Cooking and drying as effective mechanisms in limiting the zoonotic effect of *Mycobacterium bovis* in beef

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ABSTRACT

For this study 48 non-infected muscle, lymphatic and visceral bovine tissue samples were collected from an approved red meat abattoir and spiked with 8×10^7 cfu/mℓ of *M. bovis*. The different spiked samples were subjected to cooking and drying (drying through the process of biltong-making) processes in a controlled laboratory environment. Mycobacterial isolates confirmed as *M. bovis* by means of polymerase chain reaction (PCR) were observed in 17 of a total of 576 samples that were exposed to the secondary processing method of cooking. The study showed that not only can *M. bovis* survive the cooking process but the survival of the bacterium will be determined by its unique adaptive changes to the surrounding composition of the environment. The results for the samples exposed to the drying process (n = 96) did not show any growth, suggesting that the process of biltong production as used in this study is likely to render infected meat safe for human consumption.

Keywords: biltong, beef, food processing, food safety, *Mycobacterium. bovis,* tuberculosis, tissue, zoonotic.

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INTRODUCTION

The World Health Organisation (WHO) reported in 2004 that 14.6 million people had active tuberculosis with 9 million new cases per annum[27]. The WHO furthermore stated that *Mycobacterium bovis (M. bovis*) is the causative bacterium in 3 % of all tuberculosis cases[26].

The potential zoonotic importance of *M. bovis* infection in an extremely wide host spectrum including domestic animals, wildlife and humans was cited by other researchers as a cause of public health concern in Africa[2,5,12]. Despite the paucity of information on *M. bovis* in Africa, there is sufficient evidence to suggest that it is widely distributed and is found at significantly high prevalence in some animal populations in rural and privately owned farms[10,16]. The WHO reported in 1993 that 90 % of cattle and dairy cow populations in Africa are either only partly controlled for bovine tuberculosis or not controlled at all. They expressed their concern about the safety of food of animal origin with regard to contamination by *M. bovis* because only projected global estimates of the disease are available owing to the non-availability of reliable technology for early detection of *M. bovis*[2,6,15,25].

In South Africa, bovine tuberculosis has recently been reported to occur in all of the country's provinces and is also, endemic in wildlife in at least 2 of the largest game reserves[1,13,14]. The public's exposure to bovine tuberculosis is addressed *ante mortem* by the National Bovine Tuberculosis control scheme[13] that ensures animals that tested positive for tuberculosis will not become commercially available and *post mortem* by the national meat inspection system in all registered abattoirs that identifies and condemns lesions of *M. bovis* in slaughtered animals. Infected animals or those suspected of being infected are removed from the food chain, but asymptomatic animals or localised lesions could pass through the inspection system unnoticed. When meat is obtained from uncontrolled sources (not slaughtered in an abattoir), especially in poor and rural communities,[12,22] meat inspection is not performed on these carcasses and could increase the public's exposure to *M. bovis.*

The Meat Industry in South Africa has, owing to an increasing population and protein demand from consumers, progressed to identify alternative sources of meat such as game meat and is currently supplying to both the local and international meat markets[1,13–15]. Several methods such as cooking* are applied in the processing and preserving of red meat and game meat but the reduction of the water activity (A_w) through the process of drying (including the addition of vinegar and salt) is very popular and almost unique to South Africa[23].

The zoonotic importance of *M. bovis* is theefore growing, as stated above, and secondary processing of meat and edible organs (able to effectively inactivate *M. bovis*) can be regarded as a mechanism to prevent the public's exposure to tuberculosis

MATERIALS AND METHODS

Sampling of beef carcasses

Twenty grams of each of the different tissue types i.e. muscle (diaphragm), kidney, liver, heart, lung and lymph nodes (mandibular and parotid) were collected from 8 beef carcasses on the slaughter floor directly after the primary meat inspection point. These specific tissue types were used for the following reasons:

- Organs and tissues commonly used in meat recipes and in the biltong-making process[17].
- High consumption index of these organs and tissue by humans[20].
- Availability and accessibility of the tissue.
- Organs selected infected with *M. bovis*[1,3,4].

