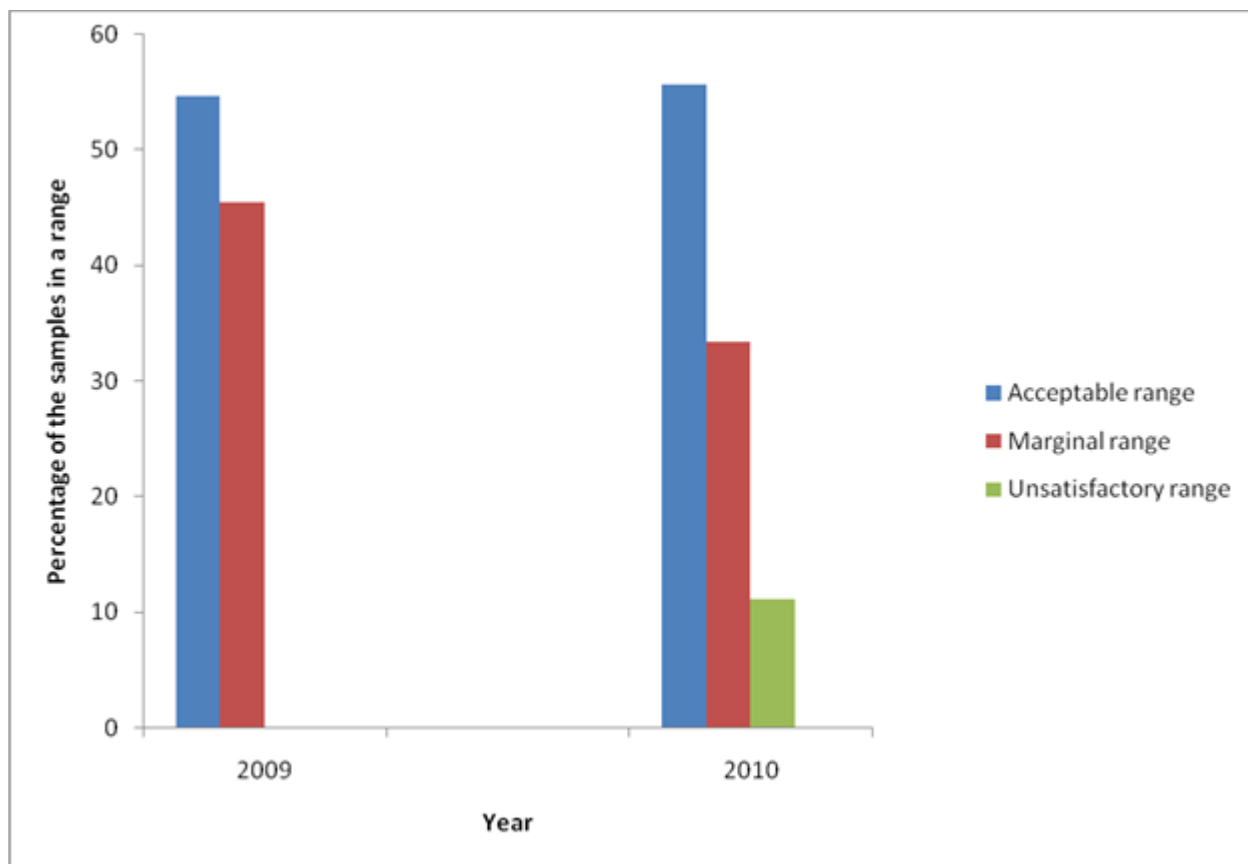
<sup>a, b, c</sup> Within columns, different superscript letters indicate that values were significantly different ( $p < 0.05$ ). 'M' is the upper bacterial contamination limit ( $> 5.0 \log_{10}$  cfu/cm<sup>2</sup> for APC and  $> 2.5 \log_{10}$  cfu/cm<sup>2</sup> for *Enterobacteriaceae*) for which batches above this level qualify for automatic export rejection (Commission regulation No1441, 2007).

Twenty five carcass samples belonging to 5 batches harvested in 2010 contained *Enterobacteriaceae* counts above the 'M' limit of  $2.50 \log_{10} \text{ cfu/cm}^2$ , contributing to a mean count of  $2.93 \pm 1.50$ . The 'M' value refers to the unsatisfactory microbiological value above the marginally acceptable quality. However, variability in the percentages of tested carcass samples for *Enterobacteriaceae* was noted when the APC and *Enterobacteriaceae* results for 2009 and 2010 were presented in accordance to the process hygiene criteria for livestock species laid out in Commission Regulation No 1441/2007/EC (Figures 2.1 & 2.2). Process hygiene criteria for mean log numbers of APC and *Enterobacteriaceae* on carcasses of springbok have not yet been defined. Typically, 76% of the samples tested in 2009 had *Enterobacteriaceae* counts within the acceptable range ( $<1.5 \log_{10} \text{ cfu/cm}^2$ ) whereas those harvested in 2010, only 29% of the samples were within the acceptable limit. Those in the marginal range ( $1.5\text{-}2.5 \log_{10} \text{ cfu/cm}^2$ ) were 24% (2009) and 0% (2010) and in the unacceptable range ( $>2.5 \log_{10} \text{ cfu/cm}^2$ ) 0% (2009) and 71% (2010) respectively (Figure 2.1). Differences were also noted in the processing hygiene criteria percentages of tested carcass samples for APC with the acceptable range ( $<3.5 \log_{10} \text{ cfu/cm}^2$ ) values of 55% (2009) and 56% (2010), marginal range ( $3.5\text{-}5.0 \log_{10} \text{ cfu/cm}^2$ ) 46% (2009) and 33% (2010), lastly the unacceptable range ( $>5.0 \log_{10} \text{ cfu/cm}^2$ ) 0% (2009) and 11% (2010) respectively (Figure 2.2). It is not known how effective is the presence of indicator bacteria, specifically TVC, *Enterobacteriaceae* and *E. coli*, at predicting the simultaneous presence of pathogenic *Salmonella* and to answer this question, Generic *E. coli* isolated from eighteen out of 25 (72%) carcass samples with *Enterobacteriaceae* exceeding the stipulated 'M' limit and pooled colonies were kept and later analysed for the STEC genotype (chapter 4).



**Figure 3.1:** The percentage of carcasses with different levels of bacterial contamination (*Enterobacteriaceae*) in 2009 and 2010 following application of process hygiene criteria laid out in Commission Regulation No 1441/2007/EC.

Acceptable range (m)  $\leq 1.5 \log_{10}$  cfu/cm<sup>2</sup>; Marginal range (m-M)  $1.5-2.5 \log_{10}$  cfu/cm<sup>2</sup>; Unacceptable (unsatisfactory) range (M)  $> 2.5 \log_{10}$  cfu/cm<sup>2</sup>.



**Figure 3.2:** The percentage of carcasses with different levels of bacterial contamination (Aerobic Plate Counts) in 2009 and 2010 following application of process hygiene criteria laid out in Commission Regulation No 1441/2007/EC.

Acceptable range (m)  $\leq 3.5 \log_{10}$  cfu/cm<sup>2</sup>; Marginal range (m-M)  $3.5-5.0 \log_{10}$  cfu/cm<sup>2</sup>; Unacceptable (unsatisfactory) range (M)  $> 5.0 \log_{10}$  cfu/cm<sup>2</sup>.

### 3.1.1 Temperatures of carcasses on delivery to the abattoir

The mean temperature (°C) of the carcasses (table 3.3) in the front of the truck was  $8.18 \pm 4.07$ ,  $10.72 \pm 3.99$  in the middle and  $13.14 \pm 4.20$  in the rear. Irrespective of the position in the truck, the mean carcass temperature for 2009 was  $9.76 \pm 4.24$  compared to  $11.59 \pm 4.69$  for 2010. Statistically significant differences in carcass temperatures ( $p < 0.05$ ) were obtained between the years and also between the positions in the truck. The influence of the position of carcass in the truck on the temperature of the carcass did not vary

( $p < 0.05$ ) by year. Despite this, there was no interaction between year and position in truck with respect to temperatures of the carcasses.

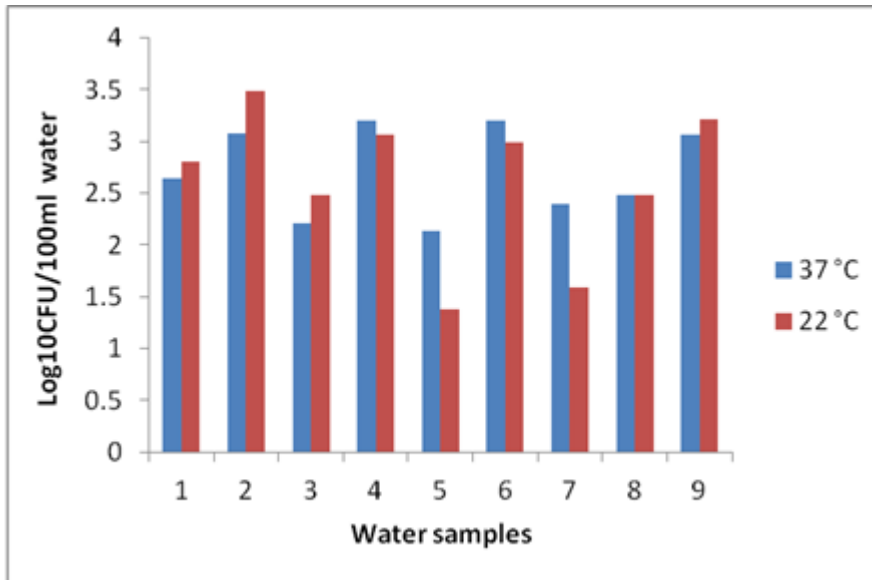
**Table 3.3:** Mean carcass temperature (degrees Celsius) ( $\pm$ SD) by year and position in truck upon arrival at the abattoir 16-26 hours post harvesting.

Year	Game carcass position in the truck			Overall mean
	Front	Middle	Rear (back)	
2009	7.83 <sup>a</sup> $\pm$ 3.79	9.62 <sup>b</sup> $\pm$ 3.99	11.85 <sup>c</sup> $\pm$ 4.07	9.76 <sup>d</sup> $\pm$ 4.24
2010	8.53 <sup>a</sup> $\pm$ 4.44	11.81 <sup>b</sup> $\pm$ 3.73	14.43 <sup>c</sup> $\pm$ 4.00	11.59 <sup>d</sup> $\pm$ 4.69
Overall mean	8.18 $\pm$ 4.07	10.72 $\pm$ 3.99	13.14 $\pm$ 4.20	

a, b, c, d, Values within rows and columns without a common superscript letters indicate that values that differ significantly ( $p < 0.05$ ).

### 3.1.2 Microbiological analysis of field water samples

To exclude processing water from contributing to high *Enterobacteriaceae* and APC counts on the carcasses, the farm tap water used during the slaughter processes was analysed for HPC. Nine out of 23 samples (39%) had HPC ranging from 1.38 to 3.48  $\log_{10}$  cfu/100ml of water after incubation at 22°C for 7 days, but these ranged between 2.13 and 3.20  $\log_{10}$ cfu/100ml when incubated at 37°C (Figure 3.3), indicating the farm tap water to be within the acceptable HPC level (the limit is 500cfu/ml). More importantly, none of the water samples contained any of the faecal pollutants tested – all were negative for *E. coli*, *Clostridium perfringens* and enterococci, but one sample yielded 2.15  $\log_{10}$  cfu/100ml of coliforms, which was not *E. coli*.



**Figure 3.3:** Heterotrophic plate counts of 9/23 positive farm tap water samples after 7 days of incubation at 22°C and 37°C.

### 3.2 Night harvesting

The edible offal and the carcasses pH measurements of harvested game during winter time are summarized in Table 3.4. In the livers, the pH ranged from 5.89 to 6.57, in the hearts it ranged from 5.43 to 6.89 and for kidneys pH was from 6.18 to 6.83. In the spleen and lungs, the pH ranged from 5.94 to 6.67 and 6.69 to 7.76 respectively.

In decreasing order, the pH was higher ( $p < 0.05$ ) than the reference pH of 6.0 in the lungs, kidney, spleen and liver and heart. Springbok carcasses had a pH lower ( $p < 0.01$ ) than 6.0. However, there was no difference between the heart pH and the regulatory reference pH of 6.0



**Table 3.4:** Summary of pH readings measured 16 to 34 hours (pH<sub>16-34hr</sub>) post-slaughter for springbok edible offal organs and carcasses supplied to the export abattoir.

N=55							
Reference	Lamb	Springbok	liver	spleen	heart	lung	kidney
pH value	carcass	carcass					
6.00 <sup>a</sup>	5.83 <sup>b</sup> ±0.10	5.73 <sup>c</sup> ±0.15	6.30 <sup>d</sup> ±0.14	6.35 <sup>d</sup> ±0.15	6.08 <sup>a</sup> ±0.39	7.10 <sup>e</sup> ±0.23	6.54 <sup>f</sup> ±0.15

Mean pH values with different superscript within row were significantly different (p<0.05).

Mean pH values with different superscript within row were significantly different from pH 6.0 (p<0.01).

### 3.3 Daylight harvesting

Harvesting took place during the summer period. Time from chasing to shooting, shooting to bleeding time, offal temperature at four hours post-slaughter, liver congestion, lung congestion, body condition score, environmental temperature, humidity, species, liver and lung pH at four hours post-slaughter were recorded to examine the relationship. Baseline predictor variables are shown in Table 3.5.

**Table 3.5:** Descriptive statistical values of the measured baseline variables/parameters of the four game species under study. N is number of game species

Species	Springbok		Red Hartebeest		Oryx		Kudu		
	N	Mean±SD	N	Mean±SD	N	Mean±SD	N	Mean±SD	
Parameter									
Chasing-shooting(min)	21	34±20	6	28±12	6	27±19	3	3±02	
Shooting- bleeding(min)	21	3±02	6	5±02	6	2±01	3	2±01	
Offal temp at 4hr	16	31.72±0.57	-	-	-	-	-	-	
Liver pH at 4hr	17	6.15±0.14	6	6.11±0.59	6	6.25±0.20	3	6.22±0.22	
Lung pH at 4hr	17	6.53±0.12	6	6.83±0.17	6	6.68±0.18	3	6.71±0.07	
Liver pH at 24hr	9	6.10±0.22	-	-	-	-	-	-	
Lung pH at 24hr	9	6.61±0.14	-	-	-	-	-	-	
Liver congestion(ml)	16	0.61±0.71	6	0.46±0.81	6	0.22±0.19	3	1.16±1.76	
Lung congestion (ml)	16	0.57±1.21	6	0.44±0.56	6	0.11±0.04	3	0.09±0.00	
Body condition score	21	3.02±0.29	6	3.5	6	3±0.55	3	3	

### 3.4 Uni and Multi-variate analysis

In the univariate linear regression analysis looking at the independent associations between pH and measured parameters, there were significant associations between liver pH at 4hrs and liver congestion by 0.08 ( $p<0.05$ ) (the more the congestion, the higher the pH) and shooting to bleeding by -0.03 ( $p<0.05$ ) (the longer the shooting to bleeding time, the lower the pH). The final multiple logistic regression model included only the variables relating to congestion in liver and liver pH at four hours post-harvesting. Species and body condition was introduced into the multivariate model as potential confounders. In the multivariate linear regression analysis, irrespective of species, the pH of game meat 4

hours after slaughter, increased by 0.11 units ( $p < 0.05$ ) per ml increase in liver congestion (Table 3.6), Irrespective of species, the pH of game meat 4 hours post-slaughter, decreased by 0.04 units ( $p < 0.05$ ) per minute increase in the shooting to bleeding interval (Table 3.7).

**Table 3.6:** Results from a multivariable linear regression model of the effect of congestion on pH of game liver 4 hours post-slaughter (N=31).

Predictor variable	Category	p	SE	Coefficient	95% CI
Liver congestion	-	0.002	0.03	0.11	0.05-0.18
Species	Ref: 6.15±0.14				
	Red hartebeest	0.433	0.08	0.07	-0.11-0.24
	Oryx	0.096	0.07	0.12	-0.02-0.27
	Kudu	0.652	0.09	-0.04	-0.22-0.14
Body condition	Ref: 1				
	2	0.141	0.16	-0.25	-0.59-0.09
	3	0.380	0.14	-0.13	-0.42-0.17
	4	0.079	0.15	-0.27	-0.57-0.03
Constant	-	0.000	0.146	6.26	5.96-6.56

Ref: reference

**Table 3.7:** Results from a multivariable linear regression model of the effect of shooting to bleeding time on the pH of game liver 4 hours post-slaughter (N=31).

Predictor variable	Category	Coefficient	SE	p	95% CI
Shooting to bleeding	-	-0.04	0.02	0.060	-0.08-0.002
	(Reference)	3±0.2			
Species	Red hartebeest	0.10	0.10	0.344	-0.11-0.320
	oryx	0.04	0.08	0.616	-0.12-0.201
	kudu	0.0003	0.09	0.997	-0.196-0.196
Body condition	1 (Reference)	-			
	2	-0.26	0.18	0.175	-0.634-0.122
	3	-0.12	0.16	0.458	-0.453-0.210
	4	-0.20	0.16	0.225	-0.543-0.135
Constant	-	6.40	0.168	0.000	6.53-6.748

#### 4. DISCUSSION

The microbiological condition of meat derived from livestock depends upon the types of microorganisms carried on the hide, gastro-intestinal tract or systemic organs, the circumstances in which the animal is slaughtered and abattoir hygiene whereas the microflora that develops after dressing is influenced by the storage conditions and its intrinsic biochemical qualities (Bacon *et al.*, 2000; Newton *et al.*, 1978; Gill, 2007; Jericho *et al.*, 1996; Small *et al.*, 2003; McCleery *et al.*, 2008). In general, hide surfaces are significant sources of microorganisms with total microbial counts for cattle hides ranging from 3.53 to 12.50 log<sub>10</sub> cfu/cm<sup>2</sup>, *E. coli* levels from 2.08 to 7.50 log<sub>10</sub> cfu/cm<sup>2</sup>, and 5.38 log<sub>10</sub> cfu/cm<sup>2</sup> on sheep fleece (Gabis & Faust, 1988; McCleery *et al.*, 2008; Cox *et al.*, 1988). However, raw meat from gemsbok, kudu, springbok, zebra and beef used in the production of salami had an acceptable mean total aerobic count of 5.2 log<sub>10</sub> cfu/g, 2.8 log<sub>10</sub> cfu/g for coliforms and

2.7 log<sub>10</sub> cfu/g for *E. coli* (van Schalkwyk *et al.*, 2011), considering that meat used for further processing, usually originates from meat of low value cuts while high quality meat is sold frozen or chilled. Although the reduction in carriage of pathogens is likely to lower the incidence of carcass contamination, there are concerns that shot game animals which invariably fall on dirty contaminated ground may be a source of a higher transfer of microorganisms from hides to carcasses during the carcass dressing process (Van der Merwe *et al.*, 2011).

