Pilot investigation of selected milk-borne pathogens in communal cattle in the uMkhanyakude district of KwaZulu-Natal, South Africa

By

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- To God Almighty for granting me this opportunity and strength to carry on when I felt like giving up.

DECLARATION

I, Jescah Munjere, do hereby declare that the research presented in this dissertation, was conceived and executed by myself, and apart from the normal guidance from my supervisors, I have received no assistance.

Neither the substance, nor any part of this dissertation has been submitted in the past, or is to be submitted for a degree at this University or any other University.

This dissertation is presented in partial fulfilment of the requirements for the degree MSc (Animal/Human/Ecosystem Health).

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AU-IBAR –African Union-Interafrican Bureau for animal Resources

BTB – Bovine tuberculosis

BMRT – Brucella milk ring test

DAFF – Department of Agriculture, Forestry and Fisheries

FAO – Food and Agricultural Organization

FMD – Foot and mouth disease

IDF- International Dairy Federation

OIE- Office International des Epizooties (World Organization for Animal Health)

PCR- Polymerase Chain Reaction

SEs – Staphylococcal enterotoxins

SFP – Staphylococcal food poisoning

TB – Tuberculosis

TSST-1 – Toxic shock Syndrome toxin 1

WHO – World Health Organization

THESIS SUMMARY

Pilot investigation of selected milk-borne pathogens in communal cattle in the uMkhanyakude district of KwaZulu-Natal, South Africa

By

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ABSTRACT

This study was a pilot investigation of selected milk-borne pathogens in communal cattle in the UMkhanyakude district of KwaZulu-Natal, South Africa. Fifty seven milk samples were collected from 12 bovine tuberculosis (BTB) positive cattle herds. Udder hygiene assessment was also carried out during sample collection. Convenience sampling of milk samples was done during a BTB diagnostic pilot project involving selected BTB positive herds at the Nibela diptank in the uMkhanyakude district. The milk samples were tested for the presence of the following milk borne pathogens: *Mycobacterium bovis (M. bovis), Brucella abortus (B. abortus)* and *Staphylococcus aureus (S. aureus)*. In addition, the microbiological quality of milk in the study population was assessed by means of total bacterial counts, total coliforms counts and total *E. coli* counts. A questionnaire survey to determine the level of knowledge, milking hygiene and milk consumption behaviour of 12 households that participated in the BTB pilot study was administered.

A total of 21 cattle representing 6 cattle herds, tested positive for *B. abortus* antibody on Brucella Milk Ring (BMRT) test. The detection of *S. aureus* in the milk samples was done by bacterial culture, catalase tests, oxidase tests and staphylase tests. The prevalence of *S. aureus* was found to be 49%. The isolation of *B. abortus* and *M. bovis*

was attempted but compromised by constraints that were beyond the investigator's control (drought related decrease in milk production and inadequate laboratory facilities). The constraints included inability to collect adequate quantities of milk as required for tests due to a sharp decrease in milk production of cows during the severe prevailing drought. Lack of adequate laboratory facilities for *B. abortus* and *M. bovis* culture in the study area and the resulting long time lag between collection of milk samples and identification of *B. abortus* and isolation of *M. bovis* at the designated bacteriology laboratory of DVTD further decreased the probability of successful culture. The seroprevalence of *B. abortus* in milk was determined by Brucella Milk Ring test and was found to be 38%. The presence of *M. bovis* was confirmed by PCR in one pooled milk sample from 5 cows. On quantification of total coliforms, 21% of the milk samples had more than 20 cfu/ml and 59% of milk samples contained *E. coli*. It was found that 59% of the milk samples yielded 100 cfu/ml and 26% of milk samples had results recorded as 'too numerous to count' and 15 % milk samples had total bacterial count of less than 100 CFU/ml.

All 10 respondents who reported consumption of milk from their own cattle confirmed that all household members consumed milk on daily basis. With regards to treatment of milk before consumption, 10 respondents indicated that they either boil or sour the milk before consumption. Consumption of raw milk was reported by 1 respondent and only 1 respondent indicated that they sold excess milk. As treatment of milk reduces the risk of zoonotic pathogens transmission from milk to humans the fact that the majority of respondents applied his intervention before consumption can be seen as risk reduction behaviour for the transmission of zoonotic pathogens from milk to humans.

Although the results on the presence of *B. abortus* were inconclusive, the overall findings in the study indicated that the raw milk in the study population posed a high risk of transmitting zoonotic diseases to humans. The cows' milk in this study was found to be of poor microbiological quality because of the presence of *M. bovis*, high prevalence of *S. aureus* and the counts for coliforms and *E. coli* that exceeded the limits set by the South African standards under the Foodstuffs, Cosmetics and Disinfectants Act, No. 54 of 1972: Regulations relating to milk and dairy products.

1. INTRODUCTION

Literature Review

General Introduction

Cow's milk is considered to be one of the main food groups important in a healthy balanced diet (Pereira, 2013). Milk from cows is considered an important high quality protein source in the human diet, supplying approximately 32 g protein/L and this includes whey proteins and caseins (Haugh *et al.* 2007). Whey proteins and caseins have various biological functions in human health (Mills *et al.* 2011). The functions offered by some of the milk proteins, include antibacterial, antiviral, antifungal, antioxidant, anti-hypertensive, antimicrobial, antithrombotic and immunomodulatory roles in addition to improving absorption of other nutrients (Mills *et al.* 2011). Calcium is also present in high amounts in milk with an average of 1200mg/L of milk and is also considered to be essential for a high bone density in humans (Little & Holt, 2004). Several other nutrients that are found in milk include fatty acids, liposoluble vitamins A, D, E and water soluble B complex vitamins such as thiamine and riboflavin (Pereira, 2013). Micro elements like zinc, phosphorus, selenium, magnesium and potassium can also be found in milk as well as other dairy products (Gaucheron, 2011).

Though milk is an important food product, milk and milk by-products can harbour a variety of zoonotic pathogens which cause zoonoses (Oliver *et al.* 2005). The World Health Organization (WHO) defines zoonosis as diseases that can be transmitted between humans and animals. Zoonotic pathogens may be bacterium, virus, fungus or other communicable disease agents (WHO 2014). These zoonotic pathogens may contaminate milk either whilst the milk is still in the udder or post milking (Schoder *et al.* 2013). Regardless of the route in which zoonotic pathogens enter the milk, transmission to human mostly occurs through consumption of raw milk (Bramely & McKinnon, 1990). There are several reasons to why raw milk is consumed in rural areas. One of the reasons for consumption of raw milk is that it is less expensive than to buy retail pasteurised milk. Some people believe that raw milk has extra nutrients which are beneficial to the health.

1

The prevalence of pathogens in milk is determined by factors that include, the size of the farm, number of animals on the farm, cleanliness of the farm, farm management practices, types of samples collected, detection methodologies used, geographical location of study area, and the season when sampling was done (Oliver *et al.* 2005). Zoonotic pathogens that may contaminate milk may originate from the farm environment for example Salmonella *species*, pathogenic *Escherichia coli* strains (including *E. coli* O157:H7), *Listeria monocytogenes* (*L. monocytogenes*) and *Staphylococcus aureus (S. aureus)*. Other pathogens may originate from infected animals for example *S. aureus* (mastitis), *Brucella arbortus (B. arbortus), Mycobacterium bovis (M. bovis)* and *Mycobacterium avium* subsp. *paratuberculosis* (Johne's disease) (Kousta *et al.* 2010). Unhygienic practices which can lead to contamination of milk include the contact with contaminated utensils, contact surfaces, floors, and packaging material (Kamana *et al.* 2014). Hands of personnel milking cows can also serve as source of human pathogens being introduced into the milk.