The sampling equipment was sterilised between sampling in a steriliser at the inspection point at 82 °C for 20 minutes[19]. The samples were placed in separate sterile plastic bags (Whirlpack), labelled

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*Cooking is a form of preservation but is essentially used to make food more palatable and safe for immediate consumption.

and placed on ice in an insulated cool box (7 °C). Temperatures were maintained during transport to the laboratory and on arrival samples were frozen to –18 °C within 4 hours[16]. Samples were stored at –18 °C until analysed.

Tissue spiking and processing

Samples received were defrosted in a biohazard cabinet and size, abnormal consistency, colour, smell and the presence or absence of visible lesions in the samples were recorded. Visible fat was trimmed away and samples cut into 20 g pieces using sterilised instruments.

These tissue samples were spiked with a field isolate of *M. bovis* which had been cultured in the laboratory from an infected cow. A Mac Farland Standard 1 solution was prepared in phosphate-buffered saline (PBS) and centrifuged at 1600 g. The pellet was resuspended in 50 % of the original volume of PBS to achieve a double-strength solution (8×10^7 cfu/mℓ). Spiking was performed by injecting 1 mℓ of the *M. bovis* suspension into multiple sites in each sample using sterile syringes and needles.

Control experiments

Two pools of non-infected samples (*i.e.* not infected with *M. bovis*) were included and consisted of lymph nodes, muscle and kidney tissue as well as liver, lung and heart tissue, respectively. The pooled samples were processed similarly.

Viability of the *M. bovis* field strain used for spiking was confirmed by streaking directly onto LJ (TB slope) medium.

The drying process

Twenty grams (20 g) of the muscle tissue ($n = 8$) was put into a biltong mixture (salt, vinegar and biltong spice) for 12 hours, and then hung in a biohazard cabinet (class II) to dry.

The cooking process

To establish the cooking time to be used in the laboratory, 10 restaurants were randomly selected and visited to observe and measure the cooking time and temperature applied to meat. Without exception the meat dishes subjected to the process of cooking were done at a temperature of 100 °C. This study did not include dishes such as 'Carpaccio', which is consumed as thinly sliced raw beef, or other processing methods such as roasting or frying. Grilled beef could be eaten from 'rare to well done' according to individual taste and the exposure of the meat to high temperature could be minimal.

Owing to the vast differences in cooking times that were used; the average longest and average shortest cooking times were established. The average of the 10 shortest cooking times was used to obtain the result of 8.9 minutes and the average of the 10 longest cooking times was 21.2 minutes. Therefore it was decided to use 10 and 20 minutes, respectively, as the cooking times to be applied in the laboratory study.

Samples cooked for 10 minutes were cut into smaller pieces and put into sterile homogenising jars using sterilised tongs and homogenised for 3 minutes at 5000 rpm (Servall omni-mixer, Ontaria, Canada). This was repeated with the 20 minute cooking time samples. Seven mℓ of each sample homogenate was transferred into each of 2 sterile 15 mℓ centrifuge tubes, thus making up a pair for each sample.

Bacterial culture of processed samples and identification of isolates

Two different decontamination procedures were applied to increase the likelihood of recovering *M. bovis* from the tissues. The paired homogenates in the centrifuge tubes were decontaminated (killing organisms other than *M. bovis*) by adding 7 mℓ of 2 % hydrochloride (HCl) to the one and 7 mℓ of 4 % sodium chloride (NaCl) to the other. Treatment of *M. bovis*-containing samples with NaCl is a very stringent method which provides better protection from contamination at the cost of sensitivity, while HCl does not kill off contaminants as effectively, but allows for a higher recovery rate of mycobacteria[14]. A standing time of 10 minutes was allowed for decontamination before the sample was again centrifuged for 10 minutes at 3500 rpm. The supernatant was then decanted and the remaining content was neutralised by adding 7 mℓ of sterilised, distilled water. The tubes were centrifuged for a further 10 minutes at 3500 rpm. The supernatant was again decanted and the remaining pellet mixed using the back of a sterilised inoculation loop handle. One loopful from each tube was spread evenly onto 2 TB-slope + glycerol in McCartney bottles (DMPA 0710) and 1 TB-slope + pyruvate in McCartney bottles (DMPA 0711) (Diagnostic Media Products, Johannesburg) respectively and incubated at 37 °C for 10 weeks and monitored for growth on a weekly basis.