There was no evidence that the mean APC differed significantly between the years and these were generally within the limit of process hygiene criteria despite 10% of the carcasses harvested in 2010 being in the unsatisfactory range. However, the statistically difference in the mean *Enterobacteriaceae* counts, with the 2010 value exceeding the M limit suggested a problem during processing and or harvesting. APC and *Enterobacteriaceae* counts are standard methods for estimating the microbial contamination of carcasses especially by the excision or swabbing methods (Atanassova *et al.*, 2008; Holds *et al.*, 2008; Van Schalkwyk *et al.*, 2011; Bernardo *et al.*, 2010). Whilst most studies use aerobic plate counts and *Enterobacteriaceae* to assess microbial quality, hygiene and good handling indicators, *Clostridium perfringens* has been found to also give a reliable indication of contamination since the organism is abundant in soil and faeces (Membré *et al.*, 2011). In one study, the contamination level of meat from a wild boar, red and roe deer was estimated to range between 0.81 and 1.52 log<sub>10</sub> cfu/cm<sup>2</sup> (Membré *et al.*, 2011). The high levels of *Enterobacteriaceae* exceeding Commission Regulation 1441/2007 stipulated limits prompted a check for the presence of generic *E.coli* and potential pathogens, in particular STEC (chapter 4).

The temperatures of the carcasses differed significantly by year and position in the truck with no interaction between year and position in truck. The cooling units of all the trucks used for harvesting were located at the front hence it was not surprising that this position was the coolest (Van Schalkwyk, 2011). Although statistically significant differences in the carcass temperatures was seen between 2009 and 2010, there was no clear evidence

indicating this as the cause of high APC and *Enterobacteriaceae* counts in 2010. On the other hand, the HPC of nine field water samples were not high enough to trigger any concerns. In the USA, HPC levels greater than 500cfu/ml are considered significant enough to warrant further investigations on the safety of drinking water, since these potentially interfere with coliform detection system (Reasoner, 2004). The frequency of coliform detection decreases when HPC levels exceed 500cfu/ml (LeChevallier & McFeters, 1985). It is therefore very unlikely that the HPC could have contributed to the high contamination levels of the carcasses harvested in 2010. Furthermore, no faecal pollutants were detected in the water, ascertaining the quality and removing its possibility as a source of contamination. Factors possibly impacting on hygiene were noted earlier.

Variability in the visible contamination levels of harvested game carcasses and edible organs was observed with different contamination levels between the harvesting years. This variability in visible contamination could have been as a result of changes in hunting management and harvesting practices (Van der Merwe *et al.*, 2011). Though not fully substantiated, significant changes were noted in the registration records of the harvesting teams in terms of; i) field harvesting infrastructure upgrade, ii) re-assessment of the food safety system, iii) field mobile abattoir management and iv) changes in harvesting personnel assistants. Subjecting carcasses to temperatures above 7°C after 24 hours, a regulatory precautionary measure for Foot and Mouth disease prevention was seen to affect the ability of the game harvesters in manipulating cooling truck temperatures. Cooling truck temperature manipulation helps to achieve carcass temperatures below 7°C within 24 hours of harvesting which is critical in controlling bacteriological hazards in game meat and offal. These changes noted could have contributed to a high contamination level, carcass temperatures above 7°C after 24 hours of chilling and consequently unacceptable bacterial levels.

One of the objectives of the study was to assess pH levels in selected chilled springbok and other commonly harvested and exported game species edible offal so as to establish whether they meet the requirement (pH<6.0) for safe food (Anonymous, 2010). The pH

values of the carcasses were all below 6.0 which is consistent with requirements of the European Commission regulation 206/2010 which stipulates the attestation to the effect that the consignment contains boneless meat, obtained only from de-boned meat other than offal that was obtained from carcasses which have been submitted to maturation at a temperature above +2°C for at least 24 hours before the bones were removed. However significant variations in pH ranges were observed for different offal organs originating from the renal, cardiovascular, respiratory and gastrointestinal systems. These results can be applied directly to a risk management strategy as the study was run under ideal temperatures and water activity conditions in the presence of competitive microorganisms under field conditions (IFT/FDA, 2003; Michael *et al.*, 2011).

Muscle pH of the springbok from this investigation, were similar to that measured for other wild species. Deer shot in the field and deer penned without handling had muscle pH values lower than 5.74 and above 5.74 respectively, with only four of the 66 male deer transported to a slaughterhouse having a muscle pH above 6.0 (Smith & Dobson, 1990). In another study, the mean pH<sub>24hr</sub> measurement values were significantly higher in females than males for springbok originating from four production regions in South Africa and ranged between 5.4 and 6.3 (Hoffman *et al.*, 2007). In a black wildebeest (*Connochaetus gnou*) meat study, animals harvested during the colder months of winter and autumn had a higher mean muscle pH<sub>45min</sub> between 6.8 and 6.7 compared to animals harvested in spring at 6.2, while the pH<sub>24hr</sub> tended to be lower in winter at 5.4 than in either spring and autumn at 5.6 (Hoffman *et al.*, 2009). The rate of pH decline in Impala (*Aepyceros melampus*) carcasses harvested at night is slower compared to those harvested in the day (Hoffman *et al.*, 2003). Male game animals generally tend to have a higher pH than females due to a more active response to disturbances especially when harvesting occurs during the rutting season (Lewis *et al.*, 1997; Hoffman, 2000b). However, gender was not taken into account during this investigation. Lymph nodes examined by Cottral *et al.*, (1960) maintained pH readings between 6.4 and 6.9, a favourable range for the survival of the FMD virus (at 4°C for 72 hours). Liver, kidney, rumen, lymph node and blood from diseased cattle were

shown to be highly infective and to remain so if stored frozen (Henderson & Brooksby, 1948).

There are many variables that could have influenced the observed pH variations in different organs under consideration. However, irrespective of the potential limitations of the current results, the lack of statistically significant association between some selected variables and pH changes in this study suggests that either the factors may have a small effect which is only detectable with large data-sets and/or the effect may have been modified by other unidentified risk-modifying factors. This large data set gap may be elucidated by further investigations. Factors that impact the hunting duration and time from shooting to bleeding includes type of hunting terrain, species behavior, experience and the number of the hunters, size of the harvesting camp wherein the animals are found. The role of the environment, genetics and their interactions could also influence the extent to which the pH decreases in these offal organs (Brown *et al.*, 1990; Knee *et al.*, 2004; Lomiwes, 2008, Scanga *et al.*, 1998; Young *et al.*, 2004; Zuliani *et al.*, 2007).

The ultimate pH of meat is not only dependent on the muscle glycogen reserves at mortem but also on other factors such as fibre type which differs between carcasses and offal organs, ante-mortem condition on the animal such as nutrition, health, stress and behavior after shooting, the later conditions may all contribute to glycogen depletion which may cause a high ultimate muscle pH (Atanassova *et al.*, 2008; Fink, 1992; Forrest, 2011; Hoffman *et al.*, 2007; Kappelhof, 1999; Smith & Dobson, 1990; Van Rijswijk & Vorster, 1995; Wiklund & Malmfors, 2003; Young *et al.*, 2004a). In this study, springbok carcasses and hearts had a pH close to 6.0 due to the fact that both are muscles that have glycogen reserves and thus enter anaerobic metabolism with lactic acid as end product, an effect that could also have influenced shooting to bleeding time pH levels in other offal organs. Observation on seasonal difference and effect of gender and age in ultimate carcass 24 hour pH values had been reported previously (Kim *et al.*, 2003; Van Schalkwyk, 2011; Warriss, 2000). Also little or no glycogen is found in the lungs, however post mortem pH decline can also be slowed by the buffering effect of blood and ammonium generated by the



deamination of adenosine monophosphate (AMP) (Bendall & Davey, 1957) hence, non-pathological haemorrhage (petechial and ecchymotic) and or congestion sometimes observed at the post mortem inspection of the harvested springbok liver, lung and spleen may have influenced the specific organ pH post-harvest. pH decline of muscle in a carcass deviates from a linear function because when the muscle enters rigor, hydrogen ion production decreases as the rate of anaerobic glycolysis and myosin ATPase activity decrease during muscle cooling (Bruce *et al.*, 2011). The initial pH fall in muscles does not depend on the glycolytic potential but at 5 to 6 and 24 hr post mortem muscles with the lowest glycolytic potential have the highest pH (Maribo *et al.*, 1988).

In muscles, time taken to reach pH 6.0 from initial pH values of around 7.1 varies between 8 and 16 hours, and as low as 3.5 hours in some extreme cases, some of this variability has been attributed to different rates of cooling between muscles in the same animal (Bendall, 1978). The rate of pH fall is determined by the rate of ATP-turnover, so variability can be due to varying intracellular free  $\text{Ca}^{2+}$  levels exerting a stimulating effect on the actomyosin ATP-ase with some of this extra  $\text{Ca}^{2+}$  arising from calcium released from the mitochondria which become anaerobic after death (Bendall, 1978; Honikel *et al.*, 1983; Maribo *et al.*, 1988). pH readings 24 hours post-slaughter which are greater than 6.5 are indicative of dark firm and dry (DFD) carcasses, a problem frequently associated with ante-mortem stress. Apart from lack of visual appeal, dryness and abnormal flavor, DFD meat has increased susceptibility to bacteriological spoilage (Homer & Matthews, 1998). Mean pH values have also been reported to correlate linearly with the coliform and *E.coli* counts (Van Schalkwyk *et al.*, 2011).

A linear relationship of lower pH and greater bacteria reduction has been reported in treated organs due to induced sub lethal injuries through alteration of the pH gradient of the food-borne pathogens (Dorsa *et al.*, 1997; Flowers, 2006). The effect of lactic acid on food borne pathogens is assigned to its ability to penetrate the cell membrane in its non-dissociated form and dissociate within the cell thereby decreasing the pH and disturbing trans-membrane proton motive force and causing an inhibition of acid sensitive enzymes

(Dorsa *et al.*, 1997; Rajkovic *et al.*, 2010). In this study where the hearts are the only edible offal organs likely to have a pH close to or below 6.0, it can be concluded that the wild game organs' natural pH cannot be relied on as a natural mechanism to inactivate endogenous food-borne pathogens such as RVF virus in some edible offal. Although international standards set a maximum pH of 6.8 as capable of inactivating RVF virus in carcasses, this cannot apply to offal as the virus has been isolated in chilled livers (kept for 3 to 5 days at 4°C) of healthy sheep that aborted after being transported from the farm to the abattoir (Magwedere, unpublished data). In this study, none of the offal presents a hazard if thoroughly cooked, however, the consumption of fresh blood either alone or mixed with milk, raw or undercooked meat/organs in some cultures should therefore be discouraged (Anonymous, 2011; Cima, 2012; Corbel, 2006; Fatma & Mahdey, 2010; John *et al.*, 2010; Mfinanga *et al.*, 2003). As part of meat safety risk management, it may be important to consider the use of generally recognized as safe (GRAS) substances to treat edible offal in order to inactivate endogenous and exogenous microbial pathogens susceptible to low pH (Berge *et al.*, 2001; FSIS directive, 2011; FVE, 2011; Harrington, 2011).

## 5. CONCLUSION

In conclusion, the data from this investigation therefore provide some of the microbiological monitoring of game meat and process hygiene criteria guidelines using *E.coli*, *Enterobacteriaceae* and APC count which should be exploited to ensure the wholesomeness and safety of game meat. Cooling regimes in the chiller truck need to be investigated further in relation to loading densities, species and time.

Treatment with GRAS should only be carried out after the official meat inspection process has been completed to prevent interference with process hygiene criteria monitoring. Further research should be done to establish the normal competitive microflora of the wild game offal that renders the surface of the offal susceptible to preferential growth of pathogens as a result of contamination. Further investigations with large data sets may help elucidate risk-modifying factors for pH changes in harvested wildlife. The prevalence and extend of congested carcasses and organs in harvested wildlife need to be investigated

to evaluate the possible maintenance and transmission of some pathogens in different livestock systems due to the exploding demand for livestock products.

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## CHAPTER 4

**Assessment of shiga toxin *Escherichia coli* (STEC) and *Salmonella* spp in meat derived from Springbok (*Antidorcas marsupialis*), Deer (*Cervus elaphus*, *Odocoileus virginianus*, *Rangifer tarandus*), Boar (*Sus scrofa*), Bison (*Bison bison*) and Rabbit (*Oryctolagus cuniculus*)<sup>1, 2</sup>**

### 1. INTRODUCTION

Microbiological analysis on game meat (Chapter 3) indicated a high level of Enterobacteriaceae counts in 5 batches harvested in 2010. Shiga toxin *Escherichia coli* (STEC) and *Salmonella* are the most common enteric food-borne pathogens which pose a significant threat to public health. The objective of this study was therefore to ascertain the microbial safety of game meat examining for the presence of these organisms.

A wide variety of wild and farmed game animals has been an important source of protein for humans world-wide for many millennia. In some countries, the main game meat consuming sectors are the traditional ones where most of the game meat produced and processed for own consumption are under regulatory derogation, evading sanitary scrutiny. *Salmonella* is a multi-host pathogen of economic and public health importance. The occurrence of *Salmonella* in meat from harvested game is different according to region and game species, ranging from <5 (wild ruminants) to 20% (wild boar) in faecal samples

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<sup>1, 2</sup> Parts of this chapter were published or submitted for publication as:

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<sup>2</sup>**Magwedere K, Dang HA, Mills E, Cutter CN, Roberts E, DebRoy C (2012)** Incidence of the presence of shiga toxin-producing *Escherichia coli* (STEC) strains in beef, pork, chicken, deer, boar, bison and rabbit retail meat. *Journal of Veterinary Diagnostic Investigation*. In print.

and recovery (<1%) from carcasses or meat cuts of wild ruminants (Paulsen *et al.*, 2011). However, in several studies, no *Salmonella* spp has been isolated in wild game mammals (Atanassova *et al.*, 2008; Deutz *et al.*, 2006; Van der Merwe *et al.*, 2011).

Farmed and wild game animals may be contaminated with shiga toxin-producing *Escherichia coli* (STEC) serogroups, thus food of game origin may be a vehicle for infection. Approximately 20 to 70% of STEC infections throughout the world are due to non-0157 STEC (WHO, 1998) and of the recovered isolates; 71% of these belonged to the six serogroups; O26, O45, O111, O103, O121 and O145 (Brooks *et al.*, 2005; WHO, 1998; Fratamico *et al.*, 2011). In Germany, more than 60% of STEC serotypes isolated from food were also found in human patients, implicating food as a source of human infection relative to other forms of transmission (waterborne, animal-to-person, and person-to person) (Beutin & Martin, 2011). In wild game, prevalence rates of 10.5 to 16.3% have been reported for non-0157 STEC in Japanese wild animals (Asakura *et al.*, 1998; Fukuyama *et al.*, 1999), 5% in fecal specimens from United States deer (Ishii *et al.*, 2007), 38.5% excretors from wildlife ruminant species in Argentina (Leotta *et al.*, 2006), high rates of ruminant wildlife excretors in Europe, 51.8% of wildlife ruminants carriers in Germany (Lehmann *et al.*, 2006), and high rates of carriage for STEC-excreting animals from different deer species in Spain (Sánchez *et al.*, 2009). In a study in Germany, STEC positives were frequent among meat samples from sheep (11.1%), wild game (9.9%), and cattle (4.5%), pork (0.68%) and dairy products (0.6 and 1.8%) (Hartung, 2007; Beutin & Martin, 2011). 0157 positives in South Africa were 67.7% in pigs and pork, cattle and beef (27.7%), water (2.3%) and humans (0.77%) respectively (Ateba & Mbewe, 2011).

Little information on STEC is available for southern African countries, however extrapolations of various 0157:H7 prevalence or incidence rates against the populations of Angola, Botswana, Swaziland, South Africa, Zimbabwe and Zambia is available (RD, 2012). Serotypes 0157:H7, O6:H21, O76:H19, O76, O113 and O8, O158 have been detected in healthy cattle, in contaminated meat, in diarrheic children and meat products in Morocco and Uganda (Elisha *et al.*, 2008; Badri *et al.*, 2011). Non-0157 STEC strains (O146:H28,

O146:H21, O26:H11, O11:HNT, O51:H49, O91:H6, O98:H21, O103:H28, O8:H10, O128:H2, O2:H6, O5:HNM, O21:H21, O36:H14, O110:H45, O174:H8, ONT:HNT, O11:H48, O74:H28, O145:H28, ONT:H21, ONT:H28, and O75:H8) have been isolated from 52.5% of roe deer, 8.4% of wild boars, and 1.9% of foxes sampled in Spain while the prevalences of STEC O157:H7 detected were 0.56% for roe deer, 0.38% for wild boar, and 0% for fox (Mora *et al.*, 2012). In the United States, non-O157 shiga toxin-producing *Escherichia coli* (*E. coli* O103:H2 and O145: NM) and *E. coli* O157:H7 have been isolated in venison, venison jerky and deer fecal pellets (Keene *et al.*, 1997; Rounds *et al.*, 2012).

Different exposure and transmission mechanisms of STEC among extensively managed range operations, intensively managed and confined systems has been speculated on but little is known about STEC in wildlife meat produced under different environments. Another primary question posed by this study is to what degree can potentially pathogenic *Salmonella* spp and STEC be found in our springbok meat?

## **2. MATERIALS AND METHODS**

*Salmonella* and STEC tests were done in Namibia and Animal Health Institute in UK respectively. The project for testing STEC in exotic meats was carried out at Penn State University, *E.coli* reference centre at Wiley lab with a grant from Norman E. Borlaug International Agricultural Science and Technology Fellowship Program under the mentorship of Dr Chitrita DebRoy, Elisabeth Roberts, Dr Edward W. Mills and Dr Cathrine C. Cutter.

### **2.1 Collection of faecal samples from springbok**

To assess whether harvested springbok harbour zoonotic STEC in their intestinal tracts, rectal contents were collected at evisceration from nine randomly selected farms. Rectal contents from ten different animals were pooled to give a total of 18 samples (i.e. 2 pooled samples per farm), each weighing approximately 400 grams.

## 2.2 Sampling and processing for *Salmonella* strains from springbok

Carcasses, in the same batches previously sampled for APC and *Enterobacteriaceae* determination (chapter 3), were randomly swabbed for *Salmonella* detection using sponges as described in the European Commission Regulation No 1441/2007 at a frequency of 10 to 20 carcasses per batch. Briefly, peptone water-moistened sterile sponges were rubbed vertically, horizontally and diagonally for 20 times over a minimum of 400cm<sup>2</sup> on the flank surface. The swabs were aseptically bottled and transported to the laboratory under chilled conditions. These samples were enriched at 37°C for 2-4h in Buffered Peptone Water (BPW; Merck, Darmstadt, Germany) followed by an overnight enrichment for *Salmonella* in selenite cystine broth (Merck) and Rappaport-Vassiliadis soya (RVS) peptone broth (Scharlau Chemie S.A. Barcelona, Spain). The enriched broth cultures were spread on two selective medium for *Salmonella* spp; xylose-lysine-deoxycholate agar (XLD; Scharlau Chemie S.A.) and brilliant green agar (Scharlau Chemie S.A.) and incubated at 37°C for 24h. Any presumptive *Salmonella* colonies were initially subjected to the following biochemical tests; Triple sugar iron, urease production, oxidase reaction and lysine decarboxylase before serologically testing with Anti-*Salmonella* Omnivalent serum (Abcam, Cambridge, UK).