These zoonotic organisms may lead to health problems in the human population such as tuberculosis (TB), brucellosis, haemorrhagic enteritis, salmonellosis and listeriosis (Addo *et al.* 2011; Schoder *et al.* 2013). The population that is at higher risk of being infected with these milk borne pathogens include people who are immunocompromised, the elderly, pregnant women and children (Schoder *et al.* 2013). Other effects of these zoonotic pathogens include causing economic losses in dairy production such as decreased milk yields and causing trade restrictions on animals and animal products and this has got an effect the growth of the economy in developing countries in Africa (Mosalagae *et al.* 2011).

Selected milk-borne zoonotic pathogens

Tuberculosis

Tuberculosis (TB) is a major threat to public health in South Africa and is amongst the 22 high TB burden countries in the world (WHO, 2015). The Global Report 2015 ranks South Africa on the second place in the world in terms of incidence rates for TB, behind Lesotho (WHO, 2015). Furthermore, in their report published in 2011, Statistics South Africa revealed that TB is the number one killer diseases in South Africa (Statistics SA, 2011).

2

Tuberculosis (TB) is a zoonotic bacterial disease caused by members of the *M. tuberculosis* complex, including *M. tuberculosis*, *M. africanum*, *M. microti*, *M. bovis BCG*, *M. caprae*, *M. canettii*, *M. pinnipedii*, *oryx bacillus*, *dassie bacillus*, *M. mungi* and *M. suricattae* (Parsons *et al.* 2013; Malama *et al*. 2013; Alexander *et al.* 2010; Aranza *et al.* 2003).

Mycobacterium tuberculosis is the main causative agent of human tuberculosis, whereas *M. bovis* mainly causes bovine tuberculosis (BTB) in cattle (Modise, 2012). *M. bovis* which causes bovine tuberculosis is a slow-growing, aerobic bacterium which can also cross species barrier and causes tuberculosis in humans (Grange *et al.* 1994). This bacterium can be transmitted between cattle via the cutaneous, oral ingestion or the teat canal (Cosivi *et al.* 1998; Grange, 2001). However, the main route of infection between animals is by inhaling infected droplets from other animals or secretions in soil, grazing etc. Human beings can be infected with *M. bovis* by the consumption of non-pasteurized (raw) milk (Hassanain *et al.* 2009) from infected animals (e.g. cattle goat, deer, buffalo, sheep and camel) (Challu, 2007). Transmission can also occur directly through inhalation of airborne droplets (Challu, 2007). Humans infected with open tuberculosis due to *M. bovis* can transmit the bacteria to animals via the aerogenous route by spitting or coughing (Grange, 2001). Reports showed that infection is principally by respiratory route but farmers with genito-urinary TB caused by *M. bovis* could infect cattle by urinating in cow sheds (Grange, 2001). It may take a long time before the signs of TB are seen in livestock. General signs include: emaciation, weakness, poor condition of skin coat, mastitis and chronic coughing (Olivier, 2013).

In humans, immune suppressed individuals may develop active TB after infection with *M. bovis* irrespective of its origins (Challu, 2007). *Mycobacterium bovis* is also associated with cervical lymphadenopathy, intestinal lesions, chronic skin tuberculosis (lupus vulgaris) and other non- pulmonary forms such as bones and joints, but not all extrapulmonary TB cases are caused by *M. bovis* (Thoen *et al.* 2006).

Bovine tuberculosis, caused by *M. bovis*, is also a zoonotic disease of concern all over the world due to the impact it has on livestock farming and trade restrictions and can persist in wildlife reservoirs and thus has an effect on the entire ecosystems (Renwick *et al.* 2006; Musoke, 2016). OIE recommends that cattle that are positive for bovine tuberculosis on intra-dermal tuberculin test should be slaughtered and the carcasses

should be condemned, and this results in a loss of income for the farmers (Michel *et al*. 2006).

In communal area settings, close physical contact between humans and potentially infected animals is encouraged where humans reside close to their cattle kraals (Cosivi *et al*. 1998; Ameni *et al*. 2006). This increases the possibility of humans being infected by *M. bovis* from cattle through aerosol transmission. A much higher zoonotic risk is, however, assigned to the consumption of untreated milk from infected cattle because livestock owners in communal area consume unpasteurised fresh and soured dairy products on daily basis (Michel *et al.* 2015).

Brucellosis

Brucellosis is caused by bacteria of the genus *Brucella*, and the species are defined based on the animal host specificity (Boschiroli *et al.* 2001; Joint WHO/FAO/OIE. 2004). Current Brucella species are Brucella *abortus* (cattle), *Brucella melitensis* (sheep and goats), *Brucella suis* (swine), *Brucella ovis* (sheep), *Brucella canis* (dog), *Brucella neotomae* (desert woodrat), *Brucella pinnipedialis* (seal), *Brucella ceti* (dolphin, whale), *Brucella microti* (common vole), *Brucella inopinata* and *Brucella sp*. NVSL 07-0026 (Mayer-Scholl *et al*. 2010; Atluri *et al*. 2011; Godfroid *et a*l. 2011). Brucellosis is most commonly spread between herds by the movement of infected animals. The disease is also spread between animals by contact of susceptible animals with infective discharges at the time of calving or abortion of infected animals for up to 1 month thereafter (DAFF Brucellosis Manual, 2003). Humans become infected with brucellosis when they come into contact with infected excretions of cattle, foetuses, foetal membranes or with infected carcass material in abattoirs. Humans may also be infected with brucellosis during informal slaughters of infected animals and by ingesting contaminated unpasteurized raw milk (DAFF Brucellosis Manual, 2003). Consumption of raw milk is very common in rural and communal areas. Milk that is commercially sold is normally screened for *B. abortus* using the milk ring test and pasteurised before it can be consumed and the same does not happen in rural and communal areas. This increases the chances of transmission of *B. abortus* from infected cattle through consumption of raw milk.

Symptoms of brucellosis in cattle include abortion in late pregnancy, usually at 5-7 months gestation and cows may become infertile and animals present with swollen joints (Olivier, 2003). Brucellosis in humans presents as a febrile 'flu-like' illness with frequent chills and people also often complain of headaches and general weakness (Krause & Hendrick, 2010).

Endemic brucellosis in developing countries in Africa has major economic implications in agriculture, public health and social development sectors (Dermott *et al.* 2013). Even though there is unreliable data reported, 18 countries had outbreaks of brucellosis in 2011 as per African Union- Interafrican Bureau for Animal Resources (AU-IBAR) report. The highest numbers of outbreaks were in Algeria (367), in the second place was South Africa (282 outbreaks) (AU-IBAR, 2011). This means that economic loss from brucellosis due to decreased productivity was huge and the probability of humans being infected with brucellosis was also high (AU-IBAR, 2011). Communal and rural areas are generally regarded as areas with poor resources and have a high unemployment rate and people in those areas rely on subsistence and cattle farming (Hesterberg *et al.* 2008). Since *B. abortus* causes a reduction in cattle productivity it means that this effect will cause rural and communal areas to become poorer.

Coliform bacteria

Coliforms are facultative anaerobic, gram- negative, short rods and include organisms like *Escherichia coli, Klebsiella pneumonia*, and *Citrobacter* species. These organisms are found in human and animal as part of the intestinal flora (Neill *et al.* 1994). Coliforms have similar phenotypic characteristics, making them not easily distinguishable. Coliforms are used as an indicator of faecal contamination (Feng *et al.* 2013). This is because most coliforms are mainly found in human and animal faeces and not usually found in other niches (Feng *et al.* 2013). Presence of coliforms in food, milk or water is indicates faecal contamination (Feng *et al.* 2013). The existence of detectable levels of coliforms in dairy products suggests the existence of unsanitary conditions during milk production and processing (DRINC, 1996). In South Africa according to South African Standards, a coliform level of more than 50 coliforms/ml in milk is considered a cause of alarm.

