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Use of Molecular Typing to Elucidate *Mycobacterium bovis* Transmission in South Korean Beef Cattle

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ABSTRACT

In this study we have characterized *Mycobacterium bovis* (*M. bovis*) isolates from a herd of Korean beef cattle in Hongsung, Korea. Of the 30 skin test positive cattle samples, isolations of *M. bovis* were made from 11 animals. Seven animals out of 11 were comprised of Korean native cattle purchased from herd of southern regions in 2008 and the others (n = 4) were natural additions (offspring) of these cattle. Using the spoligotyping and Variable Numbers of Tandem Repeat (VNTR) typing, we demonstrated that bovine tuberculosis was transmitted to a herd by the introduction of animal belonging to a geographically separated location.

Key words: *Mycobacterium bovis*, spoligotyping, VNTR

INTRODUCTION

Bovine tuberculosis (BTB) is an infectious disease caused by *Mycobacterium bovis* (*M. bovis*) that mainly affects cattle. Although cattle are its primary host, *M. bovis* can also infect other mammals, including humans. Since 1964, the National BTB surveillance program of South Korea has been using the Single Intradermal Test (SIT) to screen cattle for BTB (Wee *et al.*, 2010). However, these measures have failed to eradicate BTB in beef cattle. This failure has been attributed to the surveillance relying mainly on meat inspection in slaughterhouses; moreover, BTB tests are carried out on beef breeds only. Field surveillance, including the annual Purified Protein Derivative (PPD) tuberculin tests, is conducted on dairy cattle and beef breeds that are more than a year old. The prevalence of infected dairy and beef cattle was 0.14 and 1.3%, respectively in 2012.

Molecular epidemiology studies on BTB can provide insights into the mechanism underlying its bovine transmission and the role of wildlife reservoirs in disease transmission and maintenance (Skuce and Neill, 2001). Spoligotyping and the minisatellite technique (also called Variable Numbers of Tandem Repeat (VNTR) typing) are methods that are commonly used to identify and classify *M. bovis* strains (Comas *et al.*, 2009; Skuce *et al.*, 2005). These techniques have helped to characterize a number of species belonging to the *Mycobacterium tuberculosis* complex and have played an important role in the detection of the ongoing BTB transmission between animals (Hang'ombe *et al.*, 2012).

MATERIALS AND METHODS

Cattle samples: To characterize the epidemiology of an outbreak of tuberculosis (TB) in a beef cattle farm in Hongsung (southern Korea), *M. bovis* isolates obtained from PPD positive animals were analyzed by mycobacterial interspersed repetitive unit [MIRU]-VNTR and spoligotyping. The beef cattle comprised 181 adult Korean native cattle (Hanwoo, *Bos taurus coreanae*). Twenty-five cows and 5 steers tested positive for BTB in the caudal fold skin test.

Culture of *M. bovis*: Samples from the lungs and lymph nodes of the 30 SIT positive animals were collected and cultured, irrespective of the presence or absence of tuberculosis lesions in the animals. Each sample was cultured on three types of selective media (7H10 medium [BD, USA], 7H10 without glycerol and mycobacteria growth indicator tubes [BD, USA]) at 37°C. After incubation for 1-3 months, each isolate was identified using the polymerase chain reaction (Wilton and Cousins, 1992).

Spoligotyping: Spoligotyping was performed using an REBA Spoligotyping Strip® (Molecules and Diagnostics, Korea) as described previously (Kamerbeek *et al.*, 1997). A reverse blot hybridization assay was performed by following the manufacturer's instructions. The resulting spoligotype patterns were compared with the patterns deposited in the *M. bovis* spoligotype database (<http://www.mbovis.org/>; Smith and Upton, 2012).

Variable Numbers of Tandem Repeat (VNTR) typing: Fourteen MIRU-VNTR primers (MIRU4, MIRU16, MIRU26, MIRU27, MIRU31, ETR-A, ETR-B, ETR-C, QUB11b, QUB18, QUB26, QUB3336, QUB2401 and QUB3171) were selected from the database. DNA amplification was performed using these primers, as described previously (Jeon *et al.*, 2008; Le Fleche *et al.*, 2002). PCR fragments were analyzed using electrophoresis with a 1.5% agarose gel (Bioneer, Korea).