## 2.3 Processing and analysis for *E. coli* strains from springbok

Between year 2003 to 2010, 38 viable *E. coli* isolates from unidentified human samples were found to be positive for STEC by multiplex-PCR at a referral laboratory in South Africa (Crowther-Gibson *et al.*, 2011). However, the sources of the infection and transmission routes were not described in the report and in this regard, carcasses exceeding the stipulated 'M' limit for *Enterobacteriaceae* (European Commission regulation 1441/2007) were re-sampled and cultured for *E. coli*. To assess the presence of pathogenic *E. coli*, 0.1ml of each homogenate was separately inoculated onto Tryptone Bile X-glucuronide (TBX) agar (Merck) and incubated at 44°C for 24h. Since some 3-4% of *E. coli* from foods, notably serogroup O157 strains are glucuronidase-negative (Ratnam *et al.*, 1988) and cannot grow at 44°C, homogenized samples were cultured based on Andrews' method (Andrews, 1992). Briefly, 1 ml of the homogenate was enriched in BPW, then transferred into Lauryl



Tryptose (LT) broth (Scharlau Chemie S.A.) and examined for gas production after 24h of incubation at 37°C. Samples producing gas were transferred into EC medium (Scharlau Chemie S.A.) for a further 24h at 37°C and then finally streaked on sorbitol MacConkey agar (Oxoid).

Pooled faecal samples were directly plated on sorbitol MacConkey agar and the plates incubated overnight at 37°C. A total of 16 representative pink and pale colonies on both meat and faecal plates were confirmed to be *E. coli* by minimal biochemical tests; positive reactions for indole production, motility, methyl red and negative for Voges-Proskauer (VP) and citrate. Five colonies obtained from each of the 18 meat samples were pooled and pelleted. Similarly, colonies from faeces were pooled and pelleted to give a total of 15 samples.

Chromosomal DNA was extracted from the pellets using the Qiagen DNA extraction kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Purified DNA was eluted in 200 µl of elution buffer into eppendorf tubes and frozen at -20°C until required. Thirty-three DNA samples were transported on dry ice to the Institute for Animal Health (Compton, United Kingdom) and subjected to a PCR for the conserved portion of the intimin gene and also a multiplex PCR the gene sequences encoding for intimin, haemolysin, shiga toxin 1 and 2 as described (Paton & Paton, 1998) against a positive control. PCR products were resolved on 1% agarose as per standard procedures for agarose gel electrophoresis.

#### **2.4 Sample collection and culture for deer (*Cervus elaphus*, *Odocoileus virginianus*, *Rangifer tarandus*), boar (*Sus scrofa*), bison (*Bison bison*) and rabbit (*Oryctolagus cuniculus*) retail game meat**

Recovery of bacteria from meat samples was performed as previously described with some modifications (Valadez *et al.*, 2011; Fratamico *et al.*, 2011). Cut game meat samples (275g of ground game meat and meat cut sub-samples of deer, bison, wild boar and whole rabbit carcasses) were obtained from retail supermarkets and farmer markets across different

territorial States in the USA and held at 4°C until analysis (Table 4.1). Briefly, 5 grams were weighed from each meat sample and added to 45mL of TSB containing 8mg/l of novobiocin and 16mg/l of vancomycin (Sigma-Aldrich Corp.) followed by pummeling for 2 minutes using a stomacher lab blender (Cooke Laboratory Products).

**Table 4.1:** Number, type of samples and subsamples obtained and assessed from each species

Sample type	Number of sample type for each game species assessed					
	Deer		Bison	Rabbit	Wild boar	
	white tailed	reindeer				
ground	3(15)	1(5)	15(75)	21(105)		1(5)
stew (cuts)		1(5)	1(5)	1(5)		1(5)
steak			4(20)	2(10)		
whole carcass					4(20)	
Total	3(15)	2(20)	20(100)	24(120)	4(20)	2(10)

The rabbit was processed by aseptically transferring the whole carcass into a sterile stomacher bag and rinsing with 400ml of bufferfield's phosphate diluents (BPD) according to USDA/FSIS microbiology Laboratory guidebook 3rd edition protocol on whole bird rinse (FSIS, 1998). Upon removal of the rabbit carcass in the stomacher bag, 50ml of the liquid rinse was transferred into plastic test tubes and centrifuged at 7 400rpm for 10 minutes at 4°C. After centrifuging, 45ml of the supernatant was discarded and the pellet was re-suspended in 20ml of pre-enriched TSB broth and then transferred into another stomacher bag with 25ml TSB. The samples were pre-incubated statically for 6hrs at 37°C. After the 6hrs pre-enrichment step, bile salts (1.5g/l; Difco Laboratories), rifampicin (2.0mg/l), and

potassium tellurite (1.0mg/l; Sigma-Aldrich) were added, and further incubated for 18hrs at 42°C. STEC reference strains, obtained from the *E. coli* reference center at the Pennsylvania State University (University Park), were used as positive controls for all batches.

## **2.5 DNA isolation and purification**

All bacterial DNA was extracted with the MasterPure DNA Purification Kit (EPICENTRE Biotechnologies, Madison, WI), essentially according to the manufacturer's instructions with minor modifications. Briefly, 0.5ml of broth culture was centrifuged for 30sec at 13 000/g in a microcentrifuge tube to pellet the bacteria. The pellet was suspended in 300µl of lysis solution, gently mixed by inversion of the tube after which 1µl proteinase K was added. Digestion of bacterial protein material was allowed for 15min at 65°C, with a 10s vortexing period after every 5min. for 10s. Samples were cooled to 37°C for 10min, and 1µl of RNase was added to each sample, mixed thoroughly by vortexing, and incubated at 37°C for 30min. Samples were then placed on ice for 5min, and 175µl of MPC Protein Precipitation Reagent (EPICENTRE Biotechnologies) was added to the lysed sample and vortexed vigorously for 10sec. The debris was pelleted by centrifugation for 10min at 13 000 rpm in a microcentrifuge. The supernatant was transferred to a sterile microcentrifuge tube containing 500µl isopropanol. The tube was inverted 40 times before the total nucleic acid was pelleted by centrifugation at 13 000rpm at 4°C for 10min, and the supernatant was carefully poured off without dislodging the pellet. The pellet was then rinsed twice with 200µl of 70% ethanol, dried at room temperature for 5min and re-suspended in 35µl of TE buffer solution. All DNA preparations were stored at -20°C until required.

## **2.6 Multiplex PCR assays**

Frozen DNA preparations were thawed at room temperature with occasional vortexing before use. Multiplex PCR (mPCR) assay targeting (O26, O45, O111, O113, O103, O145, O121 and O157) serogroups was performed. The primer sequences for the forward (F) and reverse (R) reactions for O-groups were as described by Valadez *et al.*, 2011 (Table 4.2).

**Table 4.2:** List of O-group, *stx1* and *stx2* PCR primers used in this study

Primer designation	Primer sequence (5'-3')	Target gene	Primer concentration ( $\mu$ M)	Amplicon size (bp)	Reference
026	F, caatgggcggaattttaga R, ataatttctctgccgtcgc	wzx wzx	0.17	155	Valadez <i>et al.</i> , 2011
045	F, tgcagtaacctgcacgggdcg R, agcaggcacaacagccactact	wzx wzx	0.063	238	Valadez <i>et al.</i> , 2011
0103	F, ttggagcgtaactggacct R, gctcccagacacgtataaag	wzx wzx	0.03	321	Valadez <i>et al.</i> , 2011
0111	F, tgtttcttcgatgttgcgag R, gcaagggacataagaagcca	wzx wzx	0.029	438	Valadez <i>et al.</i> , 2011
0113	F, tgccataattcagagggtgac R, aacaaagctaattgtggccg	wzx wzx	0.029	514	Valadez <i>et al.</i> , 2011
0121	F, tccaacaattggctcgtgaaa R, agaaagtgtgaaatgccctg	wzx wzx	0.029	628	Valadez <i>et al.</i> , 2011
0145	F, tcattgttttgcttgctcg R, ggcaagctttgaaatgaaa	wzx wzx	0.03	750	Valadez <i>et al.</i> , 2011
0157	F, tgcaggtacctgaatcttctctgt R, accagtcttggtgctgctctgaca	wzx wzx	0.015	894	Valadez <i>et al.</i> , 2011
Stx1	actggtgatctcagtggtg ctgaatcccctccattatg	-	0.5	600	Paton & Paton, 1998
Stx2	ggcactgtctgaactgctcc tcgccagttatctgacattct	-	0.5	255	Paton & Paton, 1998

The mPCR assay was carried out in a 50 $\mu$ l reaction mixture containing 2x multiplex PCR mix (25 $\mu$ l) (HotStarTaq® DNA polymerase, multiplex PCR buffer with 6mM MgCl<sub>2</sub>, dNTP mix), 10x EHEC primer mix (5 $\mu$ l), 3 $\mu$ l of the DNA template as described in the qiagen PCR kit protocol (Qiagen, City , USA). The laboratory *E. coli* K-12 strain was used as a negative control and a mixture of (5 $\mu$ l 026, 045, 0111, 0113; 10 $\mu$ l of 0121; 20 $\mu$ l of 0145 and 0157) was used as positive control. The PCR mixtures were subjected to the following cycling conditions: 95°C (15min, 1 cycle); 30 cycles of 94°C (0.5min), 57°C (90sec), 72°C (90sec); and 72°C (10min, 1 cycle), using an iCyclerthermocycler (BioRad Laboratories, Hercules, CA). 5 $\mu$ l of the PCR product was mixed with 1.5 $\mu$ l of sample dye and the resultant mixture (6.5 $\mu$ l) was resolved on 1.5% (wt/vol) agarose gels in 0.5XTris-borate-EDTA (TBE) buffer alongside 1kb molecular weight markers, and visualized after staining with 1.5 $\mu$ l ethidium

bromide. Samples that were positive for the *wzx* (the gene determining the O serogroup) were subjected to a further the multiplex PCR assays targeting *stx1* and, *stx2*, genes.

## **2.7 Statistical analysis**

Data from Salmonella and STEC analyses were reported as numbers of samples testing positive for each of the tested pathogens and virulence genes.

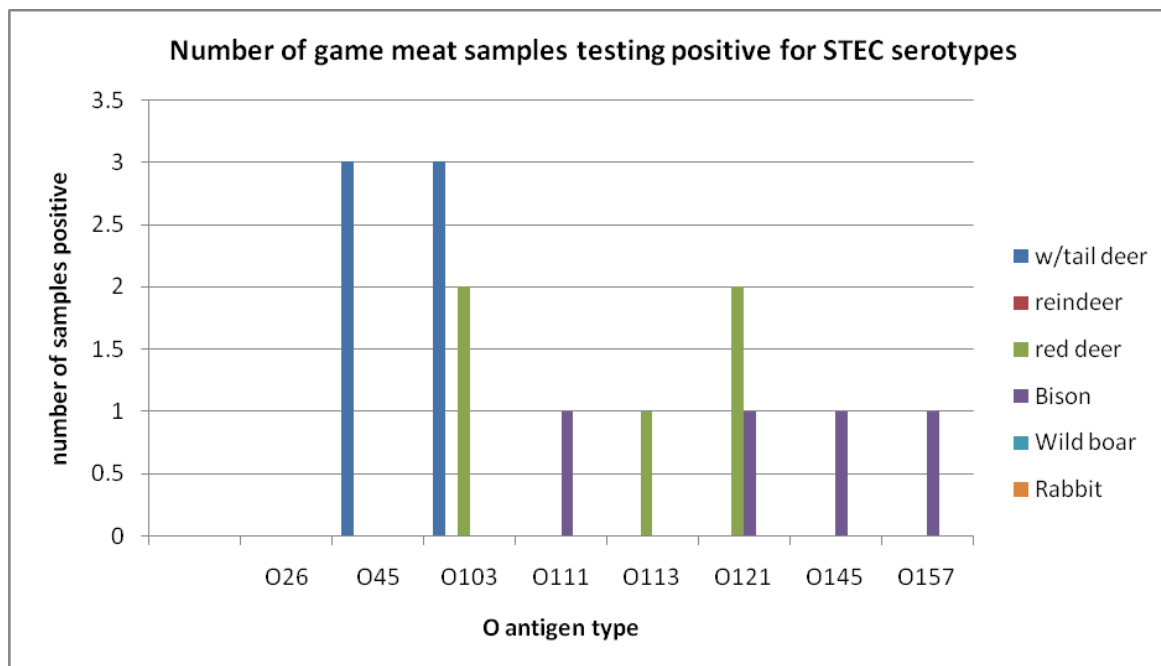
## **3 RESULTS**

### **3.1 Springbok meat and faecal samples**

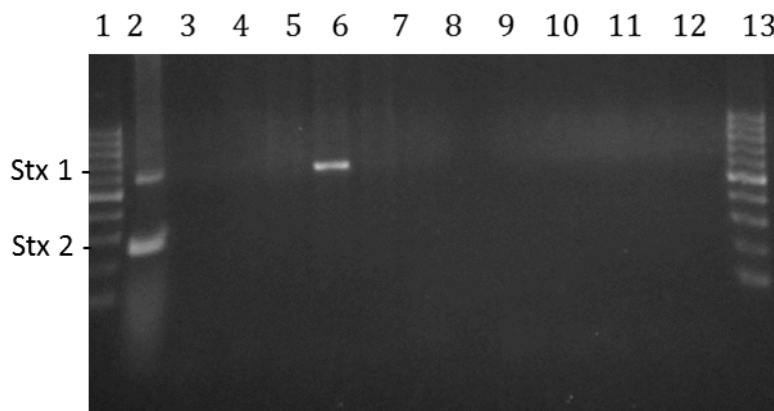
All the pooled *E. coli* strains from springbok originating from TBX and sorbitol MacConkey agar were negative for the principal STEC virulence genes (*stx1*, *stx2*, *eae* and *hlyA*). To examine if intestinal *E. coli* comprised pathogenic strains, 15 pooled *E. coli* colonies from rectal contents (faeces) were screened for STEC virulence genes. Five out of 15 pools were positive for the intimin gene but negative for three other STEC virulence genes (*stx1*, *stx2* and *hlyA*).

### **3.2 meat from deer, boar, bison and rabbit retail game meat**

The number of game meat samples that tested positive for different STEC strains and electrophoresis showing the presence of *stx1* gene in one white tailed deer ground meat sample are shown in Figures 4.1 and 4.2 respectively.



**Figure 4.1:** Number of game meat samples positive for different STEC strains serotype.



**Figure 4.2:** Electrophoresis on 1.5% agarose gel of PCR products, showing the presence of *stx1* gene (lane 6) in one white tailed deer ground meat sample positive for *E. coli* O45 and O103. Lanes 1 and 13: molecular weight markers, Lane 2: positive controls for *stx1* and *stx2*. Lane 3: negative control. Lanes 4-12: samples identified as O serotype-positive.

#### 4 DISCUSSION

The most important component of shiga toxigenic *Escherichia coli* (STEC) virulence is the production of one or both *stx1* and *stx2* (Müller *et al.*, 2001; Paton *et al.*, 1998). The first *E. coli* serotype to be associated with Shiga toxin producing was O157:H7 following an outbreak of diarrhoea and haemorrhagic gastroenteritis in the USA (Riley *et al.*, 1983). Today, non-O157 strains are increasingly being encountered in clinical cases, food, water and reservoir hosts, including food-producing wildlife species (Mora *et al.*, 2012). Samples of ground bison, white tailed deer and red deer were positive for non- O157 STEC serotypes, but only one sample turned out positive for the *stx2* gene. Despite the low prevalence, such findings were not surprising since *stx* genes are transferred by horizontal gene transfer. Furthermore, this low frequency was reflected by the absence of STEC in springbok rectal contents and export meat. However, previous reports indicate that wild animals may be carriers and transmitters of STEC strains (Wahlström *et al.*, 2003; Miko *et al.*, 2009), though none could be detected from either the meat or the pooled faecal samples in the present study.

The STEC pathotype is commonly associated with food-borne illnesses in humans and the gastrointestinal tract of ruminants is the primary reservoir for many STEC serotypes. Contamination of carcasses often occurs during the slaughtering process if sufficient precaution is not taken. Therefore, *E. coli* isolates from rectal contents were similarly screened by PCR to exclude potential carriage of STEC by Springbok. The multiplex PCR technique is sensitive and able to detect any positives using colonies or crude faecal suspensions as a template (Paton & Paton, 1998; Paton & Paton, 2003). To further increase sensitivity, purified chromosomal DNA from pooled colonies was used. It is unlikely that the culture media influenced the negative results however the examination of a small sample may result in false negative results if the contamination level with pathogenic *E. coli* is low. None the less, the absence of STEC in pooled rectal contents suggests a relatively low risk of contamination of meat derived from those animals.

Whilst sorbitol MacConkey agar is recommended for STEC O157:H7 (March & Ratnam, 1986), there is no universal media catering for all STEC serotypes (Hussein & Bollinger,

2000). In this respect, subjecting the samples to a pre-enrichment step in BPW or TSB without antibiotics is considered a vital step towards successfully isolating STEC from food, faeces or environment (Hussein & Bollinger, 2000). In this investigation, samples were pre-enriched in BPW and all presumptive *E. coli* colonies were pooled to increase the likelihood of detecting STEC since shiga toxins can be associated with unusual strains as recently seen in a German outbreak of haemolytic uraemic syndrome caused by an enteroaggregative *E. coli* O104:H4 (Rohde *et al.*, 2011). However, the detection of five intimin-positive strains from faeces implied the presence of potentially pathogenic *E. coli* strains in springbok, most likely EPEC, but not the zoonotic pathotype. EPEC is a leading cause of neonatal diarrhea in the developing world and is regarded as of a very low zoonotic potential (Kaper *et al.*, 2004).

All springbok meat samples were negative for *Salmonella* consistent with other previous reports in other wild animals (Atanassova *et al.*, 2008; Holds *et al.*, 2008; Rounds *et al.*, 2012; Wahlström *et al.*, 2003). Despite the limited sample size, these findings imply that springbok may not be a reservoir for STEC and *Salmonella* species. Therefore, a larger survey is required to define the role played by springbok in the epidemiology of such infections and also to provide insights into potential public health risks posed by springbok meat.

Only one ground bison sample was positive for O157 serotype. Mixed serotypes were detected in some of the positive ground meat samples. However one sample belonging to O45 from white tailed deer confirmed positive for *stx1* gene while the remaining positive samples were negative for *stx1* and *stx2* genes. Serotypes O26, O45, O103, O111, O121, O145 and O157 account for the majority of STEC infections consistently associated with increased incidence of disease in humans, and more frequently associated with Haemorrhagic colitis (HC) and Haemolytic uremic syndrome (HUS) in the United States (Lin *et al.*, 2011; Wang *et al.*, 2012). A range of serotypes (O157, O26, O111, O117, O115 and O5) associated with shiga toxigenic *E. coli* (STEC) have been isolated in South African



patients and the commonest isolated serotypes associated with EPEC were O55, O111, O119, O127, O145 and O109 (NICD, 2011).