Most coliforms do not cause diseases in humans except for *E. coli* strains (IDF, 1994 and Cliver, 1999). Enterohaemorrhagic *E. coli* like *E. coli* O157:H7 may produce Shiga-like toxins (Karch *et al.* 2005). Transmission of pathogenic *E. coli* O157:H7 from animals to humans often occurs via faecal-oral route. Cows suffering from mastitis may discharge, among other pathogens, *E. coli* in milk leading to infection in humans if contaminated raw milk is consumed (Cawe, 2006).

In humans, *E. coli* O157:H7 infection in most cases causes, severe, acute, bloody diarrhoea and stomach cramps. In children less than 5 years of age, immunocompromised people and the elderly, *E. coli* infection can cause haemolytic uremic syndrome (Corrigan & Borneau, 2001). Studies done in Sudan *E. coli* species were isolated from raw milk of dairy cattle in a communal area in the Khartoum State (Asmahan & Abdelgadir, 2011). In the study, 63% of the samples obtained from 100 cattle were confirmed *E. coli* positive (Asmahan & Abdelgadir, 2011). In a 2013 study in Dakahlia Governorate, Egypt, researchers were able to identify *E. coli* and *S. aureus* bacteria in the milk from 85 out of 150 cows from the rural farms (Gwida & EL-Gohay, 2013).

There are studies that showed that raw contaminated milk encourages the proliferation of *E. coli* O157 when stored at a room temperature for 2 hours (Alhelfi *et al.* 2012) which emphasises the need for refrigeration in ensuring food safety but is inaccessible in many rural areas. In the absence of electricity, souring of milk is a very common practice but on the other hand storing contaminated raw milk at 8°C, for 1-2 weeks allows *E. coli* O157 to survive and multiply increasing the risk of infection when the milk is consumed (Massa *et al.* 1999).

Staphylococcus aureus infection

Staphylococcus aureus produces several virulence factors, including enterotoxins and toxic shock syndrome toxin (TSST-1) that cause staphylococcal food poisoning in humans (Asperger & Zangerl, 2001). *Staphylococcus* spp. may be found on skin and mucous membranes of healthy warm-blooded animals, as well as in soil, air, and water (Asperger & Zangerl, 2001). This pathogen easily spreads in the environment, requiring careful procedures during milking and sanitization to avoid the transmission among cows, equipment, and utensils (Akineden *et al.* 2001).

Staphylococcus aureus in humans occurs as a result of ingestion of numerous heat and protease stable staphylococcal enterotoxins (SEs) produced under specific environmental conditions when the population density of the pathogen reaches 105 CFU/ml (Le Loir *et al.* 2002). Human-to-bovine transmission of *S. aureus* has been demonstrated by molecular studies that have shown that similar strains may be isolated from handlers and the milk of cows with mastitis (Jørgensen *et al.* 2005). Possible sources of bulk milk contamination includes, personnel and equipment involved in milking of cows, the environment where the cows reside, and the udder and teat health of dairy animals that are milked (André *et al.* 2008; Dufour *et al.* 2012).

Staphylococcus aureus is one of the causes of bacterial mastitis in lactating animals (D' Amico & Donnelly, 2009). In humans *S. aureus* causes severe diarrhoea, nausea, vomiting, and abdominal pain 1 to 6 h after consumption of infected material (Balaban & Rasooly, 2000).

A large number staphylococcal food poisoning (SFP) cases have been reported worldwide (Rosec *et al.* 1997; Akineden *et al.* 2001; Rizek *et al.* 2011). In France, 25 out of 149 foodborne staphylococcal outbreaks reported in 1999 were due to the consumption cheeses made from raw milk (WHO, 2000). A study done in South Africa in 1985 found that 18.9% of all *S. aureus* isolates from milk were toxigenic (Bolstridge & Roth, 1985). Mastitis caused by *S. aureus* is a major concern because of its capability of being resistant to antibiotics and capability of recurrence (Makovec & Ruegg, 2003).

 Production of staphylococcal enterotoxins in milk occurs when the milk is stored at temperatures of 37ºC to 42ºC or when there are fluctuations in storage temperatures (Jørgensen *et al.* 2005). Bearing this in mind, in rural areas, summer temperatures can be high and most milk in rural areas is not refrigerated, the production of staphylococcal enterotoxins in the milk is likely. Since rural areas are generally regarded as areas with poor resources, Staphylococcal food poisoning in rural areas can lead to high mortality rates due to lack of adequate medical facilities (Hesterberg *et al.* 2008).

Control and prevention of milk-borne pathogens

Certain practices such as boiling, fermenting, pasteurization and ultra violet radiation of milk have been shown to reduce the number of pathogenic bacteria in the milk. Lowering the number of microorganisms in the raw milk enhances the level of safety, quality and shelf life of the milk (Cawe, 2006).

Fermentation or natural scouring milk can be used to control the growth of milk borne pathogens especially in small holder farmers as it is a cheaper way of preserving milk. In natural scouring, the low pH retards growth of pathogens however it does not retard the growth of moulds (O'Mahony, 1988).

Boiling milk inactivates viruses, bacteria and protozoa and other pathogens. Boiling is more accurately characterized as pasteurization (New York Department of Health, 2011). Pasteurization is the most common process used to reduce the amount of pathogenic microorganisms such as *B. abortus* and *M. bovis*, to levels that do not constitute a significant health hazard (Hassanain *et al.* 2013). This was proven in a study that was carried out in Italy where the effects of pasteurization of milk on the microbiological quality of raw milk were compared to effects on milk after microwaving, boiling and refrigeration of raw milk from vending machines microbes (Tremonte *et al.* 2014). Two methods of pasteurization include the high temperature short time (HTST) and the holder method (Cawe, 2006). The holder method is when milk is held in tanks for at least 30 minutes at not less than 62.8⁰C and at no more than 65.6⁰C and in HTST milk is heated to a temperature of 72⁰C for 15 seconds followed by rapid cooling below 10⁰C (Cawe, 2006).

Justification

In South Africa, there is limited information with regards to the prevalence of zoonotic diseases of cattle in rural areas. Information about the prevalence of zoonotic diseases in rural areas is essential when prioritising and implementing disease control schemes in rural areas (Hesterberg *et al.* 2008). Studies conducted in rural areas in South Africa's KwaZulu-Natal province, outlined that 97% of households that own cattle consume milk on daily basis (Geoghegan et *al.* 2013). Considering that it is a common to consume raw milk in these rural communities, it is important that cattle owners and local veterinary and health authorities are aware of milk borne zoonoses that are

prevalent in their areas. It is also important for cattle owners to have knowledge about the risks that zoonotic diseases pose and how they are transmitted to make informed decisions on their control. Unless more publications and studies were to become available to prove otherwise, we assume that milk-borne diseases in rural areas in South Africa are as prevalent as in other developing countries with communal area farming and animal husbandry practices. Hence, the investigation into the prevalence and transmission risk of milk pathogens in a communal set up in South Africa will be beneficial to harnessing public health.

In this study, the Nibela diptank was chosen because it represented a communal area setting in South Africa at the wildlife/livestock interface where both bovine tuberculosis and brucellosis are known to occur in the community's livestock (Hluhluwe State vet. 2015, personal communication.). The results of this study will contribute important data with regards to the current status of bacterial milk- borne pathogens at the Nibela diptank which serves as a model for other communal areas in South Africa where humans and livestock share an interface. Knowledge of the prevalence of these selected bacterial milk- borne pathogens is valuable in determining the risks of possible exposure of humans, other domestic animals and wildlife. High prevalence levels of the bacteria being investigated signal the need for local authorities including the Department of Health to implement control measures and educate the communal farmers on how to prevent infections with zoonotic pathogens through milk. Low prevalence of bacterial milk-borne zoonotic pathogens will have an added value in terms of local trade in cattle and cattle products.