Twelve slants per sample were spread (2 aliquots per sample cooked for 10 and 20 min, respectively, decontaminated with HCl and NaCI, and spread onto 1 LJ-pyruvate and 2 LJ-glycerol slants. A total of 576 slants were prepared and incubated for 10 weeks.

Slants yielding Ziehl-Neelsen positive, dome shaped, off-white colonies were further analysed by polymerase chain reaction (PCR). Bacterial cell suspensions were prepared and boiled for 20 minutes before amplification using the protocol reported by Cousins[6]. This primer set amplifies a 372 base pair product within the MPB70 region of *M. bovis.*

Statistical analyses

For the statistical analyses Stata's *tabi* command was employed in performing chi-square tests. The (null) hypothesis of independence between the two categorical variables, namely cooking times, which were 10 min and 20 min, and slants, which were either positive or negative, was tested. The analysis was performed at the 95% confidence limit, *i.e.* a *P*-value of less than 0.5 declared an association between the two variables.

RESULTS

Positive experiment on cooked samples

Seventeen (17) of the 576 slants (2.9 %) showed growth of typical of *Mycobacterium bovis.* PCR amplification of single bacterial colonies confirmed them as *M. bovis.* In the samples decontaminated with sodium chloride (NaCI) and cooked for 10 and 20 minutes respectively, growth was detected in all tissues except for lung and muscle samples. In the samples decontaminated with hydrochloric acid (HCI) and cooked for 10 and 20 minutes respectively, *M. bovis* was only detected in lymph node and muscle samples.

Muscular tissue samples exposed to the biltong process (drying)

None of the tissue samples ($n = 96$) showed any growth.

Negative control experiment

None of the tissue samples ($n = 24$) exposed to the cooking process nor the muscular tissue samples exposed to the biltong process (drying) ($n = 12$) showed any growth.

Statistical analyses

The observed *P*-values (Table 1) indicated that the 2 variables were statistically independent. That is, the null hypothesis is not rejected at the 95 % confidence limit for all tissue types.

Table 1 shows the isolation of *M. bovis* from the different tissue types for the 2 cooking times.

DISCUSSION

Cooking is a form of preservation, but is essentially used to make food more palatable and safe for immediate consumption[18]. Although temperatures achieved

Table 1: **Isolation of *Mycobacterium bovis* from different tissue types cooked for 2 different periods.**

Tissue type	Cooking time (min)	Slants		*P*-value
		Positive	Negative	
Lung	10 min	0	48	
	20 min	0	48	
Muscle	10 min	2	46	0.646
	20 min	3	45	
Lymph node	10 min	1	47	1.000
	20 min	1	47	
Liver	10 min	2	46	0.557
	20 min	1	47	
Kidney	10 min	1	47	0.315
	20 min	0	48	
Heart	10 min	3	45	1.000
	20 min	3	45	

during cooking are usually sufficient to ensure an effective reduction of bacteria, core temperatures of at least 75 °C should be achieved to ensure bacteriological safety[21]. On the other hand, the ability of bacteria to survive heating is also influenced by the matrix (the micro-environment), the initial number (the spiking process added viable logarithmic numbers of the bacteria) and the distribution of especially the more temperature tolerant organisms[20]. *Mycobacterium bovis* will, in reaction to a favourable environment, replicate and increase the dose presented to the host on consumption. In reaction to an unfavourable environment, *M. bovis* can produce coccal forms that can, under favourable conditions, again elongate and return to standard growth forms[3,7]. In addition, the composition of the substrate in which the bacteria are heated, the moisture content, and the hydrogen-ion concentration (pH) have an effect on the tenacity of bacteria[17,24]. Other factors such as the material, size and shape of the container, the size and shape of the pieces and rotation or agitation can also have an effect on the ability of bacteria to survive secondary processing[9]. During the cooking process the core temperature should be reached more quickly in lung tissue than in heart muscle (L C Hoffman, Department of Animal Sciences, University of Stellenbosch, pers. comm., 2008). owing to the lower density of the tissue. Apart from the matrix density, the thickness of a tissue sample is an equally important determinant of effectively inactivating bacteria by cooking, as observed in the muscle samples from the diaphragm in this study. It is speculated that the findings from heart muscle samples are the result of a combination of higher density and thickness of the matrix compared to the results obtained from the other tissue types.