Principal virulence factors of these strains are linked to either plasmids or phages, and consequently they are likely to be subject to horizontal gene transfer between species or serotypes. Moreover, the presence of infectious *stx*-phages isolated as free particles in the environment and their high persistence in water systems suggest that they may contribute to the spread of *stx* genes (Muniesa *et al.*, 2006). Studies on *stx* phages indicate that they are transmitted between different bacteria in vivo, in vitro and extra-intestinally which may lead to evolution of *stx2* producing strains (Muniesa & Jofre, 2004; Deng *et al.*, 2011). A large variability in the prevalence of *E. coli* O157:H7 has been noted in cattle populations due to among others factors, differences in environmental suitability for growth, animal movement onto and away from farms, and carriage and excretion levels and persistence in some animals (Arthur *et al.*, 2010). Analytical methods have been to influence the recovery of STEC from various sources. It is possible that the recovery of some STEC could have been below the detection limit of our study (Mora *et al.*, 2012). Rifampicin has been reported to decrease SLT-I and SLT-II expression at both the transcription and protein release levels and novobiocin may prevent optimal growth of STEC O111 (Matar *et al.*, 2011; Vimont *et al.*, 2007; Fratamico *et al.*, 2011). It has been ascertained that novobiocin (8mg/l) in the modified TSB (mTSB) medium can slow the growth of STEC O111 strains and inhibit non-O157 STEC strains to a larger extent than *E. coli* O157:H7 and at 20mg/l novobiocin, false negative results can be obtained when testing for non-O157 STEC in food (Vimont *et al.*, 2007; Fratamico *et al.*, 2011). In this study, limitations were encountered related to diversified sample acquisition leading to difficulties in designing sampling strategies that adequately represented the population of interest. In this non ideal condition, the sample collection represented a compromise associated with availability (Stallknecht, 2007). In this study, no *Salmonella* were detected on any of the samples tested and the low incidence of STEC highlights either the effectiveness of the risk based meat safety management system adopted by the regulatory authorities with regard to STEC control or limitations in the sampling and detection methods.

## 5 Conclusion

Deer colonized by STEC can be sources of human infections. Conditions necessary to ensure the safety of fresh and dried game meat deserve further review (Keene *et al.*, 1997). In this study, presence of indicator bacteria, specifically TVC, *Enterobacteriaceae* and generic *E. coli*, are not always effective at predicting the simultaneous presence of pathogens such as *Salmonella* and STEC (Ahmed *et al.*, 2008; Krometis *et al.*, 2010; Schriewer *et al.*, 2010). Future research should focus on intervention strategies that reduce the persistence of STEC in the animal population and game harvesting and processing environment. There is need to define the population effects of bacteriophages on STEC and mixed microbial flora populations. Advanced diagnostic methods for the detection of *Salmonella* and STEC in game are needed. It is concluded that game meat carry STEC O groups that may be a potential source of emerging strains. Further work need to be conducted with larger sample size in different wildlife species to make definitive conclusions.

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## CHAPTER 5

### **Brucellae through the food chain: transmission route and the role of springbok (*Antidorcus marsupialis*), sheep (*Ovis aries*), goats (*Capra aegagrus hircus*) and oryx (*Oryx gazelle*) into sources of human infections in Namibia<sup>1</sup>**

#### **1. INTRODUCTION**

Wild and domestic animals are principal sources of *Brucella* infection to humans. A human case of brucellosis in February 2009 in Namibia triggered an investigation into the potential source of infection. The emergence or re-emergence of zoonotic diseases is complex and multifactorial, often driven by evolving ecology, microbial adaptation, human demographics and behaviour, international travel and trade, agricultural practices, technology and industry (WHO, 2010). The World Organization for Animal Health (OIE) is mandated under the Sanitary and Phytosanitary Agreement (SPS) of the World Trade Organization (WTO) to develop minimum scientific or evidence based international standards, guidelines and recommendations to facilitate safe trade in animals and their products (WTO, 2010). In the case of zoonotic diseases such as brucellosis, it is believed that protection of human health can be achieved through control of the disease in the animal population. The OIE/FAO (Food and Agriculture Organization)/WHO (World Health Organization) Global Early Warning System (GLEWS) provides for rapid notification of major animal diseases, including zoonoses of which *Brucella melitensis* infection is a priority disease (WTO, 2010).

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Brucellae are small, non-motile, Gram-negative coccobacilli organisms that are responsible for one of the most widespread zoonotic infections of medical significance worldwide (Maipa & Alamanos, 2005; Staszkiwicz *et al.*, 1991). Humans are accidentally infected through direct or indirect contact with infected material and are invariably dead-end hosts of *Brucella* infections, while some wildlife species can act as potential reservoir hosts (Ferroglia *et al.*, 1998; Garin-Bastuji *et al.*, 1990; Godfroid, 2002). The pathogenic species are; *B. melitensis* which predominantly infects goats and sheep, *B. abortus* which principally affects cattle, *B. suis* infecting swine and *B. canis* dogs (EC, 2006; Hall, 1991; Tustin & Coetzer, 2004; Young, 1997; Young, 2000). Although any of the four species of *Brucella* can cause systemic disease in humans, *B. melitensis* has the lowest infective dose, requiring as low as 10 organisms to cause infection (Buchanan *et al.*, 1974; Dogany & Aygen, 2003; Kunda, 2005). Human brucellosis, commonly referred to as undulant fever or Malta fever, often coincides with livestock infection (OIE, 2008; Russo *et al.*, 2009). The disease in humans presents as a multi-systemic, acute to chronic disease characterized by non-specific signs such as fever, headache, joint pains, musculoskeletal pains, sweating, malaise, myalgia, abdominal pain, lymphadenopathy, skin rash, pneumonitis, back-ache and body wasting (Dogany & Aygen, 2003; Hall, 1991; Kunda, 2005; Oomen, 1976). The non-specific nature often presents a tremendous challenge in clinical diagnosis of brucellosis since these signs can also occur in common diseases or conditions like malaria, typhoid, rheumatic fever and pyrexia of unknown origin (Hall, 1991). Unlike in Tanzania, the disease's status in Namibia is not precisely known as most health centres do not routinely test for brucellosis (Kunda, 2005; Swai & Schoonman, 2008). Sporadic cases and even small clusters are often difficult to identify owing to an extremely variable incubation period (weeks to months) and a lack of pathognomonic clinical features or manifestations (Maichomo *et al.*, 2000; Staszkiwicz *et al.*, 1991).

Of the zoonotic species, *B. melitensis* contributes up to 70% of human brucellosis cases worldwide with sheep, goats and camels being the main sources of infection (Dogany & Aygen, 2003; Feng, 1992; Kolar, 1987; Memish, 2001). The difficulty in clinically diagnosing brucellosis coupled with the weakness of human health services in developing countries,

often contribute to human brucellosis being poorly treated (Muriuki *et al.*, 1997; Thimm & Wundt, 1976). Bacteria gain entry into the body through; ingestion, inhalation, penetration through skin abrasions as well as through conjunctival mucosa (Salari, 2002). Once in the body, *Brucella spp* survive and multiply within cells of the reticuloendothelial system. Notably, human neutrophils exhibit variable antiphagocytic activity on some *Brucella* strains but are virtually ineffective against *B. melitensis* (Young, 1997; Young, 2000). However, once excreted, brucellae rarely survive for long periods in arid tropical environmental conditions. Risk factors for infection in humans include handling of infected dead or live animals, ingestion of contaminated animal products especially unpasteurized dairy products, poor handling of Brucella Rev 1 vaccine and cultures of *Brucella spp* (Buchanan *et al.*, 1974). By virtue of increased contact with animals and their products, abattoir workers are at a greater risk of brucellosis infection compared to other professional groups (Buchanan *et al.*, 1974; Swai & Schoonman, 2008). Whilst notification of diseases in wildlife is not a pre-condition for imposing bans on members of the WTO who implement the OIE terrestrial code (OIE, 2008; Young, 2000), the ever changing game farming systems create a need to establish and distinguish between a spill over infection from domestic animals and maintenance of infection in wildlife species.

A single human case of undulant fever or Malta fever was suspected in a community surrounding the interface of wildlife farming and domestic animals in Namibia. At clinical examination, brucellosis was considered a differential diagnosis and a blood sample was collected for serological testing at a national laboratory in Namibia and at a reference laboratory [National Institute for Communicable Diseases (NICD)] in South Africa. The serum sample tested positive for brucellae IgG and IgM antibodies by enzyme linked immuno-sorbent assay (ELISA), providing a definitive diagnosis of brucellosis. This prompted us to undertake a large serological survey to provide evidence of brucellosis in domestic animals, springbok (*Antidorcus marsupialis*), [a small ungulate frequently found in close proximity to farmed livestock] and abattoir workers. Our findings suggest direct or indirect contact with goats or their unpasteurized products as the most likely source of infection to humans, whilst farmed wildlife presented the least likely threat.

## 2. MATERIALS AND METHODS

### 2.1 Background data, study area and population

A human case of brucellosis triggered an investigation into the potential source of infection in this area and three other surrounding regions in the foot and mouth disease free zone. The main farming activities in these regions include extensive sheep, goat and game rearing. These regions contribute 88.3% of the 2.7 million sheep in Namibia. A single abattoir caters for animals from these four regions and has an annual turnover of 219 929 sheep and 6 251 springbok (*Antidorcus marsupialis*). Relevant background data in the study regions was collected through a questionnaire which included these main categories including Rev 1 vaccination status: i) animal trace-back and trace-forward movements to and from the suspected farms within a year, ii) farms and other places visited by family members within a year, and iii) sources and type of animal products consumed. Farms presumed to be at risk were identified by the track back system and designated A, B, C and D. These farms held varying numbers of small ruminants and farmed wildlife. Information retrieved from the computerized Livestock and Traceability System (NAMLITS) indicated that farm A had two milking Jersey cows, 130 Boer goats, 1 300 sheep. The farm serum samples had previously been tested using Rose Bengal Test (RBT) and declared brucellosis free through annual serological testing. Farm B was inherited and had been subdivided into portions Ba and Bb between family members. Portion Ba held 984 sheep comprising of Dorper, Damara and Persian breeds, 39 Boer goats and 3 Saanen dairy goats and portion Bb had 160 Boer goats and 11 Saanen dairy goats. Farm C held 285 Boer goats and an undisclosed number of sheep. Farm D held 106 dairy goats, the largest Saanen dairy goat operation in the region which supplied most of the milk to all the four regions. Goat movement from farm Ba's grazing camps within one year from the date of initial investigation to farm C was tracked through the Livestock Traceability system (NAMLITS) system. However, discrepancies in the actual numbers of animals were noted at some of the farms at sampling. A human case of brucellosis that triggered the investigation resided in farm A. Commercial harvesting of springbok (*Antidorcus marsupialis*) was done in three of

the four regions. To examine the trend of this infection over previous years, data on the reported serological incidences of brucellosis using RBT and Complement Fixation Test (CFT) in animals between 2004 and 2009 in Namibia were retrieved and analyzed.

## 2.2 Sampling techniques

The sample sizes for farmed wildlife were determined by the formula:  $n = \log \{\alpha\} / \log (1 - p_{\max} \times \text{sensitivity})$ , where  $n$  = the required sample size,  $p$  = the prevalence (based on livestock data),  $\alpha$  = the probability (confidence level) of missing a diseased animal at a prevalence of up-to  $p_{\max}$  in a large population. The population size refers to the total number of springbok in the three regions exercising commercial harvesting. The sampling protocol was designed to detect at least one positive animal in the flock at 95% confidence limit, that is, if the disease was present at a prevalence ( $p$ ) of 5% in harvested springbok when the test sensitivity is 95% and animals are randomly selected. In this particular study on farmed wildlife, the three regions were defined as an epidemiological unit. A total of 900 adult springbok and 50 oryx from farmed flocks in three regions were sampled by systematic random sampling where a starting and interval number was picked at random from one to eight during commercial harvesting operations with a minimum farm blood sample size of eleven. Only adult male and female animals were selected for harvesting.

For sheep and goats, the sampling size was determined by the formula:  $n = \{1 - (1 - a)^{1/D}\} \{N - (D - 1)\} / 2$  as described in Cannon and Roe (Cannon & Roe, 1982) where  $n$  is the required sample size,  $a$  is the error probability (confidence level) of observing at least one diseased animal when the disease affects at least  $D/N$  in population,  $D$  is number of diseased animals in a population and  $N$  is population size. The flock size was in reference to the number of animals in the group being sampled. The sampling protocol was designed to detect at least one positive animal in the flock at 95% confidence limit, that is, if the disease is present at a prevalence of 5% in animals of breeding age (six months old and above). If an animal was picked and not subjected to blood collection, it was placed in a different holding pen.

## 2.3 Preparation of serum from domestic animals and farmed wildlife

The samples sizes of domestic ruminants differed from farm to farm. All animals were sampled by venipuncture of the jugular vein. On farm A, 77 sheep, 25 goats and two milking cows were bled. On farm subdivision Ba, 39 goats and 984 sheep were bled whereas 34 goats on subdivision Bb. After initial positive results of samples from farm Ba, all the 285 Boer goats on farm C, all sheep and goats on farm Ba and 106 goats from farm D were bled and serologically tested for brucellosis. Blood was allowed to clot at ambient temperature and placed in a cooler box in transit to the laboratory. Serum was separated from the clot by centrifugation (13,000 x g) for 10min, collected into fresh tubes and stored frozen at -20°C until required for analysis.

Blood samples were collected randomly from springbok (*Antidorcus marsupialis*) and oryx at harvesting periods; April to August 2009 and July to August 2010. Typically, blood was freely collected in test tubes upon severing of both carotid and jugular vessels, allowed to clot at ambient temperature and then placed in cooler box before processing. Sera were prepared, collected and stored in a similar manner as described above for domestic animals.

#### **2.4 Serological testing of domestic animals and farmed wildlife**

All sera were subjected to serological testing following standard protocols (Alton *et al.*, 1988). In our serial testing, we assumed that both the RBT and CFT had 100% sensitivity at 95% confidence in detecting *Brucella* antibodies in sera from culture-positive animals. Sera from domestic animals and farmed wildlife were initially screened for brucellosis by the RBT and positive sera samples were also verified by CFT using antigens, complement and amboceptor (Onderstepoort Biological Products, South Africa and Siemens Healthcare Diagnostics Products GmbH, Germany) as per manufacturer instructions. Briefly, serum was inactivated at 56°C for 30 min. 25µl of Veronal buffer (VB) was added to each well to be used for serum samples and serum references. 25µl of the test serum, positive and negative controls were added to the appropriate wells and a twofold dilution made. 25µl of the prepared antigen (depending on the dilution of the particular antigen) was added followed by 25µl of the prepared complement (depending on the disease) to all the wells.

25µl of VB was added to first row to equalize the volume in the wells and the plate was incubated together with 3% sheep red blood cells (SRBC) suspension at 37°C for 30min. 25 µl of 3% SRBC was added to all the wells, shaken and then the plate incubated at 37°C for one hour while shaken twice at 10 minutes intervals before the results were read.

## **2.5 Serological testing of abattoir employees**

The human sera were collected as part of the company's occupational health surveillance and regulatory monitoring of worker's health in the meat industry. Approval to use such sera was obtained from abattoir management (in consultation with their Medical doctor) and the workers' representatives. A total of 137 human serum samples were collected from individuals involved in buying, slaughtering and deboning small ruminant and game animal carcasses. The relative proportion of each age group and sex of the employees sampled are given in Table 5.1. Serum samples were processed at an accredited laboratory and stored at -20°C prior to use. Serum samples were screened by the standard tube agglutination test (STAT) using *B. abortus* and *B. melitensis* antigens (Linear Chemicals SL, Spain) as per manufacturers' instructions where a titre of 1:80 or greater was considered positive. Positive samples were confirmed by enzyme-linked immuno-sorbent assay (ELISA) using the Panbio *Brucella* IgG ELISA kit (Plasmatec Laboratory Products, Dorset, UK).

**Table 5.1:** Distribution of abattoir workers tested for brucellosis on the basis of age and sex

Age (years)	Sex				Total		Positive for Brucellosis
	male		female		No	%	
	No	%	No	%	No	%	
<20							
20-30	31	22.63	6	4.38	37	27.01	0
30-40	63	45.99	11	8.03	74	54.01	1
40-50	19	13.87	2	1.46	21	15.33	2
50-60	4	2.92	1	0.73	5	3.65	0
>60	0	0	0	0	0	0	0

No: number

## 2.6 Postmortem analysis and bacteriological culture

All sero-positive animals were slaughtered separately at a non-export local municipality abattoir at the end of the slaughter week and subjected to post-mortem examination according to local health and safety guidelines for handling suspected zoonotic cases. Tissue samples were collected from the liver, kidney, spleen, lymph nodes (iliac, oropharyngeal, inguinal, mesenteric and supra mammary), testicles and udder for the detection and isolation of brucellae organisms. Where pregnant animals were involved, the foetal lung and abomasal contents, uterus, and placenta membranes were sampled and processed following standard techniques (Alton *et al.*, 1988). No abortive material or vaginal discharges were obtained. Briefly, impression smears were made from a cut surface of the organs by first blotting on clean absorbent paper and then gently pressing onto a clean glass slide. Smears were air-dried, heat-fixed before staining by a modified acid fast technique. Fixed smears were flooded with dilute carbol fuchsin for 10 min, rinsed in tap water, differentiated with 0.5% acetic acid for 30s and counter-stained with 1% methylene blue for 20s. Slides were air-dried and then viewed with a light microscope under oil immersion.

Tissue samples were cultured on agar on Farrell's selective medium for brucellae (Farrell, 1974). Briefly, samples collected at post-mortem were homogenized in sterile phosphate buffered saline (PBS) using a stomacher before being inoculated on to the solid media. 100 ml of each tissue homogenate was streaked on *Brucella* selective agar and the plates incubated aerobically in enriched CO<sub>2</sub> with daily examination for Brucellae colonies up to 7 days.

### **3. RESULTS**

#### **3.1 Data from a questionnaire**

Interviews revealed that apart from the brucellosis positive individual that triggered the investigations, three additional family members from the Hardap region (where farm A is located) and an unrelated person from another region had previously been treated for Malta fever. Interestingly, all humans diagnosed with brucellosis shared a common history of consuming raw goat milk, home-made goat cheese and coffee with raw milk and prior contact with dairy goats. None of the farms used Rev 1 vaccine in sheep or goats, but heifers were vaccinated with *B. abortus* strain 19. Reported clinical signs suggestive of brucellosis in small ruminants of farms A and C included; abortions, orchitis, and arthritis in small ruminants whereas those seen in dairy goats of farm B were; abortions, retained placenta and arthritis.

Unpasteurized cow milk from farm A, was mainly consumed on the farm with small quantities occasionally sent to farm B. Raw goat milk was consumed at subdivisions of farm B at least once per week. In general, the substantiated links between farms A and B were; i) cow milk ii) individuals from farm A visiting and drinking coffee with unpasteurized goat milk on farm B and iii) a ram introduced on farm A from farm B. We were unable to substantiate clear links between these farms (A and B) and farms C or D, except people movement between them. Nevertheless, these findings provided some clues on the possible route of transmission of brucellosis to humans.