Information from the questionnaire survey will indicate the level of knowledge people have with regards to milk hygiene and milk consumption behaviour in study area. Information from the udder score card will determine the general hygiene of the cattle pens used in the areas being investigated. Total bacterial count will give an insight in the general microbiological quality of milk in the study area.

Aims and objectives of the study

The aim of this study was to conduct a pilot investigation to determine the microbiological safety of milk including selected milk-borne pathogens in communal cattle in UMkhanyakude district, KwaZulu-Natal, South Africa.

The objectives of the study were:

1. To investigate the microbiological safety of milk and to determine the seroprevalence of milk–borne pathogens including *M. bovis*, *B. abortus, S. aureus* and coliform bacteria in milk samples from cattle presented at the Nibela dip tank, northern KwaZulu-Natal.

2. To conduct a questionnaire survey on milking hygiene and milk consumption behaviour among farmers at Nibela dip tank towards an evaluation of the risk of zoonotic transmission at the livestock/human interface.

2. Materials and methods

Study area

The study was carried out in the cattle population registered at Nibela diptank which is located near Hluhluwe in the Big 5 False Bay Municipality, the southern part of the uMkhanyakude District, northern KwaZulu Natal province, South Africa. The study area is adjacent to False Bay and Isimangaliso Wetland Park (Figure 1) (Ntatiso *et al.* 2014).

Nibela diptank serves an estimated 2 233 cattle belonging to 130 owners, as determined by the stock count carried out by the uMkhanyakude State Veterinary office in January 2015. This information shows the importance of cattle keeping in the area. According to the records provided by the Hluhluwe state veterinary office the prevalence of BTB in the cattle population of Nibela diptank, as determined by intradermal tuberculin test, was 13% in January 2015. In 2008 the prevalence of brucellosis in rural areas of KwaZulu Natal was 1.45% (Hesterberg *et al.* 2008).
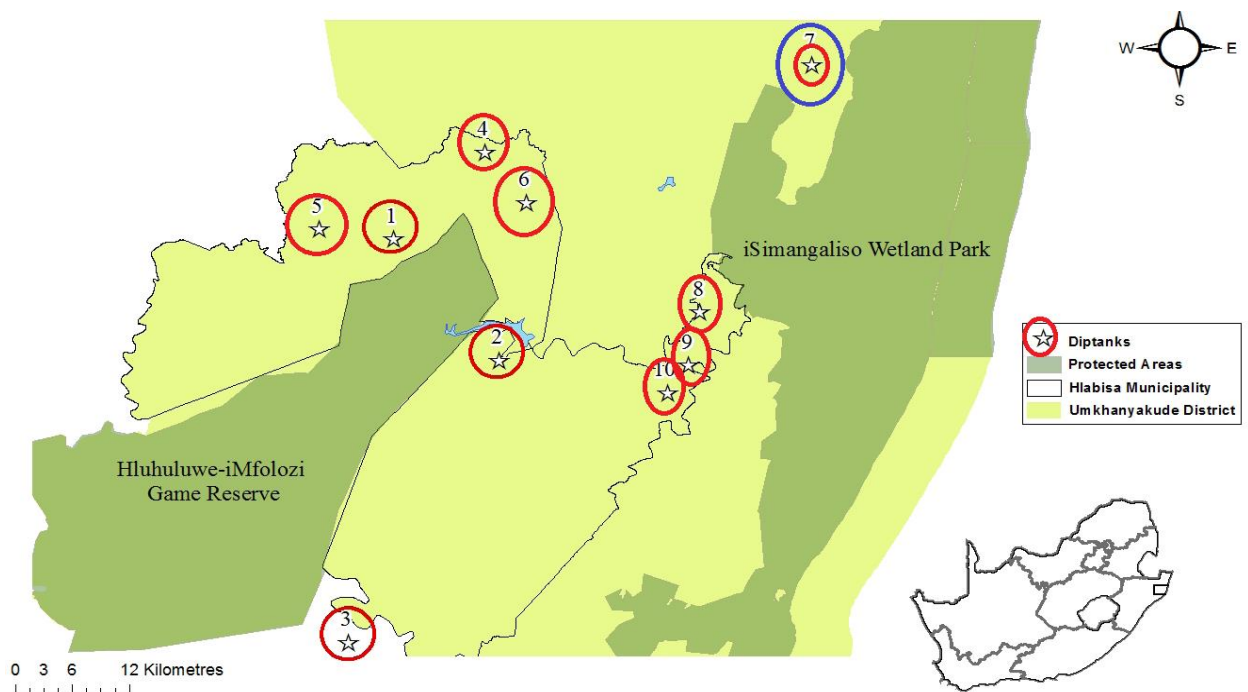
Source: Ntantiso et al 2014

Figure 1   Study area: Nibela diptank (7)

(GPS coordinates: 27° 51' 09, 9" S, 32° 26' 43,4")

Animals and sampling

Convenience sampling of milk was applied during a diagnostic pilot project involving selected BTB positive herds at the Nibela Diptank in the uMkhanyakude district from 18 May 2015 to 21 May 2015. A total of 344 cattle from 12 herds were available for this study while being tested for BTB. From those, lactating cows were selected for milk sampling on condition that consent from the owner was given. All animals were identified by individually numbered ear tags.

Collection and preparation of milk samples and udder hygiene scoring

The cattle were driven into a crush and then restrained using a rope around the hind legs. The udder scoring was based on the scoring chart from scoring chart from University of Wisconsin –Extension (Cook & Reinemann, 2007). The scores were applied as follows: Score 1: Free of dirt, Score 2: Slightly dirty (2-10% of surface area), Score 3: Moderately covered with dirt (10-30% of surface area) and Score 4: Covered with caked-on dirt (>30% of surface area) (Cook & Reinemann, 2007) (udder hygiene score Appendix 1).

A minimum of 50 millilitres of milk were collected from all functioning quarters of the udder. The sample collecting tubes (sterile 50 ml screw-cap centrifuge tubes) were labelled according to the ear tag number of the animal. The milk samples were transported on ice to the Hluhluwe state veterinary laboratory where the microbiological analysis including total bacterial count, coliform count, brucella milk ring test and primary milk culture was performed within 2-3 hours after sample collection. *Mycobacterium* cultures*, S. aureus identification test*s, and *B. abortus* cultures were conducted in the BSL2 + laboratory at the Faculty of Veterinary Science of University of Pretoria, South Africa, 14 days after milk sample collection.

Microbiological analysis

Total bacterial count

The 3M™ Petrifilm™ Aerobic Count Plates (manufactured by 3M, South Africa) was used to determine the total bacterial count as per manufacturer's instructions (Total bacterial count methodology Appendix 2). Briefly, one millilitre of each milk sample was

put in the centre of the film. The top lids were then released down onto the milk samples and the samples were evenly distributed using a spreader with gentle downward pressure and the spreader was removed. Plates were incubated for 48 hours at 37º C. Determination of the total bacterial count was done as per South African regulation relating to milk and dairy products (R1555). After incubation the Petrifilm plates were enumerated by counting the number of red dots on the circular growth area on the film. Estimates of total bacterial counts on plates containing greater than 250 colonies were done by counting the colonies in one square and multiplying the number by the number of squares in the circular growth area. In cases were the entire growth area was red or pink in colour the total bacterial count was recorded as "too numerous to count" (TNTC).