RESULTS

Of the 30 SIT-positive samples, 11 yielded mycobacterial colonies. Apart from the 1030-bp genus-specific signal that is common to all *Mycobacterium* species, a 372-bp fragment specific for *M. bovis* was also amplified in the multiplex PCR reaction, thus confirming the identity of the isolates as *M. bovis*. Only one spoligotype (SB0140-42542755434343) was identified among the 11 *M. bovis* isolates. SB0140 is the most common spoligotype reported to infect animals in South Korea Ireland and the United Kingdom (Reyes *et al.*, 2012). The MIRU-VNTR PCR analysis revealed 5 different profiles; 3 of the strains were unique, while 8 others clustered into two groups (Table 1). Of all the 14 MIRU-VNTR loci, 11 exhibited no polymorphism in the isolates, while the other 3 (MIRU-27, MIRU-31 and ETR-A) were distinct. An analysis of the spoligotyping and MIRU-VNTR results revealed that the largest cluster contained six isolates, while the other cluster contained two isolates. Three isolates showed unique genetic profiles.

DISCUSSION

Molecular typing revealed that 8 isolates could be classified into 2 clusters (Cluster I contained 3596, 9570, 6679, 1099, 5069 and 8013 and Cluster II contained 4765 and 9607), while the remaining exhibited unique genetic profiles. Epidemiology investigations revealed that 7 cattle out of 11 *M. bovis* culture positive cattle were introduced in the herd belonged to the southern regions of Korea in 2008. Moreover, prior to 2013, 7 cattle had never been tested for BTB during

Table 1: Molecular characteristics of *Mycobacterium bovis* isolates obtained from beef cattle in Hongsung, South Korea

Isolates	Spoligotype	MIRU-VNTR	Age	Sex	Origin of region*	Origin of herd	Combined cluster
9570	SB0140	4 2 5 4 2 7 5 5 4 3 4 3 4 3	69	F	GB	A	Cluster I
3596	SB0140	4 2 5 4 2 7 5 5 4 3 4 3 4 3	68	F	GB	A	Cluster I
6679	SB0140	4 2 5 4 2 7 5 5 4 3 4 3 4 3	19	M	CN	A	Cluster I
1099	SB0140	4 2 5 4 2 7 5 5 4 3 4 3 4 3	13	M	CN	A	Cluster I
5069	SB0140	4 2 5 4 2 7 5 5 4 3 4 3 4 3	38	F	CN	A	Cluster I
8013	SB0140	4 2 5 4 2 7 5 5 4 3 4 3 4 3	23	M	CN	A	Cluster I
4765	SB0140	4 2 5 3 2 7 5 5 4 3 4 3 4 3	59	F	GN	B	Cluster II
9607	SB0140	4 2 5 3 2 7 5 5 4 3 4 3 4 3	59	F	GB	C	Cluster II
3400	SB0140	4 2 5 3 3 7 5 5 4 3 4 3 4 3	57	F	GN	D	Unique profile
5868	SB0140	4 2 5 3 3 8 5 5 4 3 4 3 4 3	56	F	GB	E	Unique profile
4836	SB0140	4 3 5 4 2 7 5 5 4 3 4 3 4 3	59	F	GN	F	Unique profile

*GB: Gyeongbuk, CN: Chungnam, GN: Gyeongnam

the annual PPD tuberculin tests. In January 2013, a lesion was detected in a cow at the slaughterhouse, following which the entire herd (comprising 181 cattle) was quarantined. Among the cluster I group, 2 cattle (infected with the strains belonging to 3596 and 9570) appeared to have surfaced in 2008 in a herd from the Gyeongbuk province, while the others (infected with the strains belonging to 6679, 1099, 5069 and 8013) infected the animals born on the farm after 2010. This implied that the Cluster I strain was endemic to the farm and was the most likely the source of infection. The SB014-42542755434343 strain is responsible for the majority of infections in South Korea. Two cattle infected with the strains belonging to cluster II (4765 and 9067) were introduced in the herd in 2008. These cows had the same cluster despite originating from two different herds. Previously published spoligotyping and MIRU-VNTR data showed that TB likely spreads within a farm among the livestock (Zanardi *et al.*, 2013). Like in most other Korean farms, the cattle we studied were raised indoors. Molecular fingerprinting has also been previously used to demonstrate the persistence and spread of *M. bovis* within a herd (Perumaalla *et al.*, 1999).

CONCLUSION

To summarize, we showed the genetic diversity present in *M. bovis* isolates obtained from native Korean farm cattle. The outbreak of BTB in beef cattle has witnessed a steady increase in recent years (31 cases in 2007 as opposed to 254 in 2013; <http://kahis.nvrqs.go.kr/home/recsroom/selectLegalDissStats.do>). This may be attributable to the South Korean policy of BTB control being focused mainly on dairy cattle as opposed to beef cattle. Our study showed that BTB was transmitted to a herd by the introduction of animals belonging to a geographically separated location. Therefore, there is an urgent need to introduce a BTB control policy that includes mandatory pre-movement and annual skin tests of Korean beef cattle. These steps might prevent disease introduction in a TB-free herd.

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