### 3.2 Serological testing of domestic ruminants and wildlife

Samples of sheep (n=77) and goats (n=25) from farm A and farm subdivision Bb (n=34) were serologically negative for brucellosis using the RBT. Initial serological analysis of 39 sera from farm Ba revealed 20 positive cases by CFT [where a titre of 1:8<sup>++++</sup> (49 international standard unit) or greater was considered positive], prompting us to test the entire flock on this farm and the nearby farm C, to obtain the extent of the problem. Twenty-six out of 42 goats from farm Ba and twelve of the 285 from farm C were positive for brucellosis using the CFT. The positive cases on farm Ba included all milking goats whilst all sheep from both farms were negative. Of the 12 positive goats in farm C, six were pregnant females and a single 6-9 month-old male goat, whereas all those on farm Ba were not in lamb. The two Jersey cows on farm A were also negative by both serological tests. To contain the disease, farms B and C were immediately quarantined and the positive goats were isolated before being sent for slaughter at a local non-exporting municipality abattoir. To investigate the disease status in farms Ba and C after quarantine, the remaining animals were re-tested after 3 months and none tested positive for brucellosis, suggesting the infection had been controlled. To explain the possible cause of orchitis reported in farm C, we tested the sera of uncastrated males for *Brucella ovis* antibodies by RBT and CFT and detected three positive cases. *B. ovis* is of exclusive concern in rams where it causes epididymitis.

The annual reporting of the incidences of brucellosis in Namibia has previously focused on livestock but not farmed wildlife. With game animals increasingly entering the human food chain, the springbok and gemsbok (*Oryx gazelle*) were first included in the reporting system in 2009, but the incidence was zero (Table 5.2). To determine whether farmed wildlife play a role in the transmission of brucellosis, we tested a total of 900 springbok and 50 oryx samples harvested from 29 different farming units, but none was positive by either RBT or CFT suggesting this species was not responsible for transmission of brucellosis to humans. These findings were consistent with the absence of clinical or

pathological signs suggestive of brucellosis. As such, it was felt that no further action was necessary.

**Table 5.2:** The number of brucellosis positive samples from food-producing animals as determined by the RBT and CFT from 2004 to 2009 in Namibia.

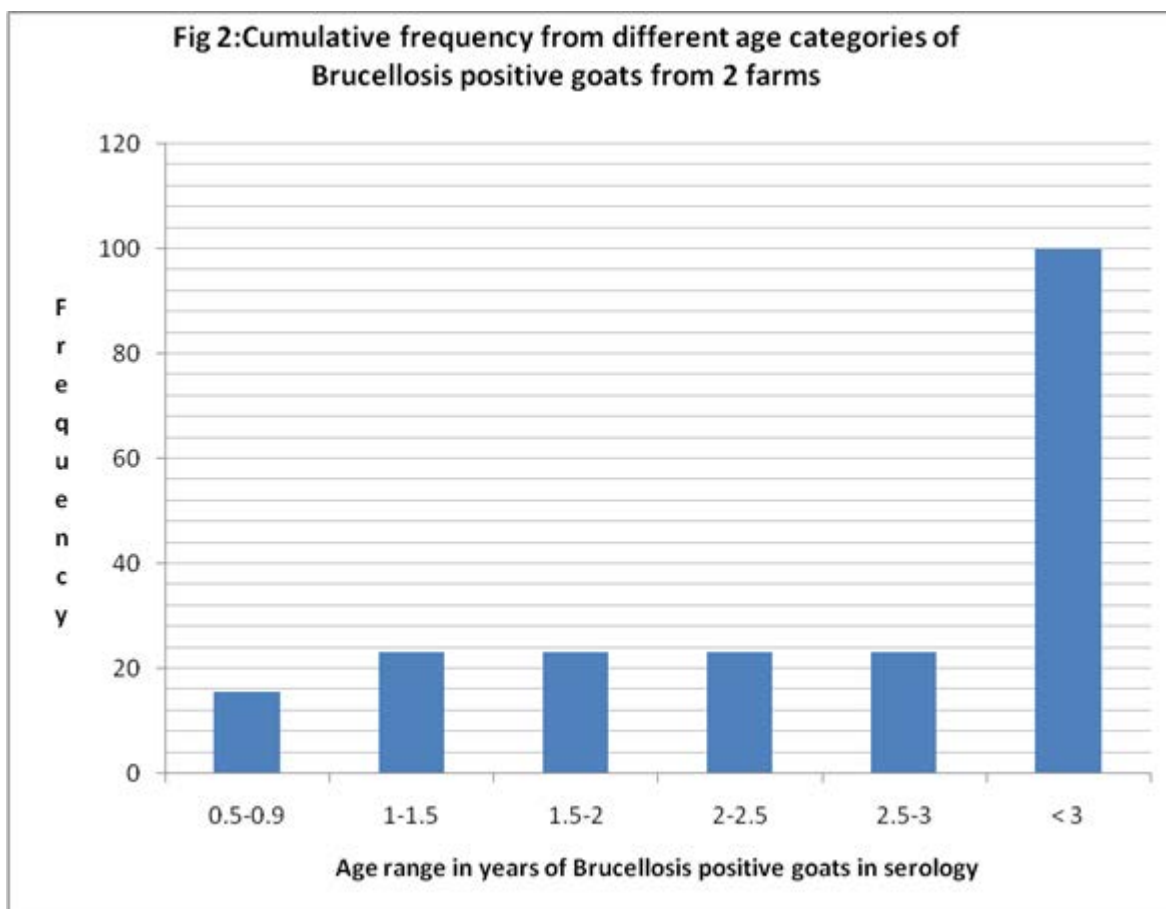
Year	Species	Total number of samples tested	Number of positive samples	% seropositive
2004	Dairy cows	4001	19	0.47
	Other bovine	311	11	3.54
	Sheep and goats	18485	19	0.10
2005	Dairy cows	2541	2	0.08
	Other bovine	246	0	0.00
	Sheep and goats	10191	15	0.15
2006	Dairy cows	2994	9	0.30
	Other bovine	401	15	3.74
	Sheep and goats	3452	21	0.61
2007	Dairy cows	1578	0	0.00
	Other bovine	587	5	0.85
	Sheep and goats	1486	31	2.09
2008	Dairy cows	Data unavailable	N/A	N/A
	Other bovine	1910	15	0.79
	Sheep and goats	13745	37	0.27
2009	Dairy cows	Data unavailable	N/A	N/A
	Other bovine	1030	20	1.94
	Sheep	7376	4	0.05
	Goats	516	32	6.18
	Springbok and gemsbok	122	0	0.00

### 3.3 Serological testing of humans

Of the 137 serum samples screened for *Brucella* antibodies, all were negative by STAT, three were positive for IGg antibodies but negative for IgM antibodies by ELISA. The distribution of tested abattoir employees on the basis of age and gender is shown in Table 5.1. The positive samples were from males aged 31 (one) and 40 (two) years. These individuals were clinically healthy and received no treatment. To examine if new cases developed, the same 137 individuals were bled as part of annual health screening and were re-tested for *Brucella* antibodies by ELISA and none was positive, suggesting the infection had cleared.

### 3.4 Post-mortem findings and bacteriology

The goats were transported under a special movement permit to the abattoir and slaughtered in accordance with local Health and Safety regulations relating to the handling of zoonotic agents. The majority of sero-positive goats were over 3 years (Figure 5.1). Post-mortem analysis and collection of tissue samples for bacteriology was undertaken by the official state veterinarian following local instructions and precautions for handling zoonotic agents. Post-mortem examination of all sero-positive goats revealed no typical brucellosis lesions except in a single ram that had swollen mesenteric and iliac lymph nodes for unknown cause. Direct microscopy on collected organs failed to reveal any bacterial cells suggestive of the presence of *Brucellae* organisms. Subsequent culturing of homogenized tissues also failed to yield any *Brucella* colonies after 7 days of incubation, suggesting the absence of an active infection in the sero-positive animals.

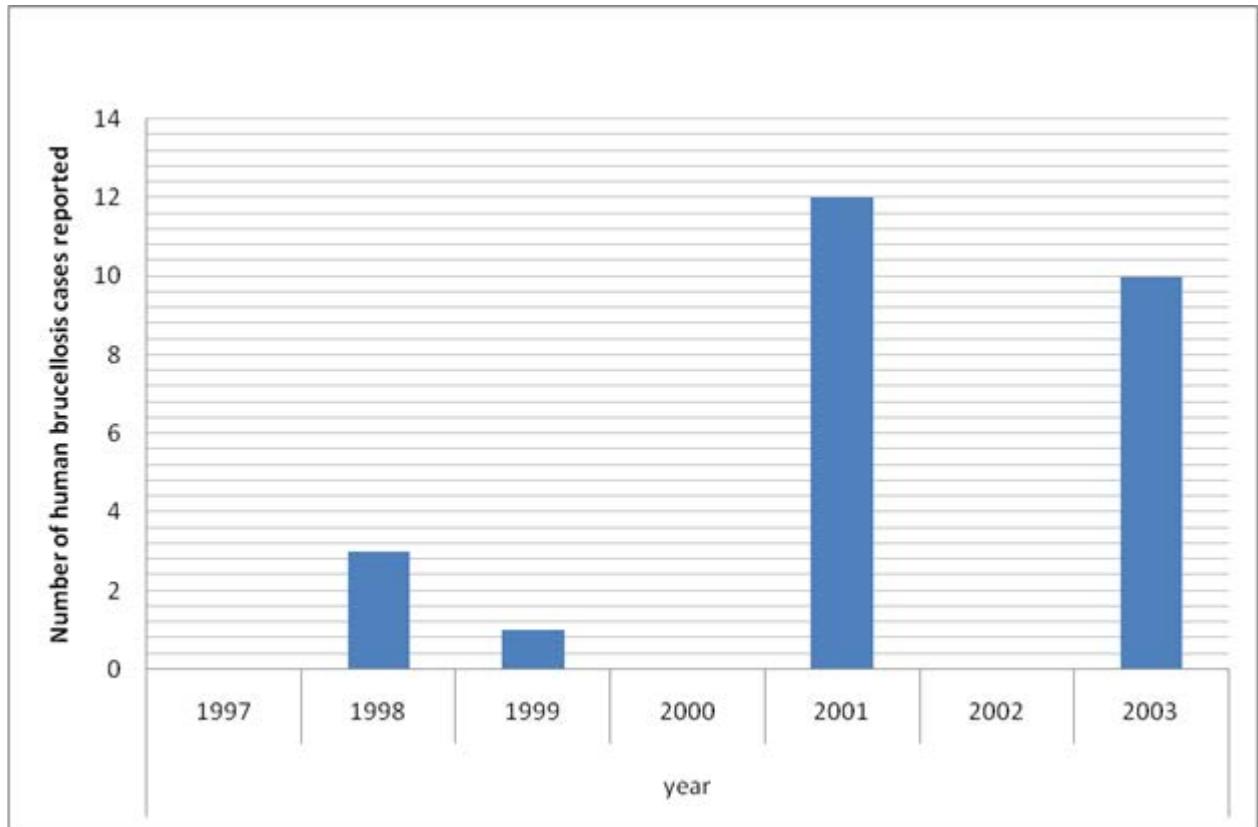


**Figure 5.1:** Cumulative frequency from different age categories of brucellosis positive goats from 2 farms.

### **3.5 Retrospective analysis of incidences of brucellosis in domestic animals and humans**

To gain insights into the trend of brucellosis in farm animals and humans, annual records for the period 2004 and 2009 were retrieved and analyzed. Sero-positive bovine cases were detected as more compared to other species for the period under consideration suggesting brucellosis to be endemic in domestic animals. The incidence of brucellosis in sheep and goats was relatively low from 2004 to 2008. An increase in the number of goat cases was observed in 2009 (Table 5.3), indicating goats as a likely leading source of brucellosis during this period. However, there is no corresponding data in humans for the same reporting period to indicate a zoonotic transmission. Human cases were only

reported for the period 1997 to 2003 (figure 5.2), suggesting either a break in the reporting system or absence of cases until those triggering the present investigation.



**Figure 5.2:** Number of official reports of human brucellosis in Namibia from 1997 to 2003 (OIE, 2012).

#### 4. DISCUSSION

The annual reporting of the incidences of brucellosis in domestic animals in Namibia (shown in Table 5.2) relies on the RBT and CFT on sera from suspected cases. However, due the cross-reactivity and interspecies infection by *B. abortus* and *B. melitensis*, it is difficult to ascertain the actual prevalence of each species. Differentiation of the species requires successful isolation and subsequent molecular characterization studies (Smits *et al.*, 2004). Indeed, a multiplex PCR assay for the identification and differentiation of all *Brucella* species including conventional vaccine strains now exists (García-Yoldi *et al.*,

2006) but requires *Brucella* colonies. Such studies were impossible in our study since we were unable to isolate *Brucella* from tissues of sero-positive cases. Failure to obtain any *Brucella* colony from sero-positive could have been due to; the inactive infection in the animals as evidenced by lack of pathological lesions or the suppressive effect of the selective medium used. Indeed, Farrell's medium has been reported to be inhibitory for *B. ovis* and some *B. abortus* and *B. melitensis* strains (Marin *et al.*, 1996). Despite this, the selective advantage conferred by cycloheximide and a range of antibiotics makes this medium a priority for primary isolation of *B. abortus* from contaminated samples like post-mortem tissues. Importantly, we have previously been successful in isolating of brucellae from organs of sero-positive goats from other regions of Namibia using this medium (Unpublished), thus confirming its quality. Although alternative selective media for the isolation of brucellae have been described (De Miguel *et al.*, 2011; Hornsby *et al.*, 2000; Kuzdas & Morse, 1953; Stack *et al.*, 2002), these have not been widely used. We did not employ non-selective media like blood agar since brucellae organisms are often overgrown by contaminating fungi and other fast-growing bacteria when plates are incubated for 7 days.

Brucellae are usually abundant in abortive material and vaginal discharges hence these are highly recommended for the demonstration of brucellae organisms by direct microscopy or culture methods. Although demonstrating the presence of *Brucella* organisms in tissues and fluids is central to confirming the diagnosis of brucellosis, serological testing remains the hub of estimating the prevalence of the infection either in humans or animals. It has recently been suggested that i-ELISA can be used for screening cattle with improved specificity, but the RBT and CFT remain confirmatory tests (McGiven *et al.*, 2008). The RBT and CFT are conventional tests widely used in serological diagnosis of brucellosis in domestic animals where their respective sensitivities have been reported to correlate with culture-positive animals (Díaz-Aparicio *et al.*, 1994). Application of the RBT and CFT serially with a simultaneous consideration of the test results increases the likelihood of detecting infected animals (Raúl *et al.*, 2005; Waghela *et al.*, 1980). In humans, ELISA is the gold standard test for diagnosis of brucellosis, where its specificity is increased when IgM

antibodies are not detected (Wright & Nielsen, 1990), though a conflicting report suggests its potential to detect false positives (Ertek *et al.*, 2006). However STAT has previously been reported to differentiate from active and inactive infections in humans based on the titres (Young, 1991). An immunocapture-agglutination test (Brucellacapt) also exists (Orduña *et al.*, 2000), but this is not widely used. The inability to detect any positive case with STAT suggests these were inactive infections, which were later confirmed by re-testing. In general, caution should be exercised in interpreting these serological tests particularly in patients with chronic brucellosis, re-infections and relapses, and in endemic areas where a high proportion carries antibodies against brucellosis.

Although brucellosis is rarely reported in wildlife, cases believed to be a spillover from small ruminants have been reported in *Rupicapra rupicapra* and *Capra ibex* in Europe (Ferroglio *et al.*, 1998; Garin-Bastuji *et al.*, 1990; Herr & Marshall, 1981). We therefore tested farmed springbok for brucellosis since these were reared in close proximity with small ruminants which could result in spillover of the infection. However, Brucellosis seroprevalences of 23% and 48% have been reported in African buffalo and other wildlife populations in southern African countries where contact with domestic sheep, cattle, pigs and goats were absent (Bekker *et al.*, 2012; Corbel, 1997; Ewalt *et al.*, 1997; Herr & Marshall, 1981; Verger *et al.*, 1989). Furthermore, several species of wildlife including African buffalo (*Syncerus caffer*), zebra (*Equus zebra*), impala (*Aepyceros melampus*), waterbuck (*Kobus ellipsiprymnus*), and hippopotamus (*Hippopotamus amphibious*) have tested positive for *B. abortus* and *B. suis* worldwide with *B. abortus* biovar 1 being isolated in the cotyledons of pregnant buffaloes (Godfroid, 2002; Herr & Marshall, 1981). In some areas where *B. abortus* had been eradicated in cattle populations, sheep and feral pigs have been implicated as sources of *B. melitensis* or *B. suis* infection in cattle respectively (Corbel, 1997; Ewalt *et al.*, 1997; Verger *et al.*, 1989).

It was unclear why none of the sero-positive goats, including pregnant females failed to show typical lesions of brucellosis on post-mortem examination. We were, however, unable to exclude the possible presence of other cross-reacting pathogens. False-positive cross-

reactions due to *Yersinia enterocolitica* serotype O: 9 infections have been reported in cattle (Gerbier *et al.*, 1997; Raúl *et al.*, 2005). Another reason for sero-positivity could be vaccine-induced antibodies, though our background investigations revealed that vaccination of goats with Rev 1 was not routinely undertaken on these farms. However, the enlarged lymph nodes found in the male goat was attributed to be *B. ovis* suggesting the presence of multiple infections within the flock. Based on the historical data gathered through a questionnaire and interviews, the animals testing positive for brucellosis, a few abattoir employees also testing positive, we strongly suspect goats as the most likely source of infection. Although, we could not ascertain this, it is likely that *B. melitensis* was involved as it is usually associated with goats since vaccination against *B. abortus* was routinely done in cattle. The consumption of unpasteurized goat milk or its products like home-made cheese is a leading antecedent to human brucellosis (Chanet *et al.*, 2005; Moraes *et al.*, 2009; Plommet *et al.*, 1988).

Generally, where there is lack of traceability of animal movements to and from the affected farms, it is frequently impossible to trace the origin of the infection (McDermott & Arimi, 2002). In this study, lack of a coordinated approach during the brucellosis case investigation across the Ministry of Health, State Veterinary region and Central Veterinary Office precluded effective planning and formulation of common strategies for food safety and control of zoonotic diseases. Consequently, there was a direct conflict in the implementation of simple preventative measures against brucellosis in abattoir workers that are usually presented with a greater risk of infection despite the existence of health and safety related handling of suspected zoonotic agents. Although contact with infected herds and/or with contaminated environmental sources have been shown to play a major role in the spread of brucellosis and despite historical sporadic outbreaks of brucellosis in humans, sheep and goats attributed to *B. melitensis*, the contribution of these and other factors to the epidemiology of brucellosis in game species in Namibia remains unclear. Consequently, more work is required to dissect the cross-species and within species transmission dynamics of brucellosis at the interface of wildlife and domestic animals.



## 5. Conclusion

Our data highlight the risk of *Brucella* transmission through goats and unpasteurized products to humans. It is very likely other human brucellosis cases were missed since the manifestations are non-specific. As such, a better control program which includes public awareness, vaccination of goats with the Brucella Rev 1 vaccine (Gradwell & Van Zyl, 1975) and pasteurization of milk should be expected to reduce the incidences of brucellosis in humans. This data also show that springbok may not be a vehicle of transmission of brucellosis despite being reared at the wildlife-domestic animal interface.