Coliform count

The 3M™ Petrifilm™ *E. coli*/Coliform Count Plates (manufactured by 3M, South Africa) was used to determine coliforms and *E. coli* count according to the manufacturer's instructions (*E. coli* methodology Appendix 3). Briefly, one millilitre of each milk sample was put onto the centre of the bottom films. The top films were rolled down onto the milk samples to prevent air bubbles. A spreader was used distribute the milk samples. The plates were left for one minute before incubating them for 24 hours at 35ºC. Determination of coliform count was done as per South African regulation relating to milk and dairy products (R1555). Blue colonies which had entrapped gas bubbles were regarded as *E. coli*. Other coliform colonies were red and had gas bubbles. The total coliform count comprised of both the red and blue colonies with gas bubbles. Petrifilm plates that had colonies with the numerous small colonies and numerous gas bubbles, were considered as "too numerous to count" (TNTC).

Mycobacterium bovis detection

The procedure of milk sample processing and isolation of Mycobacterium was used according to the procedure described by Michel *et al*, 2015. In summary, 75 ml of milk were transferred to a 250 ml bottle and an equal volume of 1% cetylpyridinium chloride (CPC) was added to make a volume of 150 ml and the solution was mixed well. The samples were incubated for a week at room temperature. Thereafter 45 ml of the sample was added into three 50 ml centrifuge tubes. The milk samples were centrifuged at 3500 rpm for 30 min, and the supernatant discarded. The sediments were neutralized by adding sterile distilled water up to a volume of 25 ml. The mixture was well mixed

with the back of a sterile inoculation loop and mixture was further centrifuged at 3500 rpm for 10 minutes. The supernatant was poured off, leaving approximately 3 ml of pellet. A loop full or two of pellet was inoculated onto four Löwenstein Jensen medium slopes, two of which were supplemented with pyruvate and two with glycerol. The cultures were incubated at 37ºC for 10 weeks and inspected weekly for growth. Single bacterial colonies were picked using an inoculation loop and suspend in a phosphate-buffered saline solution (PBS) which was heat treated at 94º C for 15 minutes. The isolates were submitted to the ARC-Onderstepoort Veterinary institute (OVI) and subjected to the *M. tuberculosis* complex polymerase chain reaction (PCR) (Cousins *et al.* 1992). For *Mycobacterium* species identification PCR assays targeting the RD4 and RD9 regions were used to distinguish *M. tuberculosis* and *M. bovis*.

Brucella species detection

Brucellosis infection was detected using the Brucella Milk Ring test (BMRT). The BMRT was done as described by Alton *et al.* 1975, using *B. abortus* antigen which was obtained from Onderstepoort Biological Products Ltd, South Africa. Test tubes were labelled to according to the number of the milk samples. 0.03 ml of the antigen was then dispensed in each test tube and 1 ml of the milk sample was also added. The test tubes were incubated in an incubator at 37°C for 1 hour. Positive reactions were samples that had formed a distinct blue ring as the cream rises while negative reactions were indicated by samples that had blue coloured milk and white cream.

The milk samples that tested positive with a ring formation were then cultured on blood agar plates. Initially the cultures were incubated in an aerobic incubator at 37°C for 24hours at the laboratory located at the Hluhluwe state veterinary offices close to the study area. Colonies that were characteristic of *Brucella* which were non-haemolytic, non-pigmented small grey colonies were subcultured to obtain pure cultures of the suspected *Brucella* colonies. The subcultured plates with suspected *Brucella* colonies were transported to the BSL2 + laboratory at the University of Pretoria (several days later on completion of the field work) where the suspect colonies were stained using the Grams' stain, oxidase and catalase tests for identification of the *Brucella* species (Basset & Thomas, 2014). Due to the lack of CO_2 the subcultures of suspected *Brucella* species were incubated in an aerobic incubator at 37℃ for 2 weeks before being transferred to a CO_2 incubator. Culture material was washed off the primary culture

plates (that initially had characteristics of *Brucella* colonies) and the subculture plates. The washings were done by pipetting 1 ml of phosphate-buffered saline solution (PBS) into the culture plates then letting the PBS stay in the culture plate for 5 minutes before gently scrapping off the colonies using the inoculating loop and pipetting 1ml of the solution with scrapings into a universal tube. Samples were then heat treated at 90 ⁰C for 15 minutes. The culture washes were sent to the ARC-Onderstepoort Veterinary Institute (OVI) Diagnostic PCR laboratory for PCR-based screening for Brucella organisms. The assay was designed to exploit a single unique genetic locus that was highly conserved in *Brucellae* targeting the 16S rRNA genes (Bricker, 2002).

Staphylococcus aureus identification test

Staphylococcus aureus was cultured by directly plating milk samples onto blood agar and incubating the samples at 37⁰C for 24hrs. After incubation, any colonies with the typical appearance of *S. aureus* (large, round, golden-yellow colonies, surrounded with beta haemolysis) were further confirmed using the Staphylase tests, Gram's stain, catalase tests and oxidase tests. Samples were considered to be positive for *S. aureus* if the positive cocci were identified on Gram's staining, and had catalase positive and oxidase negative reactions.

Questionnaire Survey

The questionnaires were handed out to 12 farmers whose animals were milked at Nibela diptank for the purposes of this study. Verbal consent from the cattle owners was requested first before the questionnaire (see Appendix 4) was completed. The questionnaire that was administered had been previously used in a survey on zoonotic TB (Musoke. 2016) and was modified to include information about milking hygiene. The questionnaire was administered face to face by a translator who was an animal health technician at Hluhluwe veterinary services, in the local language which is Zulu.

The questionnaire was structured into 2 sections, namely the respondent demographics and food consumption (questionnaire attached to Appendix 4). The first section on respondent demographics assessed the following: age, gender, occupation and highest education qualification for the respondent. Other questions were about the respondent's family size, number of people per specified age group and if there were any members of the family that were diagnosed with TB.

The second section of the questionnaire which was about the milk consumption assessed the following: consumption of milk from own cattle, who consumes the milk, how often, treatment of milk, selling of excess of milk. In the second section, the milking practices are also assessed. The answers to each of the questions on the questionnaire were entered into a Microsoft Excel spread sheet and statistical analysis was done.

Data analysis

The level of udder contamination for the diptank was calculated by determining the average percentage of different udder scores obtained for all the animals sampled.

All the bacterial results were recorded on Microsoft excel spread sheet. The prevalence of *S. aureus* and *B. abortus* in the BTB positive herds at Nibela dip tank was calculated using the formula:

Prevalence= (number of cases)/ (population size) (Le & Boen, 1995).

The data obtained from the questionnaire survey were analysed using descriptive statistics (Trochim, 2006).

16

3.   RESULTS

In this chapter the results of microbiological analyses from 57 milk samples that were collected from 12 cattle herds are presented. Furthermore the data obtained during interviews with members of 12 households at the Nibela diptank is reported.

3.1   Microbiological analysis

3.1.1   Total Bacterial Count

Total bacteria counts (TBC) yielded over 100 cfu/ml in 32 (59%) milk samples and 14 (26 %) milk samples were recorded as "Too numerous to count" (TNTC). A total of 8 (15%) samples had a count of between 1 and 100 cfu/ml. For 3 of the 57 milk samples no quantification could be performed for total bacterial counts because the sample quantities were insufficient to perform the test (table 1).

Table 1   Total bacterial count performed for 54 cows

Total bacterial Count (CFU/ml)	Number of samples (%)
0	0
1< 100	8 (15%)
>100	32 (59%)
"Too numerous to count (TNTC)	14 (26%)
Total number of milk samples	54

3.1.2   Coliform and *Escherichia coli* counts

A total of 11 (21%) milk samples had more than 20 cfu/ml of coliforms and no coliforms were detected in 20 (38%) of milk samples. *E. coli* was detected in 32 (59%) milk samples and was not detected in 22 (41%) milk samples. The recommended values given by the South African standards under the Foodstuffs, Cosmetics and Disinfectants Act, No. 54 of 1972: Regulations relating to milk and dairy products indicate that raw milk may not contain more than 20 coliforms (using the dry rehydrated film method also known as the Petrifilm plate for coliforms), or any *E. coli* per millilitre. A total of 4 milk

samples out of 57 collected milk samples were not checked for the presence of coliforms and 3 milk samples out of 57 collected samples were not checked for *E. coli* because the quantities were insufficient to perform the tests (table 2).