On the other hand, cooking at a fixed temperature (100 °C) will not be as effective as maintaining a lower core temperature for an extended period of time (e.g. 95 °C for 2 hours) (F D Mellet, Department of Animal Sciences, University of Stellenbosch, pers. comm., 2008). This could explain the lower isolation rate, especially from the softer tissue types observed at a cooking time of 20 minutes as compared with 10 minutes.

Other processing methods such as grilling and frying as well as individual preferences when consuming very rare or well done meat were not included in this study and should be considered with caution owing to the lower temperatures and shorter periods of exposure to high temperatures in terms of their possible failure to destroy *M. bovis.*

The processing method of lowered water activity (A_w) relies on the fact that all bacteria require moisture to facilitate their metabolic reactions. *M. bovis* requires an A_w of at least 0.95[7,16]. With desiccation of *M. bovis,* plasmolysis occurs when the cytoplasmic membrane partially separates from the rigid layer of the wall and leaves behind a peri-plasmic space with small bridge-like structures that withstand the retraction of the protoplast[3,10]. Bacteria in a healthy state may contain 80 % water which is obtained from the food in which they grow. If the water is removed from the food, water will also be removed from the bacterial cells and multiplication will stop. However, since bacteria can live in one part of a food that may differ in moisture from the food just millimetres away, the micro-environment of the bacteria must be considered[13]. Therefore, thicker biltong cuts and cuts with a percentage of fat should all be considered as factors potentially influencing the survival of *M. bovis.* The A_w was not determined for the biltong and could have been much lower owing to the small pieces of meat used and the drying efficacy of the flow cabinet.

In the biltong-making process, a hurdle effect of preservation is used whereby drying is combined with preservation by sugar, salt, vinegar and other spices according to taste. The optimal growth pH of *M. bovis* at 5.8 to 6.5 could allow ingested *M. bovis* to survive the pH of digestive acids in humans, where the pH may range between 4.7 and 5.9[16] and in animals, where the pH often varies from 5.7 to 6.5[15]. *M. bovis* possesses a wide variety of fatty acids that hinder penetration of an acid by the lipids barrier principle used in the Ziehl-Neelsen stain technique.

Lowering the pH with a solution of acetic acid (vinegar) is commonly used in the biltong-making process. In sufficiently high concentrations, acid modifies bacterial proteins as it denatures food proteins. Bacteria in general are sensitive to acid[16] and although most bacteria grow best at pH levels near neutrality, *M. bovis* has a tolerance for low pH levels[13]. The optimal acidity of the environment in the case of *M. bovis* was confirmed when growth was seen at pH levels of 5.8 to 6.5[17]. However, the acid fastness could be destroyed by physical, chemical as well as biological means[8]

No significant difference ($P > 0.05$) in growth was observed between the application of the 2 decontaminants (HCl *versus* NaCl). The findings suggest that, although a certain bactericidal effect of the decontaminants is acknowledged, the growth of mycobacteria was not significantly hampered by the decontamination procedures.

The laboratory process of isolating *M. bovis* takes between 4 to 12 weeks and corresponds with the bacterium's chronic disease character[11,13]

The newly discovered drug resistance ability of mycobacterium (in the absence of detailed information on how the mycobacterium changes its macromolecular composition and growth rate in reaction to an unfavourable environment), highlights not only the underestimated pathogenic phenomena of the bacterium but also the need for such studies. Effective processing methods which render beef safe for human consumption offer a simple way to reduce the risk of zoonotic diseases in humans in general and should be communicated more widely, especially in developing countries where the prevalence of zoonoses is high.

While conventional and alternative methods of producing biltong may differ in their inactivating effect on *M. bovis,* the results of the drying process in this study suggest that *M. bovis* cannot survive the

specified conditions. However, a follow-up study involving conventional meat portions would be required to evaluate their microbiological safety under commercially used biltong rendering protocols.

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