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## CHAPTER 6

### Incidence of parasitic contamination at inspection of harvested springbok (*Antidorcus marsupialis*) and gemsbok (*Oryx gazelle*)<sup>1</sup>

#### 1. INTRODUCTION

The spectrum of foodborne diseases is constantly changing and in the light of favorable microbiological safety results from game meat, careful scientific observation and research was considered in other areas as little work has been conducted on parasitic infestation of harvested wild game at game abattoirs in Namibia. Wildlife farming is increasingly becoming integrated into the mainstream meat industry for food security and income generation in most Southern African countries thus positioning the wildlife industry on a different platform from its traditional past (Vertefeulle & Benn, 2005). In recent years, Namibia has witnessed a remarkable recovery and increase in wildlife populations. The number of small communal conservancies, which bring immense benefits to many rural communities, has spiraled significantly in many parts of the country. Surveys indicate that springbok (*Antidorcas marsupialis*), oryx (*Oryx gazelle*) and mountain zebra (*Equus zebra*) populations increased over 10 times between 1982 and 2000 with about 80% of these located on privately owned farms or freehold conservancies (Weaver & Skyer, 2003). However, the characteristics of soil, vegetation, spatial distribution of resources and presence of multispecies, such as predators, all influence the dynamics of the host-parasite system (Blancou *et al.*, 2011).

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<sup>1</sup>Part of this chapter was published as:

**Magwedere K, Hemberger HY, Khaiseb S, Hoffman LC and Dziva F**(2012) Parasitic contamination incidences at inspection of harvested springbok (*Antidorcas marsupials*) and gemsbok (*Oryx gazelle*) in Namibia. *Journal of Veterinary Science & Technology* 3(3): 113.

In many parts of the free ranging farms, cattle, sheep, and goats graze together and carnivores such as jackals are common in some areas. Sheep are by far the most numerous livestock species in the southern part of Namibia, and along with goats have the greatest opportunity for livestock-wildlife disease transmission when grazing in remote lands frequented by wildlife. Due to the nature of this important emerging wildlife industry, killing practices tend to differ in many aspects from standard procedures applicable in slaughterhouses of livestock species. Small ruminant wildlife are killed by free bullet and immediately bled then eviscerated whilst suspended on mobile line hangers. However, meat inspection procedures conform to standard practices for other food-producing animals and likewise, further investigations are invariably undertaken on all seized carcasses.

The changing ecosystems impact on wildlife populations and their susceptibility to pathogens thereby calling for a systematic and thorough meat inspection process in order to prevent transmission to humans (Mukaratirwa *et al.*, 2001; Anonymous, 2005). Parasitic infestation is not uncommon in springbok (Anonymous, 2005; Boomker, 2009; De Villiers *et al.*, 1985; Horak *et al.*, 1982; Ortlepp, 1961; Round, 1968; Young *et al.*, 1973). In game species examined elsewhere, a number of helminth species and new host records for several species of nematodes have been reported (Anonymous, undated). Of particular significance, worm burdens ranging between 2 954 and 71 790 per gram and a high proportion of 4<sup>th</sup> stage larvae were reported in South African wildlife (De Villiers *et al.*, 1985; Horak *et al.*, 1982), implying that surveillance of wildlife parasitic diseases may be problematic. Not surprising, the majority of reviews on animal health monitoring systems mention wildlife disease surveillance in passing due to the difficulties in establishing population estimates (denominator data) for defining rates, such as disease incidence. As such, this becomes a key obstacle in developing systematic wildlife surveillance programs linking with those for human diseases (Childs, 2007). To determine the risk posed by parasites, the inspection incidence of parasites in springbok (*Antidorcas marsupialis*) and gemsbok (*Oryx gazelle*) carcasses for gross infestation and rectal contents from eviscerated

gut for worm egg counts were evaluated. These data provide a baseline for possible future assessments of the distribution of parasites among Namibian wild ungulates.

## **2. MATERIALS AND METHODS**

### **2.1 Pre-slaughter health assessment**

Animals from two farms located 100 km apart in different districts of the Hardap and Karas regions of Namibia were initially presented for harvesting in 2005. A further 9 251 springbok and gemsbok animals sourced from eight different farms were presented for harvesting between April 2009 and August 2010. Assessment of the general physical and health status of these animals was undertaken by an experienced hunter. This was accomplished by assessing the animal's response to attempted capture. Briefly, this involved a precise estimation of escape distances and flight zones of animals at night. Sick or exhausted animals, generally had shorter escape distances (30-100m) and flight zones compared to healthy animals which were always greater than 120m.

### **2.2 Slaughter and meat inspection process**

Animals were shot with a free bullet and immediately suspended on to mobile hangers for bleeding and evisceration. Bleeding and evisceration were completed within 10 and 60 min of shooting, and carcasses were transported to a field dressing station for an initial health inspection. Carcasses passing initial inspection were transported under chilled conditions to a slaughterhouse for skinning and final inspection as described previously (Game regulations draft, 2010; Maja, 2006). The carcasses underwent a meat inspection process conforming to guidelines stipulated in the European Commission regulation (Game regulations draft, 2010). A detailed schedule of the inspection process is illustrated in South African game regulations (European Union Regulation 854, 2004). Specific features requiring serious consideration included the following; general body condition, efficiency of bleeding, degree of gross contamination, colour, odour, external parasitic infestation, bruising and injuries. Any condemned whole or part of the carcass was seized and



subjected to further detailed examination which included macro and microscopic examination (Game regulations draft, 2010).

### **2.3 Faecal worm egg counts**

Approximately 50g of faecal contents from the posterior part of the rectum were randomly collected from each of the adult health gemsbok and springbok harvested between April 2009 and August 2010 from six different farms. Each pooled sample consisted of five subsamples collected from five animals. Forty five pooled samples from springbok and twelve from gemsbok were collected. The samples were refrigerated at 4°C within 30–60 min after collection until analysis. Parasites eggs were quantified using the Wisconsin double centrifugal sugar flotation method (Goodman *et al.*, 2007). Briefly, pooled faecal material was initially well-mixed in a small grinder and five grams weighed and placed in a 15ml glass beaker. 13ml of water was added and the faecal material thoroughly mashed with a spatula. The slurry was then passed through a tea sieve and funnel into a test tube while pressing on the remaining material. The filtered samples were centrifuged at 1500rpm for 10min, the supernatant carefully removed and discarded, leaving the fine sediment of the pelleted material undisturbed. The pelleted faecal material was re-suspended in concentrated sugar solution (specific gravity=1.275) and dispersed using a glass rod. The test-tube was then topped with sugar solution and a 22×22 mm cover slip was placed onto the meniscus. The samples were again centrifuged at 1500rpm for 10min, after which the cover slips were gently transferred to slides and the number of eggs on entire cover slips counted using a light microscope. Total count were divided by 5 and expressed as worm egg counts per gram of faeces.

### **2.4 Identification of parasitic larvae and adult worms**

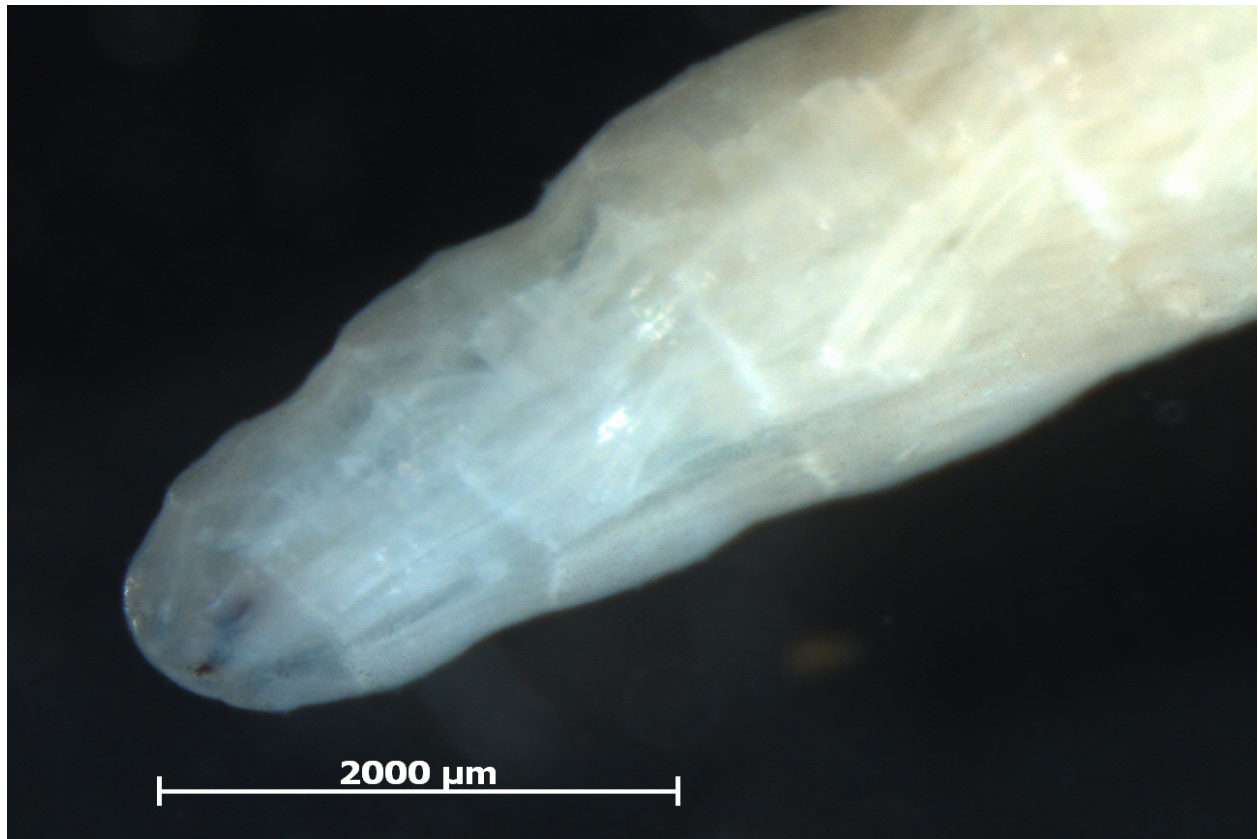
*Skrjabinodera kuelzii* was identified at the Onderstepoort Veterinary Institute, the unidentified larvae were examined at the Department of Veterinary Tropical Diseases, University of Pretoria and Department of Zoology & Entomology, Rhodes University while faecal worm egg counts were done by the technical parasitology staff from a veterinary laboratory in Namibia. The larvae infested meat samples were preserved in 10% formalin

and 70% alcohol before referring them to the Central Veterinary Laboratory in Windhoek. Identification was to the level of genus and where possible to species level, using keys and illustrations based on gross morphological features and microscopic examination using a light microscope. In the identification of *S. kuezii*, the worms were carefully separated from the muscle tissues using dissection equipment into sterile petri-dishes. The worms were preserved in 70% alcohol. Identification was based on microscopic examination using a light microscope (Chabaud & Rousselot, 1956).

### 3. RESULTS AND DISCUSSION

Shooting individual game animals opportunistically on encounter is not an ideal sampling method, and can be prone to biases; for example, towards animals with parasites of high intensity. However, in this study, it was assumed that shooting did not result in the selection of thinner animals or those with high intensities of parasites, assuming that group size itself is independent of parasite intensity and based on the post mortem findings. During a routine inspection of 279 springbok carcasses in 2005, no helminthes, arthropods or parasite larvae associated abnormalities or pathological lesions were detected at post mortem although some carcasses, whole or in part, were condemned for aesthetic reasons (bruising, insufficient bleeding and general gross contamination). Subsequent detailed examination of the passed carcasses during deboning revealed parasite larvae on 39 carcasses (Figures 6.1 and 6.2). The larvae were more concentrated in the hindquarter muscles (*Musculus biceps femoris*, *M. gluteus* and *M. semitendinosus*) and seldom in the *Longissimus dorsi* muscles. Notably, the infested carcasses comprised 36 from the 1<sup>st</sup> farm in Hardap and three were from the 2<sup>nd</sup> farm in the Karas region. Characteristically, the larvae were approximately 7-10mm (Figure 6.2) long with a few possessing a distinct dark red pigmentation, whilst the majority was creamy in color. Microscopic examination presumed the larvae to be early or late 1<sup>st</sup> stage larvae of *Chrysomya* or *Musca* species, however, further molecular identification of these larvae to species level conducted at the Department of Zoology and Entomology, Rhodes University in South Africa ruled out the larvae as of *Chrysomya* or *Musca* spp thus further molecular identification is required and

will form part of the future studies. Table 6.1 shows the prevalence of the unidentified larvae.



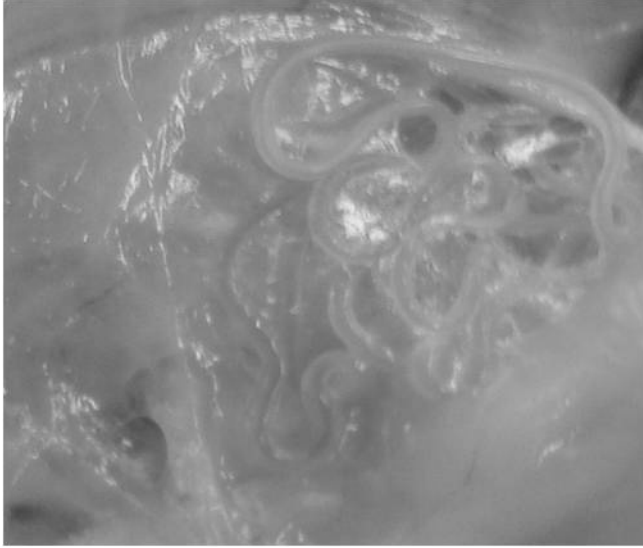
**Figure 6.1:** Shows the microscopic picture of preserved unidentified larvae recovered from the muscle of springbok (*Antidorcas marsupialis*).



**Figure 6.2:** Shows the approximate length of un-identified larvae recovered from the muscle of springbok (*Antidorcas marsupialis*).

A further examination of 9 251 springbok carcasses from different farming units failed to detect these larvae. Whilst the observations in 39 springbok carcasses are considered to be significant, it could not be explained why such larvae were not detected in 9 251 springbok carcasses from five different geographical regions over a four year-period. Nevertheless, to our knowledge, the deep muscle contamination of carcasses by these parasitic larvae is the first ever reported and hence should be considered during meat inspection of game meat.

A filarial parasite, identified as *Skrjabinodera kuelzii*, was detected at a prevalence rate of 19% on one farm and 100% on another farm, altogether representing a total of 700 springbok carcasses brought in for cutting and boning. The farms were separated from each other by approximately 50km without any history of interaction. In a total of 104 springbok carcass, *S. kuelzii* was primarily located in the inguinal region fascia, muscle fascia and renal fat (figure 3).



Dimensions 2048 x 1536

**Figure 6.3:** *Skrjabinodera kuelzii* adult worms inhabiting the inguinal region muscle fascia of Springbok (*Antidorcas marsupialis*).

Due to the lack of clinical or pathological significance of this parasite, a precautionary approach was adopted and the carcasses were condemned whole or in part depending on the level of infestation. Subsequent investigations after 3 months confirmed the inguinal region to be the predilection site of these worms. Very little is known about the life cycle of this filarial worm which belongs to the family *Onchocercidae*. An unidentified haematophagous arthropod, most likely a dipteran insect as for other *Onchocercidae*, is believed to be the transmitting vector. It is widely accepted that *Diptera* flies are mechanical and biological vectors of human enteric pathogens and hence should be of great concern from the sanitary and health point of view (Baumgartner, 1993; Siyalo *et al.*, 2007). Occupation of a specific niche within the host such as renal fat helps to evade immune mechanisms and thus seems to be an *in vivo* survival technique. The significance of these worms is not apparent, they do not seem to cause pathological lesions in the peritoneum since none of the mesenteric or inguinal lymph nodes were abnormal in the affected animals. However, the numbers and location of worms appeared vary from farm to farm and this pattern could be related to the spatial distribution of *Diptera* flies, which is poorly characterized in Namibia. Typically, in one farm 1-20 worms were found in the

pelvic area only while on another farm 15-30 worms were consistently found in the pelvic and inter-muscular connective tissue of hindquarter muscles. Interestingly, the parasite dies within 1 hour after the death of the host suggesting that it probably relies on oxygenated circulating blood or homeostatic body temperature. The parasite was restricted to three farms possibly reflecting the distribution of the vector. Undoubtedly, more work is required to define the clinical significance, the biology and epidemiology of this parasite in wildlife in order to inform on safety of meat derived from such sources. Additionally, the impact of climatic variation on prevalence, geographical distribution and more importantly, the survival of free-living larval stages of parasites (Boomker, 2009; Mas-Coma *et al.*, 2008; Patz *et al.*, 2000; Van den Bossche & Coetzer, 2008), should form part of a future investigation.

Infestation with filarial worms is not uncommon in ruminant wildlife species though. *Skrjabinodera kuelzii* was originally described as *Filaria kuelzii*. *Filaria kuelzii* was described in three antelopes (*Philantomba maxwellii*) (Khalil & Gibbons, 1975; Rodenwaldt, 1910). The parasites were found in the intramuscular connective tissue of the back and pelvis region including subcutaneous tissue and microfilariae could be demonstrated on the blood smears. Another filarial worm, *Dirofilaria asymmetrica* was described in the common duiker (*Sylvicapra grimmia*) in South Africa, but with an undisclosed site of infection. Chabaud and Rousselot synonymised this species with *S. kuelzii* (*F. kuelzii*) in specimens from the intermuscular connective tissue from the black-backed duiker (*Cephalophus dorsalis castaneus*) in the then Belgian Congo (Chabaud & Rousselot, 1956; Quentin, 1969). Moreover, another filarial worm, *S. tanganyikae* was originally described as *Gazellofilaria tanganyikae* from the peritoneal cavity and mesentery of Thomson's gazelle (*Gazella thomsoni*) and further studies showed that this parasite also occurred in the pelvic cavity of Grant's gazelle (*Nanger-granti*) (Sachs, 1976; Liang-Sheng, 1956).

Rectal contents of both gemsbok and springbok were found to contain variable numbers of different helminth eggs. Whilst the gemsbok harboured eggs of strongyle, *Trichuris* spp and coccidia, the springbok were also additionally infested with *Toxocara* spp and *Strongyloides*

*papillosus* in addition to those eggs isolated in gemsbok. Detailed worm egg burdens are given in Table 1. Parasitic load and prevalence in wild animals can be used as indicators of population and ecosystem health and a number of species from the genera *Toxocara* and *Trichuris* pose a potential anthrozoonotic transmission risk (Okulewicz *et al.*, 2012; Dado *et al.*, 2012; Teichroeb *et al.*, 2009). The significance of the egg counts in this study is not clearly discernible as the carcasses were of apparently average body condition. A possibility of variation in fecal water content confounding estimates of gastro-intestinal parasite intensity in wild African herbivores have been postulated (Turner *et al.*, 2010). Consistent with this, worm egg loads have been reported to remain constant in healthy animals under normal conditions but increase considerably upon the induction of stressful conditions such as drought, disease and serious injury (Boomker *et al.*, 1986). In impala, worm egg loads can rise from 20 000 to 60 000 per animal during drought periods, but the damage to the host largely depends on the composition and dynamics of the worm population (Boomker, 2009). The composition of the worm load is also dependent of the type of vegetation and its condition as determined by seasonal factors (Bothma, 2006; Boomker *et al.*, 1986). A significant difference in worm burden has been reported between browsers and grazers. Kudu in the Kruger National Park where they predominantly graze had a mean worm egg count of 2251 compared to 339 in those mainly browsing in Etosha National Park in Namibia (Boomker *et al.*, 1986). However, gastrointestinal nematode infections are known to reduce growth rates in domestic ruminants and decreased body mass and condition have been reported in parasitized animals in a range of wildlife species, including ruminants (Morgan *et al.*, 2005).