Table 2   Coliform and Escherichia coli results from 53 and 54 milk samples

Count (CFU/ml)	Number of Samples with *Coliforms*	Number of samples with *E. coli*
0	20 (38%)	22 (41%)
0<20	22 (42%)	20 (37%)
>20	11 (21%)	12 (22%)
Total number of milk samples	**53**	**54**

3.1.3 *Mycobacterium bovis* detection

A total of 20 pooled samples comprising of milk from 48 cows were cultured for *M. bovis*. Eleven samples yielded contaminated cultures (on both LG with glycerol and pyruvate) which prevented an assessment of whether or not *M. bovis* was present. One pooled sample representing 5 individual animals yielded a culture with typical *M. bovis* colony growth on the LG media with pyruvate. There was no growth on both the LG media with glycerol and pyruvate on 8 samples. The *Mycobacterium* species was ascertained by PCR specific for *M. tuberculosis* complex (test performed at ARC-OVI; data not shown). The prevalence of *M. bovis* could not be calculated because 55% of the samples were contaminated.

3.1.4 *Brucella* species detection

Brucellosis infection was detected using the BMRT. The milk samples that tested positive with a ring formation on BMRT were then cultured on blood agar gel.

Six pooled samples representing the milk from 21 (38%) cows were positive on the MRT. The 21 Individual milk samples from the pooled samples that tested positive on MRT were cultured on the blood agar and 9 samples showed typical *Brucella* species growth (non-haemolytic, non-pigmented small grey colonies). Gram's staining was done on the 9 culture isolates and 8 were identified as Gram negative rods. To confirm the *Brucella* species growth, oxidase and catalase tests were used on the 8 samples that

18

had Gram negative rods and 7 samples had the characteristics that exhibited the presence of *Brucella* species. Final identification of suspected *Brucella* colonies using PCR was not possible.

3.1.5   Identification of *Staphylococcus aureus*

Milk samples from 57 cows were cultured on blood agar and 31 samples had typical *S. aureus* growth. To confirm *S. aureus*, Gram's stain was used and 29 samples were identified as positive cocci. Oxidase and catalase tests were performed on the 29 samples that were positive cocci on Gram's stain and 28 had characteristics of the *S. aureus*. The Staphylase test was performed as an additional confirmatory test for *S. aureus.* The prevalence rate of *S. aureus* in the cattle that participated in the study was 49% (table 3).

Table 3   *Staphylococcus aureus* results for 57 cows

Test	Number of positive samples
Staphylococcus aureus culture (typical growth)	31 (54%)
Grams stain	29 (51%)
Oxidase test	29 (51%)
Catalase test	28 (49%)
Staphylase test	28 (49%)

3.2. Udder Hygiene Score assessment

The udder scoring was performed at the cattle crush facility of the Nibela diptank using a scoring chart University of Wisconsin –Extension (Cook & Reinemann, 2007). The scores were applied as follows: Score 1: Free of dirt, Score 2: Slightly dirty (2-10% of surface area), Score 3: Moderately covered with dirt (10-30% of surface area) and Score 4: Covered with caked on dirt (>30% of surface area). Ninety three percent of the cattle that were sampled had udder scores of 2 which indicated that they were slightly soiled while 7 % had a score of 3 (table 4).

Table 4   Results for udder hygiene scoring performed for 57 cows

Udder Score	Number of animals
Score 1-free of dirt	0
Score 2-slightly dirty (2-10% surface area)	53 (93%)
Score 3-Moderately covered with dirty (10-30% of surface area)	4 (7%)
Score 4-covered with caked on dirt (>30% surface area)	0

3.3   Questionnaire survey results

3.3.1   Household demographics

All 12 representatives from the 12 households that were asked to participate in the questionnaire survey agreed to do the interviews and 100 % of the respondents were males. The median age for the respondents was 54 years and the average cattle herd size per family was 39. Average number of people in a family was 12.

Table 5   Employment status of respondents

Employed	2 (17%)
Unemployed	10 (83%)

83% of the respondents were unemployed and 17% indicated that they were employed (table 5). In this study, being self-employed was considered as being unemployed.

Table 6   Level of education among respondents

No formal Education	Basic Education (Primary school up to Grade 10)	High school and Tertiary
3 (25%)	8 (67%)	1 (8%)

Twenty five percent of the respondent's reported that they do not have formal education and 67% had basic education. Only 8% had high school or tertiary qualifications (table 6).

Table 7   Number of family members per age group

Age category	Average number per family
Children aged below 12yrs	4
Persons aged 13yrs to 18 yrs	2
Persons aged 19yrs to 64yrs	5
Persons aged 65yrs and older	1

According to the information that was provided by the respondents, it showed that the second majority age group in the study families was children below 12 years, and the least age group was over 65 years (table 7). The average household size was 12 people per household.

3.3.2   Milk consumption

3.3.2.1   Consumption of milk from own cattle

Consumption of milk from own cattle was reported by 10 respondents and 2 (17%) respondents did not consume milk from own cattle (table 8). The reasons why 17% did not consume milk from own cattle where not determined. The respondents who consumed milk from own cattle confirmed that all members of the household consumed that milk on daily basis.

Table 8   Consumption of milk from own cattle

Consumption of milk from own cattle	Number of respondents
Consume milk from own cattle	10 (83%)
Do not consume milk from own cattle	2 (17%)

3.3.2.2   State in which milk is consumed

With regards to the state in which the milk is consumed in, 10 respondents representing 10 households indicated that they either boil or sour the milk before consumption. Only 1 respondent representing a household with an average of 12 family members indicated consumption of raw milk.

3.3.3 Milking hygiene

Sixty percent of the respondents indicated that they clean the udders of the cows before milk and 30% indicated that they never clean the udders before milking.

3.3.4 Sale of excess milk

The majority of respondents (9 respondents) indicated that they do not sell excess milk to the public and 1 respondent admitted that they do sell excess milk to the public. The reasons why the respondents did not sell excess milk to the public where not determined.

3.3.5 Diagnosis of TB cases in the families

With regards to family members that were diagnosed with TB, 5 respondents indicated that they have family members that were diagnosed with TB.

4. DISCUSSION

Introduction

In South Africa, there is limited published data on the microbial quality and prevalence of milk borne pathogens in raw milk from communal cattle. Most studies that were done in South Africa concentrated on commercial dairy cattle herds. Most of the communal farmers consume the milk produced by their cattle and rarely sell it (Dovie *et al.* 2006) in a study in Mnisi communal area, Mpumalanga province, South Africa it was found that in times of low milk production, owners don not even use the little milk they would get, the leave the milk for the calves and for young children (Musoke, 2016). This makes it difficult to determine the microbiological quality of milk from communal cattle and to also determine the economic impact of the milk quality from communal cattle (Dovie *et al.*, 2006). This project was therefore conducted to carry out a pilot investigation to determine the microbiological safety of raw milk and apparent prevalence of selected pathogens in communal cattle at Nibela diptank in UMkhanyakude district in KwaZulu-Natal, South Africa.

Total bacterial count

According to the South African standards under the Foodstuffs, Cosmetics and Disinfectants Act, No. 54 of 1972: Regulations relating to milk and dairy products 74% of the milk samples met the required standards because they yielded 100 cfu/ml or less, upon subjection to total bacterial count. Total bacterial count is a good indicator for monitoring the hygienic measures that are practiced during production and handling of raw milk (Chambers, 2002). This indicates the majority of the cattle stay in a clean area. A total of 14 (26%) milk samples out of 54 milk samples collected had results recorded as "too numerous to count" and did not meet the South African regulations with regards to microbiology of milk. Contamination of milk can take place during milking, when various bacteria enter the milk from the teat canal, milking equipment and environment (Sandholm *et al.* 1995). In this study the source of contamination could have been from the cow dung that was in the crush or from hands of people who were assisting with milk sample collection since there was no hand washing facility at the diptank.