**Table 6.1:** Prevalence of parasites eggs detected in farmed gemsbok (*Oryx gazelle*) and springbok (*Antidorcus marsupialis*) in Namibia at slaughter.

Animal species	Parasitic species	Worm egg count per gram of faeces	Number of positive cases expressed as a percentage of the total number examined
<i>Oryx gazella</i>	Strongyle	200-2000	57.14
	<i>Trichuris</i> spp	100-1000	7.14
	Coccidia oocyst	300-1000	28.57
<i>Antidorcus marsupialis</i>	Strongyle	>200	24
	<i>Strongyle papillosus</i>	11-200	7
	<i>Toxocara</i> spp	1-10	2
	<i>Trichuris</i> spp	1-5	5
	Coccidia oocyst	1-1000	44
	Unidentified muscle larvae	N/A	0.4
	<i>Skrjabinodera kuelzii</i>	N/A	77.14

#### 4. CONCLUSION

A better understanding of the ecology and spatial distribution of haematophagous arthropods and other dipteran species in Namibia is urgently needed to determine the dynamics of these larvae and filarial worms in free ranging wildlife and domestic species. It is recommended that the national veterinary laboratory consider the full time service of a specialist veterinary entomologist and or parasitologist as well as acquisition of the respective high tech equipment. Lastly an urgent need exists to define the significance of *S. kuelzii* in wild ungulates and its public health significance.

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## CHAPTER 7

### **Lead (Pb) and Cadmium (Cd) contamination values in liver, kidney and muscle of harvested wild springbok (*Antidorcus marsupialis*) in Southern and South-Eastern Namibia<sup>1</sup>**

#### **1. INTRODUCTION**

Although poorly characterised, chemical hazards present a significance public health concern. In light of the favourable microbial safety of game meat, careful scientific observation was taken into consideration as little work has been conducted on heavy metals (lead and cadmium) contamination.

Meat is a universally valued and sought-after source of human nourishment (Radder & Roux, 2005; FAO, undated). For many communities, the most common source of meat are livestock species, however in recent years, harvested wild game has formed an important component of the diets of certain social and economic groups. Across Africa, interest in game meat exists in leisure and subsistence hunters, and those with an ethical preference for consuming wild animals (Bender, 1992; Pain *et al*, 2010). Offal from harvested wild game may either be directly eaten or processed for the production of a range of foods which are nutritiously attractive. In South East Asia and Africa, food products of offal origin are on high demand and highly prized while in Australia and USA, demand is variable (Pearson & Dutson, 1988; Ali & Mahdey, 2010).

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It is estimated that 4 300 tonnes of game meat were produced annually in Namibia during the period between 2001 and 2005 (Laubscher *et al.*, 2007). Lindsey (2011) estimates between 16 to 26 000 tonnes of annual game meat production from Namibian farmlands of which Oryx (*Oryx gazelle*), greater kudu (*Tragelaphus strepsiceros*) and Springbok (*Antidorcas marsupialis*) contributes approximately two thirds of the game meat production on freehold farms in the form of trophy hunting, followed by own use with a relatively small proportion produced through harvest specifically for meat to sell (e.g. “shoot-and-sell” and wildlife export harvesting). In slaughtered animals, edible offal contributes 33% of the edible material (Aduku *et al.*, 1990).

Illness from foodborne chemical hazards is a significant global health concern but population level incidence estimates are uncertain due to underreporting and difficulty in attributing illness to food consumption (Rocourt *et al.* 2003; WHO 2005). One of the most important aspects of environmental pollution for humans is the intake of toxic elements in the diet (López-Alonso *et al.*, 2007). Livers and kidneys as offal can accumulate high concentrations of toxic heavy metals which expose human consumers to negative health effects when ingested (D’Ilio *et al.*, 2008; Petersson-Grawe’ *et al.* 1997). Pb also concentrates in bone, which is sometimes used in the form of bone meal as a livestock dietary supplement however meat and bone meal is banned in ruminant livestock species in most countries including Namibia (Fox, 1987). In game animal meats (muscle, liver and kidneys), toxic Cd and Pb occur in relatively low values in muscle and organ meats, with the exception of Pb in muscle and Cd in kidneys, which could carry relatively higher contamination values (Falandysz, 1994).

Human exposure to Pb is mainly via food and water, with some via air, dust and soil. Pb levels in blood has been correlated with the frequency and amount of meat consumed from wild game animals (Pain *et al.*, 2010; Dewailly *et al.*, 2001; Iqbal *et al.*, 2009; Saggese *et al.*, 2009). Absorption of lead from the gastrointestinal tract depends on host characteristics and on the physicochemical properties of the ingested material (EFSA, 2010). It is absorbed more in children than in adults although at a lower rate in the presence of food to

accumulate in soft tissues and over time, in bones (EFSA, 2010). Pulmonary effects such as emphysema, bronchiolitis and alveolitis may result from severe lead exposure while renal effects may result from sub-chronic inhalation of Cd (Elsayed *et al.*, 2011; European Union, 2002; Young, 2005). Developmental neurotoxicity in young children and cardiovascular effects and nephrotoxicity in adults has been identified as the critical effects in lead toxicity (EFSA, 2010).

Cigarette smoke is the most common source of exposure to Cd; other sources, include, workplaces where cadmium-containing products are made, and from the air near industrial facilities that emit Cd (Anonymous, 2012; Trading Economics, 2011). Airborne Cd particles can travel long distances before settling on the ground or water hence for the non-smoking general population, foodstuffs are the main source of cadmium exposure (EFSA, 2009). Cd absorption after dietary exposure in humans is relatively low (3–5%) but cadmium is efficiently retained in the kidney and liver in the body, with a very long biological half-life ranging from 10 to 30 years (WHO, 2011; EFSA, 2009). Zinc is used in almost all agriculture and animal feed and over the years, elevated levels of arsenic, Cd and Pb trace elements in Chinese imported zinc sulphate fertilizer and contaminated "pre-mix" in poultry feed have been reported in the South African farming community (Gosling, 2007). Several factors have an impact on heavy metals in Namibia farming systems. Trade in Pb and Cd contaminated farming inputs could have taken place due to the nature of trade agreements among Southern Africa Customs Union (SACU) members of which Namibia is a member (SACU, 1910). Unconsolidated sand (arenosols) and shallow, weakly developed soils on bedrock (lithosols, xerosols, regosols and yermosols) characterize the main groups of soils in Namibia (FAO 1973; Jenny, 1994). Some 97% of the country's soils have a clay content of less than 5%, and thus have a very low water holding capacity leading to soil runoff in the farming community (Sweet *et al.*, 2006). Borehole water is predominantly used for livestock and human consumption and underground water bodies have been reported to share the same space as base-metal ores and soluble species of heavy metals are created and released as a result of interaction between ore-bearing rocks and percolating water (Leung & Jiao 2006; Midzi, 2011; Michael *et al.*, 2011; Sweet & Burke

2006). Exposure to and acquisition of heavy metals by animals is directly related to environmental contamination in the rearing areas, and wildlife are a good bio-monitor for heavy metal contamination in extensive production systems worldwide (Petersson-Grawe *et al.*, 1997; López-Alonso *et al.*, 2007). The objective of this study was therefore to determine the levels of Pb and Cd in meat and offal (liver and kidney) of springbok harvested in some parts of Namibia.

## **2. MATERIALS AND METHODS**

Sample analysis were done by the technical team at an Agriculture laboratory, Analytical laboratory services both located in Windhoek, Namibia and Agricultural Research Council (ARC) of South Africa (ISCW).

### **2.1 Study area and sampling**

#### **2.1.1 Liver and kidney sampling**

A total of 12 310 springbok were harvested from 26 commercial farms located in the Karas, Omaheke and Hardap regions of southern and south eastern Namibia. Harvesting was focused on animals capable of producing more than 12kg of dressed carcass meat weight. Harvesting was randomly performed between April and September of 2009 as part of the national residue monitoring and surveillance program. Collection of specimens for laboratory analysis was performed at abattoirs approved to slaughter wildlife animals. Harvested animals with head and upper neck shoots only were passed for export and considered for sampling. Specimens (n=101) of 60 livers and 41 kidneys were taken from randomly selected healthy animals on the abattoir processing line. Liver specimens weighing at least 200g were randomly collected from the liver, while a whole kidney was taken from each carcass. The expected proportion of positives was set at 5% hence a sample size of 100 was targeted to be 95% certain of detecting at least one positive animal. In this regard, two to eleven specimens were collected from each batch of animals, except two batches where one specimen was collected. The samples were packed in polyethylene bags and frozen until they were delivered to the laboratory for Pb and Cd analysis.



### **2.1.2 Muscle sampling**

Harvesting was randomly performed between April and September of 2010 as part of the national residue detection surveillance follow up due to Cd and Pb positive liver and kidney samples collected in year 2009 harvesting season. About 200g of unrecognized hindquarters muscle sample was collected during boning from 52 springbok carcasses harvested from 10 different farms in three regions for Cd and Pb detection. Specimens were packed individually in polyethylene plastic bags, stored at -18°C until they were prepared for analysis.

### **2.1.3 Sample preparation and processing**

Frozen samples were defrosted overnight at room temperature. After washing, the samples were minced in a blender. The minced specimens were transferred into oven dishes and placed in an oven at 70°C for 3 days. The samples were cooled down to room temperature after which they were milled and passed through a 1.0mm sieve. The ground specimens were stored in screw-capped glass bottles and refrigerated until acid digestion in a microwave.

### **2.1.4 Sample digestion**

Unless stated, all chemicals and reagents used were of analytical grade, and were purchased from Merck (South Africa) and all digestion vessels and volumetric ware were carefully washed with acid and rinsed with reagent water. Multiple point calibration of the microwave (MARSXpress, United Kingdom) was applied and the decomposition system was able to sense the temperature to within  $\pm 2.5^{\circ}\text{C}$  and permit adjustment of the microwave output power within 2 sec. Using the analytical method EPA 3051A, an acid digestion method using pressure controlled microwave heating was used for specimen preparation (Link *et al.*, 1998). The temperature of each sample was allowed to rise to a maximum of  $175\pm 5^{\circ}\text{C}$  in approximately 5.5min and remained at  $175\pm 5^{\circ}\text{C}$  for the remainder of the 10min digestion period at 1000W maximum power of the microwave (Ramírez-Ortiz & Monreal, 2009). Half a gram of kidney specimen material was weighed into each vessel

and 5 ml of di-acid mixture of HNO<sub>3</sub> (65%) was added. For liver samples, 0.5grams weighed into the vessel were mixed with 2ml double de-ionized water and 5ml of HNO<sub>3</sub> (65%). All vessels were sealed and the vent tubes connected from the vessels to the collection vessel and pressure sensing lines connected to the control vessel. The heating program was run to completion and the samples cooled for a minimum of 30 minutes before centrifugation at 3000 rpm for 10 min and subsequently transfer of the solution to a flask with a filtration step, prior to analysis and dilution to 10ml with double de-ionized water. A low temperature (85°C) digestion procedure was used on muscle samples (25 grams) using an open vessel at atmospheric pressure by addition of HNO<sub>3</sub> followed by H<sub>2</sub>O<sub>2</sub> (30%). Digestion solution was transferred and diluted with reagent double de-ionized water to 50 ml. Although only the average values are shown, the digestion of the samples was made in triplicates and each subsample was analysed three times.

### **2.1.5 Lead and Cadmium determination**

The digested liver and kidney samples were analyzed for the presence of cadmium and lead using inductively coupled plasma atomic emission spectrometry (ICP-MS) (Thermo Scientific X series 2, Thermo Scientific). Standards (Merck, South Africa) were made at concentrations of 5, 10, 20 30 and 50ppb for cadmium and lead respectively.

### **2.2 Quality control**

Although only the average values are shown, the digestion of the samples was made in triplicates and each subsample was analysed three times. The ICP-MS was calibrated with standards prepared in the same matrices as the samples. Correlation coefficients ( $r^2$ ) for the standard lines were  $\geq 0.99$ . Blank analysis was used to check for matrix interferences and a quality control (QC) standard of known concentration was analyzed with the samples to measure machine drift and calculation for drift correction. If percentage recoveries of the continuing calibration verification (CCVs) were not within 10% of their known value, a new calibration line was generated and the necessary samples were re-analysed. For quality control (QC), an internal standard spiked in each sample was used to monitor the performance of the instrument with an allowance failure limit of 10%. The detection limits

for  $^{111}\text{Cd}$  and  $^{208}\text{Pb}$  were 0.006904ppb and 0.052303ppb and correlation coefficients were 0.999925 and 0.999937 respectively.

Cd and Pb values measured ranged from below analytical method detection limit (<dl) to quantifiable.

### **2.3 Statistical analysis**

Single positive (above the detection limit) samples were obtained from a number of farms hence statistical analysis could not be conducted on single sample results from farm 1-9 for kidney Cd, farm 1-8 for kidney Pb, liver Cd and Pb from all farms. The organ metal values (mg/kg) are presented as a mean ( $\pm$ SD). Descriptive statistical analysis was done on detected residue values in the liver and kidneys using XLSTAT 2011.4.03 (XLSTAT, 2011).

### **2.4 Questionnaire for qualitative analysis**

A questionnaire was prepared for the collection of additional information on farms where lead and cadmium had been detected in liver and kidney samples analysed (addendum 1).

## **3. RESULTS**

### **3.1 Cadmium values in kidney, liver and muscle**

The mean Cd values in the kidney and liver were  $0.33\pm 0.22\text{mg/kg}$  and  $0.10\pm 0.05\text{mg/kg}$  respectively. In 96.7% (n=60) of liver specimens and 58.5% (n=41) kidney specimens, Cd was below the detection limit. When compared to recommended maximum limits of Cd in the offal of domestic animals (bovine, sheep, pig, poultry and horse) (Ambushe *et al.*, 2012), none of the samples had Cd values above 0.5mg/kg set for the liver and 1.0mg/kg set for the kidney. Cd values in all 52 muscles samples were below the detection limit.

### **3.2 Lead levels in liver, kidney and muscle**

Mean Pb values in the kidney and liver were  $0.91\pm 0.51\text{mg/kg}$  and  $1.03\pm 0.21\text{mg/kg}$  respectively. In 96.7% of liver specimens and 58.5% kidney specimens, Pb was below the detection limit. When compared to maximum limits of Pb in the offal of domestic animals

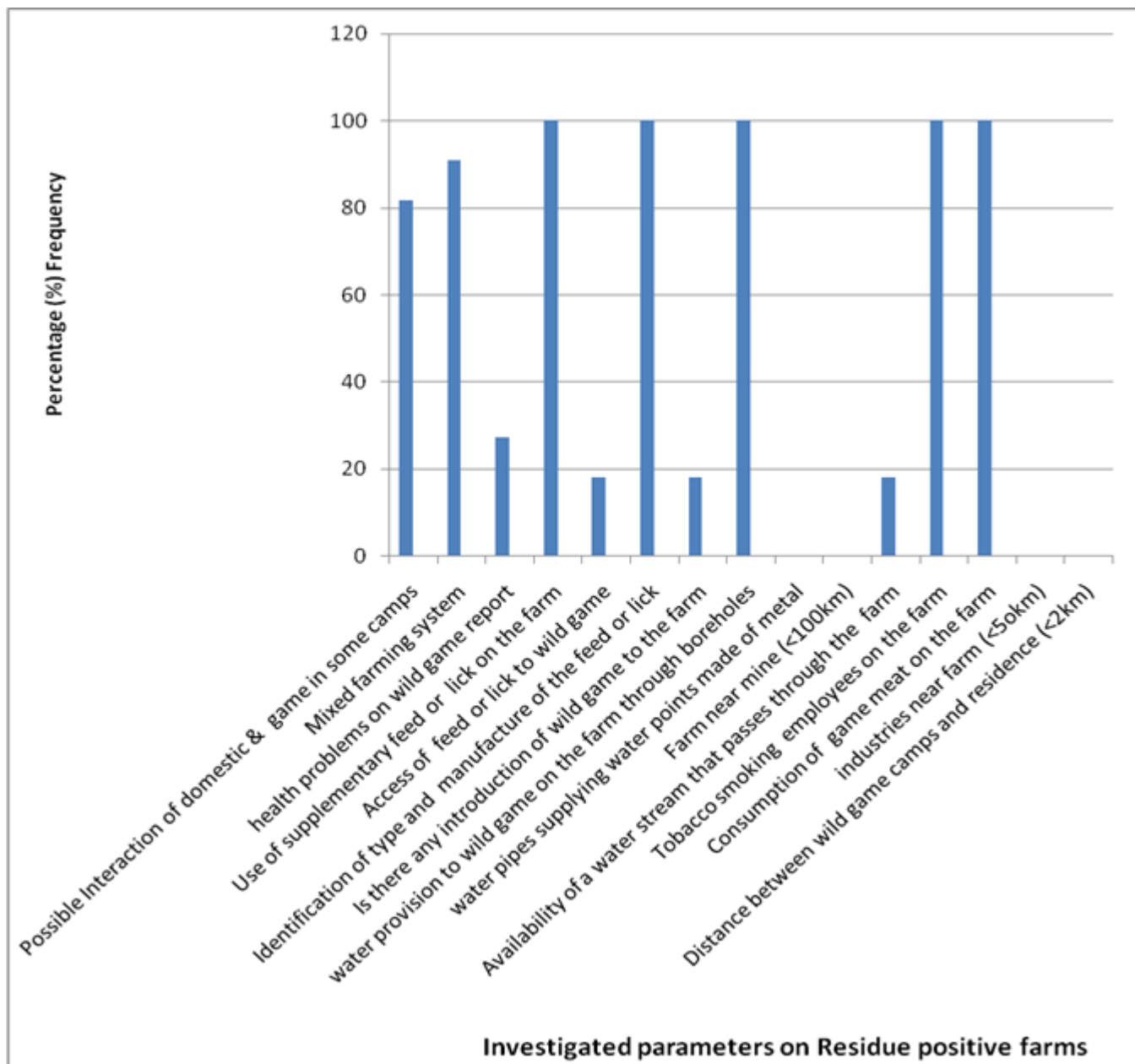
(Ambushe *et al.*, 2012), two of the samples had lead value above the maximum limit of 0.5mg/kg for the liver and twelve samples had Pb values above 0.5mg/kg for the kidney. Pb values in all 52 muscles samples were below the detection limit.

Approximate regions and locations of free hold farms where harvested springbok that had measurable (above the detection limit) Pb and Cd originated and frequency results from the questionnaire on positive farms are shown in Figures 1 and 2. Notable findings on the positive farms were the non-presence of nearby mines, the usage of supplementary feeding or lick with possible interaction of domestic and wild game in some camps/paddocks, water provision to wild game through boreholes. Table 1 shows Cd and Pb values above detection limit for springbok liver and kidney samples expressed as mg/kg on a wet mass basis.

**Table 1:** Pb and Cd contamination values above detection limit of springbok liver and kidney samples expressed as mg/kg on a wet mass basis (mean±se) for kidney samples from farm 9, 11, 12 and 13 only).

Farm number	Organ: Kidney			Organ: Liver		
	number of samples	Cd mg/kg	Pb mg/kg	number of samples	Cd mg/kg	Pb mg/kg
1	1	0.39	0.43			
2	1	0.32	0.56			
3	1	0.26	0.40			
5	1	0.32	0.29			
6	1	0.23	0.32			
7	1	0.42	0.73			
8	1	0.93	0.42			
9	2	0.52	1.11±0.26	1	0.06	1.18
11	3	0.31±0.12	1.31±0.22			
12	2	0.26±0.19	0.88±0.20			
13	3	0.10±0.02	1.45±0.31	1	0.13	0.89





**Figure 2:** Shows frequency of investigated parameters on residue positive farms for Pb and Cd.

#### 4. DISCUSSION

Information on regulatory limits on lead and cadmium in game meat and offal in southern African countries is limited except in South Africa where default values are set in regulation 545/2008 at 0.1mg/kg and 0.05mg/kg respectively in accordance to codex general maximum residue limits in meat. However, in comparison to Ambushe *et al.*, 2012 stipulating maximum Pb and Cd values for offal from domesticated animals (sheep, cattle and goats), the voluntary noncompliance incidence in this study was insignificant. Pb and Cd are of established toxicological significance for humans, and both elements concentrate to high levels in liver and kidneys of food animals (Doganoc & Gačnik, 2004; Falandysz, 1994; UNEP, 2002; 2002a & 2011).

The unequal distribution of heavy metals amongst organs is related to differences in the specific physiological functions of the elements and their relative abundance in intracellular ligands able to bind metals, such as metalloproteins (Ambushe *et al.*, 2012). The presence of Cd and Pb in livers and kidney was not surprising as previous analysis had reported results above the detection limit in other wildlife species. Notably, few cases of Pb poisoning reported in Namibian cattle and sheep in previous animal health surveillance.

In this study, the mean values and ranges for Pb (liver- $0.034 \pm 0.26$ mg/kg, kidney- $0.38 \pm 0.56$ mg/kg) and Cd (liver- $0.14 \pm 0.22$ mg/kg, kidney- $0.003 \pm 0.03$ mg/kg) were comparable with those previously established in other game species. The weighted mean concentration ranges obtained related to wet-weight (w.w) for muscle, liver and kidneys of wild boar, roe-deer and stage were 0,01, 0,11-0,21, and 1,5-2,1mg/kg for Cd; 0,086-0,16-0,19-0,21, and 0,21-0,29mg/kg for Pb respectively (Falandysz, 1994). In red deer, the value of Cd has been reported to be significantly higher in kidney in comparison with liver and muscle. Contrary to the findings of this study, the level of Pb was reported to be highest in muscle and lowest in the liver and kidney (Gasparik *et al.*, 2004). In other studies, highest values of Cd were reported in kidneys (0.213-2.387mg/kg) of brown hare (*Lepus europaeus*), yellow-necked mouse (*Apodemus flavicollis*), wood mouse (*Clethrionomys*



*glareolus*), and red deer (*Cervus elaphus*) with a 0.032-0.258mg/kg concentration of Cd in the livers (Kramárová *et al.*, 2005). Pb values were higher in kidneys of yellow-necked mouse and wood mouse (0.503-0.780mg/kg) than in liver (0.177-0.268mg/kg) (Kramárová *et al.*, 2005).

Contrary to other studies, accumulation of Pb in liver (0.221-1.904mg/kg) had been reported to be higher in comparison with Pb values in kidneys (0.115-0.561mg/kg) of brown hare and red deer (Kramárová *et al.*, 2005). Age related accumulation of Cd, in deer and Cd in boar has been observed (Taggart *et al.*, 2011). Two boar meat samples contained high Pb, at 352 and 2,408mg/g dry weight. Likewise, 19-84% of all samples (depending on species and sampling area) had Pb levels >0,0001 mg/g w.w, with 9 and 43% of samples exceeding comparable voluntary Cd limits when samples were collected from hunting estates within and outside an area that has been historically used for mining, smelting and refining various metals and metalloids (Taggart *et al.*, 2011). Kidneys of red deer contained Cd and Pb in greater concentrations than liver or muscle tissue from the same animal (Jarzyńska & Falandysz, 2011). Indeed, Cd, binds to various biological components, such as protein and nonprotein sulfhydryl groups and anionic groups of various macromolecules such as metallothionein which has little or no direct metabolic conversions and is instrumental in determining the disposition of Cd in the body (e.g. values of cadmium in the kidneys) (ATSDR, 1989; U.S. EPA, 2011).

Cd and Pb in game meat is correlated with vegetation and soil values of the elements and presence in animal tissues result from natural sources (soil and water) contamination and from anthropogenic activities (Midzi, 2012; Sharma & Shupe, 1977; WHO, 2011). Though the contamination source could not be established in the present study, it is unlikely that water was the source of contamination as collected water samples in year 2010 from four harvesting farms boreholes located in the game harvesting areas gave insignificant Pb and Cd values of 0.01mg/l and <0.01mg/l respectively (Magwedere, Unpublished data). Similarly, negligible figures of <0,01mg/l for Pb and <0.003mg/l for Cd have been reported in annual surveillance of physicochemical parameters in potable water from some

municipality areas in Namibia (Magwedere, unpublished data). Contamination of water may also result from the presence of Pb and Cd as an impurity in the zinc of galvanized pipes or Cd-containing solders in fittings, water heaters, water coolers, borehole pipes and taps. Levels of Cd could be higher in areas supplied with soft water of low pH, as this would tend to be more corrosive in old plumbing systems containing Cd (WHO, 2011). Whereas the average Pb concentrations from different locations ranged from 1.4–9.2mg/kg in the liver, 45–195 in bones, 4–283 in vegetation, and 15–1399mg/kg in soil samples and that of Cd from 1–27mg/kg in the liver, 3–77 in bones, 0.5–5 in vegetation, and 1–10 in soil, it was not possible to establish values and correlation between vegetation and soil values for the elements in the present study (Sharma & Shupe, 1977). Indeed the most notable findings on the positive farms were the non-presence of nearby mines, the usage of supplementary feeding or lick with possible interaction of domestic and wild game in some camps/paddocks, water provision to wild game through boreholes. Although the relationship between animal to vegetation and animal to soil residues levels (Bowell & Anser, 1993) was not established in the current study, the most common type of sand reported from the questionnaire information results were the kalahari sand, loose rocky soil and limestone type with the type of vegetation wild game camps ranging from long and short bushmen grass, lucern bush, *Acacia* spp, blinkhaar, saculant plants, suur and accession (Nriagu, 1992; Peryea, 1999). The kalahari consists of nutrient poor soils of aeolian origin, arranged into permanent linear dunes with the vegetation described as shrubby Kalahari dune bushveld (Hoon, 2010).

Springbok are known to survive without water for long periods and to get most of the water their body requires from the food they eat notably soft green leaves, flowers and buds. At the time of this study, one farmer confirmed having knowledge of soil, water and vegetation contamination with heavy metals on his farm following testing of soil samples to diagnose nutritional disorders (urinary calculi) in cattle on the farm (Canty *et al.*, 2009; De Abreu Faria *et al.*, 2010; Maskall & Thornton, 1996). Salt mineral blocks (as an animal supplementation feed) placed on the ground can leach salt and eventually encourage animals to ingest cadmium contaminated soil (Abrahams, 2012; Beyer & George, 2003;

Belli *et al.*, 1993; Mahaney *et al.*, 1990; Midzi, 2012; Mincher *et al.*, 2008). In the current retrospective study, interpretation of the results was limited by the small number of positive samples.

## 5. Conclusion

The potential public health risks from Pb ingested in the kidney and liver of springbok harvested in Southern and South-Eastern Namibia is unclear. To provide a measure of the potential nature and magnitude of effects that wild mammals may experience, heavy metal concentrations should be modelled using site-specific or literature-derived information and the values then compared to available literature information for concentrations of chemicals in specific tissues associated with adverse effects. It is recommended that extensive risk assessments for Pb and Cd be done in Namibia taking into consideration eating habits, smoking, environmental sources and nutritional factors since MRLs alone can not guarantee low-risk exposures in humans (Ambushe *et al.*, 2012). Medical geological studies need to be done to identify the root and source of Pb and Cd contamination in the springbok and possibly other species. Health and stakeholder awareness programs should be developed for regular monitoring of Pb and Cd in water, soil and plants on farms to assess the impact and trends of heavy metals to human and animal health.

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## Addendum 1

ITEM	Response
No of game camps on the farm	
Are domesticated animals kept in the same camps with game?	
How many wild game animals are on the farm?	
What health problems do you normally encounter with your wild game?	
Use of feed and licks on the farm	
If yes to which animals are feed and licks provided?	
Type and manufacture of the feed and lick	
Drug stock treatment register Yes/No Which drugs and to which species?	
Does wild animals have access to domestic animals feed/licks?	
Any insecticide or pesticide used on the farm?	
Is there any introduction of wild game to the farm?	
How is water provided to wild game on the farm?	
Are the water pipes supplying water points made of metal or plastic?	
Is your farm near a mine? If not how far is it from a mine?	
Is there a water stream that passes your farm?	
What type of soil is present in your game camps?	
How many employees smoke on the farm?	
How often do people consume game meat on the farm?	
What type of vegetation and grass is eaten by the wild game in your camps?	
Any industries near your farm?	
How far are the wild game camps from the main house?	
Where there game animals brought to your farm before: which species and year?	

## CHAPTER 8

### GENERAL DISCUSSION AND CONCLUSIONS

#### 1. INTRODUCTION

The global trade in wildlife has historically contributed to the emergence and spread of infectious diseases due to new host adaptation by pathogens, increased human susceptibility to diseases, changing environments, intensification of the human-animal interface, human and animal movements across international borders, and emerging antimicrobial resistance, however, minimal pathogen surveillance has precluded assessment of the health risks posed by this practice (Aguirre *et al.*, 2006; Beutin & Martin, 2012; Cima, 2012; Jori *et al.*, 2011; Siembieda *et al.*, 2011; Smith *et al.*, 2012; Thalwitzer *et al.*, 2010). Although the conversion of wildlife habitats into arable land for crops, pastures and lodging camps can be viewed as the biggest driver of emerging and re-emerging zoonotic diseases associated with wildlife species, it is the ever increasing wildlife-human-domestic animal interface including the consumption of game meat around the world that attracts recent concerns and challenges (Govender & Katsande, 2011; Cima, 2012; Molyneux *et al.*, 2011; Paulsen *et al.*, 2011). It is postulated that the wildlife/livestock interface in Namibia is largely influenced by the livestock production systems present in the country and the feeding ecology of the different species (Joubert & Evans, 1997; Karuaera, 2011; Katakweba *et al.*, 2012; Trinkel, 2009). It is important to note that a positive serological test for a zoonotic disease simply indicates that the animal has been exposed to the disease, and is not necessarily infected with the disease or capable of transmitting it to other animals or humans. The current study addressed some of the critical and emerging research questions in wildlife for the future development of better control strategies. Some of the questions answered are summarized below.

#### 2. Contribution of the study to new knowledge required to reduce public health risks arising from game meat production

Much of the veterinary public health data in Namibia is buried in unanalysed historical data and therefore such valuable information was utilized in the current study to generate peer reviewed information for use in future risk assessments, provide insight into the past and present perspective of the veterinary public health for further research by other interested scientists and building of effective current and future integrated public health strategies in Namibia. When game began having commercial value, it became a protected valuable resource, it prompted the introduction of sustainable commercial game harvesting. Sustainable game harvesting is probably the single largest factor in the success of game conservation as it allows risk based disease surveillance and management and minimization of food safety risks from environmental contaminants such as lead and cadmium. In Africa and other developing countries, zoonotic gastrointestinal diseases and brucellosis have been listed as two of the most important hazards in terms of their impact on human deaths, livestock sector and severity in people, along with their amenability to agriculture-based control. The study results act as a quick reference guide for food regulators, game risk assessors, conservationists, veterinarians, wildlife farmers, harvesting teams, processors and public health professionals. From this study, it can be concluded that wildlife industry has a potential to play a role in ensuring the country's food security, however caution should be taken to ensure that the industry is not penalised by overregulation and inappropriate conservation lobbies. Given the importance of the wildlife industry and the substantial economic costs of wildlife-related disease, government should invest more resources in the research and development of the entire wildlife value chain. It has been noted that, the current focus of harvested wildlife is not comprehensive, as all species of wild animals ranging from insect and insect larvae through rodents, antelopes, and amphibians can be exploited for food and in a considerable number of cases in rural communities of Africa. As the spread of infectious disease among and between wild and domesticated animals has become a major problem worldwide, new ways of achieving successful disease control through development of effective wildlife vaccines and vaccination of captured wild animals with the intention of relocation offers one such opportunity. A quality assurance system (seal of quality) with a possibility of

accreditation should be developed and implemented to guarantee the hygiene and make the industry a safe supply chain of game products to the consumers.

Intensification of game farming has led to a higher disease(s) burden on animals that normally have no diseases associated problems. Game meat STEC and parasitic food risks do exist and this calls upon regulators, industry leaders, consumers and scientists to come together to manage the existing complex of wildlife food safety risks. The study highlights the potential threat and impact on food security of microbial contamination, emerging parasitic larvae and filarial worms in wildlife.

Data from the current investigation game meat provided negligible values on *Salmonella* spp and comparable bacteriological values (TVC, *Enterobacteriaceae* and *E.coli*) in relation to an adopted generic risk-based sanitary certification for export meat from domestic animals (Kamwi, 2007 & 2012). The bacteriological values can be applied in the selection of sensitive indicator organisms for process hygiene criteria using *E.coli*, *Enterobacteriaceae*, *Salmonella* and APC count in the hygiene monitoring of game meat. The study highlighted the low incidence of STEC in wild game slaughtered in approved facilities which proves either the effectiveness of the risk based meat safety management system adopted by the regulatory authorities with regard to STEC control or limitations in the sampling and detection methods. In as much as HACCP based procedures can prevent, reduce and eliminate hazards, the presence of stx1 in one sample shows that wildlife colonized by STEC can be possible sources of human infections. There is no evidence to support the belief that wildlife meat is a high risk product in comparison to meat from domestic species. However, observed variability in microbiological results of the game meat samples over the two years calls upon scientists, regulators and other industrial players to focus on training harvesters in game meat harvesting and inspection to ensure effective hygiene control and consistent demonstration of the safety of the harvested, dressed and processed game. Extension advice to consumers and caterers on cooking of game meat and avoiding cross contamination should be reiterated.

Although the quality assurance system in existence in Namibia is based on domesticated livestock species, it is clear that in the case of Brucellosis, the system works well for food-producing wildlife species where no vaccination, no ante-mortem inspection and no individual identification of the animal entering the food chain takes place. The risk of *Brucella* transmission through goats and unpasteurized products to humans exists and it is very likely other human brucellosis cases are missed since the manifestations are non-specific, however better good practice control programs can reduce the incidences of brucellosis in humans. Due to the arid and semi-arid climate some infective pathogenic agents may not be able to survive. Moreover, the population of Namibia is very small in a vast country, an important factor that may explain a hinderance for the spread of some infectious diseases such as Brucellosis. The study clarified that springbok and gemsbok may not be a vehicle of transmission of Brucellosis in Namibian extensive production systems despite possible interactions between farmed domestic animals.

The lack of pH data for some game offal has been addressed and some factors influencing post-mortem pH changes established. The positive correlation between poor bleeding and increase in pH highlights the importance of effective bleeding in preventing the creation of a favourable environment for the growth of spoilage microorganisms and inactivation of certain pathogens (Omojola, 2007).

Even though source of contamination has remained elusive for Pb contamination in Namibia cattle offal, a general spatial pattern for Pd and Cd has been established in the current study. The potential public health risks from Pb and Cd in contaminated kidney and liver of food producing wildlife exists but remain unclear.

### **3. Areas requiring further investigation**

In conclusion, the increasing marketing of game meat warrants the following sanitary investigations:

- Potential threats to acquired antimicrobial resistance of *Enterobacteriaceae* and *E.coli* isolates from ready to eat game products should be established (Crowther-Gibson *et al.*, 2011)
- Little information is available in the control of meat contamination with viral hazards (FAO/WHO, 2008; Venkatesan *et al.*, 2010) and the threats of viral hazards to public health were not addressed in the present study except for the contribution to a review on the mathematical modelling of viral zoonosis in wildlife which provided insights into some mathematical models developed for the study of viral zoonoses in wildlife and identification of areas where further modeling efforts are needed (Allen *et al.*, 2012).
- Due to the presence of regional trans-frontier parks for countries with different disease status and mining activities, it is recommended that extensive trans-boundary food safety risk assessments for environmental chemical contaminants (Ambushe *et al.*, 2012) and zoonotic pathogens be done in southern African wildlife species based on multi-criteria based ranking (FAO, 2012).
- An urgent need exists to define the significance of *S. kuelzii* in food-producing wildlife and its possible public health risk potential.
- Further work on STEC needs to be conducted on different indigenous wildlife species commonly harvested for meat.
- The presence of potential public health risks in wildlife suggests a need to rapidly implement and periodically review a national integrated veterinary and public health database that allows for the timely exchange of information by Ministry of Health in collaboration with the Ministry of Agriculture, Water and Forestry through which rapid and coordinated responses to health threats can be initiated.
- Alternative measures are required to inactivate endogenous pathogens susceptible to pH reduction in edible game offal.
- There is need to strengthen existing control measures and develop other control strategies among the different organisations managing the presence of public health risks along the meat chain.

- The game harvesting teams and establishments should have working food safety management systems in place and be able to verify and act if there are shortcomings (corrective actions) identified.
- Harvested carcass cooling regimes in the chilling truck need to be investigated further in relation to loading densities, product safety and shelf life, species and time.
- A framework for managing wildlife food safety and zoonotic disease surveillance and intervention strategies based on risk assessment should be strengthened.
- In the present study, only a few Veterinary public health aspects were studied while some significant aspects were overlooked, in this regard a comprehensive detailed analysis on impact of each identified zoonotic agent should be evaluated in the currently growing wildlife industry.
- is therefore called upon within the Namibian wildlife industry so as to help in the continual goal of providing the safest wildlife edible products to the consumers.
- Most of the diagnostic methods used are not primarily made for species specific wildlife disease surveillance hence further work is needed in the development and improvement of diagnostic tests and technologies for the identification of zoonotic agents in wildlife species.
- There is possibly a need to link public health risks in humans with species specific veterinary public health to assist in the improvement of integrated public health policy strategies and determine interventions that can be employed to control pathogens.
- There is need for more primary research and access to relevant proprietary data to properly evaluate veterinary public health programs' effectiveness using modeling techniques capable of differentiating the effects of specific instruction notices from other concurrent factors.
- The wildlife industry should develop a data base to capture accurate statistics on wildlife production, production systems, health and welfare. This will assist policy makers in the allocation of adequate resources for the sector and development of progressive regulatory laws



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