Coliforms and *Escherichia coli* counts

Bacteriologically, raw milk may not contain more than 20 coliforms (using the dry rehydrated film method also known as the Petrifilm plate for coliforms), or any *E. coli* per millilitre. Presence of these higher levels of coliforms and *E. coli* in the milk makes the raw milk unsafe to consume. Coliform counts reflect hygiene and sanitation practices followed during milking of the animals. Coliforms may enter the milk supply when milking dirty cows. In this study the high coliform and *E. coli* counts in some milk samples could have been due to the fact that milk samples were collected by different people in a very unhygienic environment. That the results could also be suggesting that the cows had subclinical or clinical coliform mastitis. The samples were collected while the cows were in a crush which was not cleaned and also contained large volumes of cattle dung. *E. coli* thrives in wet, warm and organic environmental conditions, and these environmental conditions are found in dairy herds (Winfield & Groisman, 2003). Contamination of the cow's environment with faeces plays a major role in the occurrence of *E. coli* mastitis (Green, 2002; Jones & Swisher, 2009).

Mycobacterium bovis detection

Culture of *M. bovis* can be challenging, especially if the samples are contaminated with fast growing bacteria as mycobacteria take 4–10 weeks to grow. In this study culturing of bacterially contaminated milk samples was done more than 4 weeks after milk collection. It was hence to be expected that cultures of 11 out of 20 milk samples were contaminated. Another constraint was having small milk volumes for culture resulting in combining milk samples from different animals hence individual results for animals could not be ascertained and were possible affected by the dilution effect. Despite the assumption that the isolation rate for *M. bovis* could have been higher without these constraints, it is an important finding that *M. bovis* was detected in the milk. This shows that there is a risk of transmission of *M. bovis* to humans who consume contaminated raw milk. According to the literature review that was carried out by Muller *et al.*, (2013), it was clear that *M. bovis* infections in humans were prevalent and Africa had the highest (2.8%) prevalence estimates compared to other continents. There are limited publications with regards to occurrence of zoonotic TB in humans in South Africa. The fact that *M. bovis* can survive in milk for at least 5 days means that there is a possibility of other domestic animals to be infected with *M. bovis*, for example dogs and pigs that are fed on milk contaminated with *M. bovis* (Mariam, 2014; Kleeberg, 1984). These domestic animals roam freely on communal land and have the potential for transmitting *M. bovis* to humans and also wild animals (Kleeberg, 1984).

In this study 5 respondents indicated that they had members of their families that were diagnosed with TB, it is important to investigate the type of TB causative organism and its origin.

Brucella species detection

Upon subjection to the BMRT, 6 pooled samples representing milk from 21 cattle were positive and on culture 9 samples showed colony growth phenotypically consistent with B*rucella* species. It was, however, not possible to confirm the isolation of *Brucella spp*. Isolation of *Brucella* species requires 4 to 7 days of incubation and the presence of overgrowing fungi and other bacteria in the milk samples causes contamination of culture plates and also inhibits the growth of the *Brucella* species (De Miguel *et al.* 2011). In order to make a reliable diagnosis of Brucella species when using field samples like milk it is important to use a selective culture media for *Brucella* species.

Farrell's medium and modified Thayer-Martin medium are normally used as selective mediums for Brucella species (De Miguel *et a*l. 2011). In this study blood agar was used and is not a selective medium and in addition lack of proper controlled laboratory environment may have contributed to the contamination of culture plates.

During the culture process subcultured plates with suspected *Brucella* colonies were initially incubated in an aerobic incubator at 37℃ for 2 weeks and were then transferred to the CO_2 due to the absence of CO_2 in the laboratory. The use of CO_2 increases the probability of success of bacterial culture and the absence of CO_2 could have resulted in the growth failure of the *Brucella* species. Additionally it may also be possible that the cultures suggested as *Brucella* species were not *Brucella* species.

Milk Ring test is used for screening of brucellosis in cattle herds but may also give false positive results in cases were cattle have mastitis or in cases where samples are composed of abnormal milk like colostrum. During milk sample collection some abnormal milk samples were observed hence it cannot be concluded that the cattle had brucellosis. It is advisable to carry out more diagnostic tests to reach a conclusion. According to the Office International des Epizooties (OIE) the "gold standard" for the diagnosis of brucellosis is bacterial isolation, selective media of choice include Farrell and modified Thayer-Martin media.

Identification of *Staphylococcus aureus*

In this study, the prevalence of *S. aureus* in milk samples was 46%. This was very alarming because *S. aureus* is associated with disease in humans. The study did not determine if the *S aureus* that was detected in the milk was the enterotoxin producing *S. aureus*. The colour of some of the milk samples resembled puss and had clots and this is typical with milk from cattle with clinical mastitis and this might be one of the reasons why the prevalence of *S. aureus* was high. In South Africa, in the commercial dairy farming, *S. aureus* has been found to be the dominant mastitis-associated organism (Petzer *et al.* 2009). Since there are limited studies on the microbiology of milk from communal areas in South Africa, the dominant organism causing mastitis in communal cattle in is not defined so the assumption would be that it is *S. aureus*. The milk samples may also have been contaminated by *S. aureus* from people who were collecting the milk samples. This is because during milk sample collection people who

collected milk from the cattle were not washing their hands before collecting the samples. It has been found that the hands of food handlers can spread food-borne to poor personal hygiene or cross-contamination (Lues & Van Tonder, 2007). In a study by Lues & Van Tonder (2007) in delicatessen sections of a retail group, *S. aureus* was isolated from the hands of 88% of the population sampled. Different surveys have shown that between 4% and 60% of humans are nasal carriers of *S. aureus*, and that, 5% to 20% of people carry the organism as part of the normal skin flora (Asperger, 1994). Not all strains are capable of producing staphylococcal enterotoxins (SEs) but more than 20 staphylococcal enterotoxins have been found (Lindqvist *et al*. 2002). It is difficult to conclude that there is a high risk of transmission of *S. aureus* from milk to humans in the Nibela diptank area unless further studies are carried out to determine the type and origin of the *S. aureus* detected in the study.

Udder hygiene scores

Udder hygiene affects milk quality and is also related to occurrence of environmental pathogens, for example *S. aureus* and *E. coli*, in the milk (Manzi *et al*. 2012). A dirty environment results in dirtier udders (Schreiner & Ruegg, 2003) and in the current study the average udder score of 2, means the cattle are being housed in a clean environment. Teat and udder contamination also cause mastitis in cattle, which may also affect the microbial quality of raw milk (Schreiner & Ruegg, 2003).

In the questionnaire survey 60% of the respondents indicated that they clean the udders before milking the cattle. This agrees with the udder hygiene score that was also carried out in the survey where the average cleanliness of the udder was found to be a score of 2 (2-10% surface area slightly dirty). Cleaning the udder of cows before milking is important because the udder could have had direct contact with the soil, urine, and dung and feed refusals while resting and environment is a source of contamination for milk. Only portable water should be used in the process of milk production (Bramley & McKinnon, 1990). If dirty water gets into the milk or if dirty is used for cleaning udders or milk containers, any micro-organisms that are present in the water will contaminate the milk.

Questionnaire survey on milk consumption and demographics of the study population

Twelve households participated in the questionnaire survey in this study, ten of which with an average of 12 members indicated that they consumed milk from their own cattle on a daily basis. This agrees with other studies conducted in South Africa's KwaZulu-Natal province where 90% of households and in Tanzania, 97% households in communal areas consumed milk daily (Geoghegan *et al.* 2013; Rough *et al.* 2014). With regards to treatment of milk before consumption, 10 respondents indicated that they either boil or sour the milk before consumption. Studies showed that there is no risk of being infected by *M. bovis* for Masi older than 2 weeks (Michel *et al.* 2015), but people generally do not ferment milk for consumption for as long as 2 weeks. Even though 1 respondent (8%) with an average family size of 12 people indicated that they consume milk in its raw form and 1 respondent indicating that they sold excess milk means that there is a limited risk of transmission of *M. bovis* from milk to humans in the study population. This is because majority of respondents treated their milk and treatment of milk is a risk mitigating factors which largely reduces the public health risk but of course does not eliminate it completely. This agrees with a study that was carried out in rural Mnisi community, Mpumalanga province, South Africa to determine the risk of transmission of M. bovis from livestock to humans revealed that there was a low risk for that based on the community's milk consumption practices (Musoke, 2016).

It is also alarming that 5 respondents indicated that they had family members that were diagnosed with TB although the type and source of the TB were not determined. This result is alarming because *M. bovis* can cause TB in humans, hence the type of the causative organisms in these TB cases need to be identified.

The questionnaire survey results also showed that one of the major age groups in the households was children under the age of 12 years. It was also shown that there was at least one person aged 65 years and older per household. It is assumed that milk often is an important component of the diets of the young and the elderly. Young children and the elderly are at higher risk of being infected from food-borne pathogens (Wang *et al.* 1997). This is because the immune systems of these groups of individuals are often not sufficiently responsive to prevent infection by pathogenic bacteria (Johnson *et al.* 1984; Wang *et al.* 1997). For these reasons greater emphasis should be placed on the safety of milk.

Limitations of the study

The results of this current study may be biased because of the convenience selection of interviewees and study animals during a pilot study for the BTB testing that was being carried out by Hluhluwe state veterinary services. This approach may have limited the response group to cattle owners who had BTB positive herds.

Other limitations of this study included having limited numbers of lactating animals for sample collection due to the drought that was being experienced in the area at the time of the study. Lack of collection of milk samples in required amounts resulted in the pooling of milk samples in order to acquire enough samples to perform the required tests.

Another limitation was lack of a laboratory in the study area and this resulted in most tests not being done soon after collection of milk samples. Pathogens like *Brucella* species do not survive in stored milk for a long time and amounts of *M. bovis* also decrease after 2 weeks. During sample collection there was no water for washing hands and udders before milking the cattle, this may have caused cross contamination of samples. These limitations may have interfered with the results and conclusions of the current study.

Conclusions

Generally, the microbiological quality of milk in this study was poor when compared to bacteriological standards of dairy products in South Africa. This is based on high apparent prevalence of *S. aureus* which was 49% and that of *E. coli* was 56%. The important fact is that *M. bovis* was detected in the milk and legislation does not allow such milk to be consumed in raw form. The questionnaire survey indicated that milk treatment measures are in place in households which aid in the risk mitigation of zoonotic transmission of M*. bovis*. However the respondents need to be educated on the importance of hygienic milk collection which includes cleaning of udders before milk collection.

Future recommendations

1. The prevalence of *S. aureus* is very alarming and there is need to conduct further studies to determine the source of the *S. aureus*. It is also advisable to determine whether the *S. aureus* is resistant to antimicrobials

2. Sampling needs to be done during a season where cattle will be lactating and this will result in more milk samples being collected in adequate amounts.

3. Collection of milk samples needs to be done in a clean cattle crush and microbial analysis needs to be done under a proper laboratory environment to minimize biased microbiological results.

4. When conducting a questionnaire survey, it is important to explain to the respondents the importance of carrying out the survey and to assure them that their answers will be kept as confidential information. This encourages the respondents to be truthful when answering the survey questions.

5. Local authorities of the study area need to be alerted about the results of the study and need to be encouraged to intensify control measures and educate the communal farmers on how to protect their selves from being infected by zoonotic pathogens through milk.

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Appendix 1
Udder score chart

Appendix 2

Methodology for Total Bacterial count using 3M Petrifilm kit (as per manufacturer's instructions)

1. The Petrifilm Aerobic Count plate was placed on a flat surface and the top film was lifted.

2. One milliliter of milk sample was dispensed onto the center of bottom film and the top film was released down onto sample.

3. The sample was evenly distributed using a spreader and plate was left for one minute to allow the gel to solidify and plates were incubated for 48 hours at 37º C.

4. All red dots on the circular growth area were counted as colonies. Estimates on plates containing greater than 250 colonies were done by counting a representative number of squares and multiplying by the number of squares in the growth area. The presence of very high concentrations of colonies on the plates were recorded as "too numerous to count (TNTC).

Appendix 3
Methodology for *E. coli* / Coliform count using 3M Petrifilm Plates (as per manufacturer's instructions)

1. The Petrifilm E. coli/Coliform Count plate was placed on a flat surface and the top film was lifted up.

2. One millimetre of milk sample was dispensed onto the centre of the bottom film and the top film was rolled down onto the sample.

3. The sample was evenly distributed using a spreader and plate was left for one minute to allow the gel to solidify and plates were incubated for 48 hours at 37º C.

4. Blue colonies that had entrapped gas were confirmed *E. coli* and blue colonies without gas were not considered as *E. coli*.

5. Red colonies with gas bubbles were counted as other coliforms and red colonies without gas bubbles were not counted as coliforms. The total coliform count consisted of both the red and blue colonies associated with gas at 24 hours after incubation.

Appendix 4
Questionnaire

Respondent Identification:

Village: _____

Dip-tank: _____

Stock card number: _____

Household identity: _____

Respondent demographics

Gender of respondent

    Female   Male

Age of respondent: _____

What is your highest education?

    No formal

    Basic education (Primary + secondary school)

    High school and above

What is you occupation

    Employed

    Unemployed

    Farmer

What is the respondent's family size? _____

*Please complete the following table in respect of the number of members of your households for the age category **including** the respondents*

Age category	Number
Children aged below 12yrs	
Persons aged 13yrs to 18 yrs	
Persons aged 19yrs to 30yrs	
Persons aged 31yrs to 60yrs	
Persons aged than 61 yrs	

Has any family member **including** respondent been diagnosed with TB?

    Yes   No

Food consumption

Do you consume milk from your cows

    Yes   No

How do you and your household consume milk from your own cows

    Raw

    Boiled

    Soured

How often do you consume milk from own cows

    Daily

    Weekly

    When needed

Who mainly consumes milk?

    Children aged below 12yrs

    Persons aged 13yrs to 18yrs

    Persons aged 19yrs to 30yrs

    Persons aged 31yrs to 60yrs

    Persons aged than 61 yrs

    Whole household

Do you sell excess milk to the community?

    Yes   No

Where do you mostly get your milk from?

    Owns cows

    Local farmer

    Supermarket

BTB status of cattle

    Positive   Negative

Appendix 5
Animal Ethics Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	Investigating the prevalence and significance of bacterial milk-borne zoonotic pathogens in communal cattle in Mpempe and Nibela dip tanks uMkhanyakude District in Kwa Zulu Natal province. South Africa	
PROJECT NUMBER	V047-15	
RESEARCHER/PRINCIPAL INVESTIGATOR	Dr. J Munjere	

STUDENT NUMBER (where applicable)	1024 0081	
DISSERTATION/THESIS SUBMITTED FOR	MSc (W-Based)	

ANIMAL SPECIES	Milk samples	
NUMBER OF ANIMALS	To be reported	
Approval period to use animals for research/testing purposes		1 May 2015 – 1 May 2016
SUPERVISOR	Prof. A Michel	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	25 May 2015
CHAIRMAN: UP Animal Ethics Committee	Signature	

S4285-15