

# Molecular Epidemiologic and Geographic Information System Analyses of *Mycobacterium bovis* Isolates from North America

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## Introduction

*Mycobacterium bovis*, the etiological agent of bovine tuberculosis has been reported in a wide variety of domestic animals and wildlife. In the United States, the bovine tuberculosis eradication program was launched in 1917 during a period when the prevalence of disease was estimated to be 5% in cattle and 15% in swine. By 1991, 41 states plus the Virgin Islands were accredited tuberculosis free. During the past ten years bovine tuberculosis in the state of Texas has been on the rise and Texas now harbors more than 50% of *M. bovis* infected U.S. cattle. Epidemiological causes of disease are presumed to include importation of infected animals, incomplete depopulation of infected herds, movement of tuberculosis exposed animals between herds and transmission from unidentified wild life reservoirs. Identification and differentiation of various strains using recently developed DNA marker techniques would provide a better understanding of the epidemiology of *M. bovis* infections and effective control of the disease. In the present study, *M. bovis* isolates originating from North American cattle, deer and other were fingerprinted using IS6110 and DR probes. The isolates were categorized into 85 distinct RFLP types based on a combination of individual fingerprint patterns. This method revealed that bovine tuberculosis cases in North America are caused by strains exhibiting different RFLP types. A significant proportion of bovine isolates harbored multiple IS6110 copies which is a characteristic feature of isolates originating from animals other than cattle.

## Materials and Methods

Two hundred and sixty-three bovine, cervid and other mammal isolates used in this study originated from USA (n=221), Mexico (n=29), and Canada (n=31). *M. bovis* isolates were cultured immediately upon receipt in 20 ml Middlebrook 7H9 broth (Difco laboratories) supplemented with OADC enrichment media (Difco) for 3 weeks at 37°C. *M. bovis* isolates were characterized on the basis of susceptibility to 5 µg/ml of thiophene carboxylic hydrazide (Sigma Chemical Co.) in Middlebrook 7H10 agarose media supplemented with OADC. Mycobacterial genomic DNA was extracted as described previously and two micrograms of genomic DNA from each isolate was digested with 10 units of either *Pvu*II (Boehringer Mannheim) for analysis with IS6110 probe or with *Alu*I (Promega) for analysis with the DR probe. Autoradiographs were developed in an automatic film processor (M35A X-OMAT; Kodak Eastman) and computer analysis of DNA patterns was performed using the BioImage Whole Band Analyzer, version 3.1 (Millipore Corporation, Ann Arbor, MI) on a Spark 10 workstation.

## Results & Discussion

### General results

Fingerprint analysis using IS6110 with *Pvu*II digested *M. bovis* genomic DNA revealed 16 different fingerprint patterns among 79 bovine. In contrast to previous observations, a significant proportion of the bovine isolates (15 of 79) examined in this study exhib-

ited multiple IS6110 copies (2-5 copies) represented by eleven different patterns. Two different IS6110 patterns were observed in the deer isolates originating from Montana and New York. Although a majority of bovine isolates in Texas and Mexico harbor a single IS6110, a significant proportion (19%) contain multiple copies of IS6110. Twenty-five distinct DR patterns were observed among seventy-nine bovine isolates of *AluI* digested *M. bovis* genomic DNA. In most cases fingerprinting using the DR probe further distinguished among the isolates exhibiting identical IS6110 type. For example, IS6110 type 1B isolates (n=38) exhibited eight different DR patterns. These data clearly demonstrate differences in the ability of the IS6110 and DR probes to categorize *M. bovis* isolates. The DR probe was generally superior to IS6110 in distinguishing among isolates which often contained a single copy of IS6110. Based on individual fingerprint patterns generated by both probes, 79 bovine isolates could be categorized into 30 different RFLP types. The deer isolates represented two additional RFLP types (types 19,24), and each of the three *M. tuberculosis* isolates represented distinct RFLP types (types 33-35). Among the 30 bovine *M. bovis* RFLP types, only nine are represented by more than a single isolate (clustered). Each of the remaining 21 types are represented by a single isolate (non-clustered). Although it is not surprising to see varied geographic distribution among non-clustered isolates, the broad distribution observed among clustered isolates may be associated with movement of cattle between herds. The largest cluster, RFLP type 7 is comprised of 24 bovine isolates originating from Mexico (n=17) and Texas (n=7). The Texas isolates originated from herds in Comanche (n=2) and in Clint (n=3), 450 miles apart. There has been no apparent contact between the Comanche and Clint herds which contain two different breeds of cattle, Jersey and Holstein, respectively. Thus, it must be assumed that RFLP type 7 is established in Texas. The remaining Texas isolates (n=2) originated from feed-lot animals at Dumas and San Angelo. The majority of the Mexican isolates (n=15) were recovered from animals in feed-lots dispersed throughout Texas, and were traced back to different herds located in geographically disperse regions of Mexico. The remaining isolates (n=2) were recovered from animals in Mexico. These data show that isolates belonging to RFLP type 7 are widely distributed in both Mexico and Texas, presumably as a result of active or historical movement of infected or carrier animals. At this point, the presence of infected Mexican cattle in Texas feedlots suggests that transmission of RFLP type 7 was caused in part by cattle importation. The existence of infection in herds not reportedly in contact with Mexican cattle suggests active foci also exist in Texas.

#### *RFLP analysis of clustered isolates*

The next largest cluster, RFLP type 3, is comprised of 13 isolates originating from Mexico (n=12) and Texas (n=1). Mexican bovine isolates (n=9) were recovered from Texas feed-lots which were traced back to herds located in different regions of Mexico. The remaining isolates (n=3) were recovered from animals in Mexico. Although a majority of isolates are widely dispersed, two isolates originated from a single herd in Cuatrocinegas, Coa. The lone Texas isolate was recovered from a feed-lot animal of unknown history. Based on the above observations it is likely that RFLP type 3 isolates were imported from Mexico. The lone Texas isolate presumably represents an animal from Mexico or is the result of transmission from such an animal.

#### *RFLP analysis of non-clustered isolates*

Each of the remaining bovine RFLP types are represented by a single isolate. The majority exhibited unique DR patterns, but shared the same limited number of IS6110 patterns as the clustered isolates. Among these isolates the DR probe was useful for discrimination according to geographic origin. For example, RFLP type 2 exhibited by an isolate from Mississippi shared IS6110 pattern (1A) with Texas and Mexican isolates, but exhibited a unique 'DR' pattern (dr9). Similarly a Kansas bovine isolate of RFLP type 15 shared IS6110 (1C) pattern with Texas and Mexican cattle but exhibited a unique 'DR' pattern (dr17). The observed similarity in IS6110 and DR patterns of deer isolates from New York and Montana suggest a close relationship, but could not be confirmed epidemiologically.

Isolates originating from deer and cattle were shown to contain multiple IS6110 copies. Evidence reported in this paper indicates limited distribution of such organisms and it has been suggested by us and others that such organisms may be attenuated for virulence in cattle. A wild-life reservoir in which these isolates retain virulence may represent a source of infection. In the past only isolates from antelopes, oryxes, monkeys, seals or goats have been shown to harbor multiple IS6110 copies. Potential reservoirs of *M. bovis* in Texas and Mexico include deer, feral pigs and havalina. Recent isolations of *M. bovis* from deer in Texas have confirmed this suspicion. Fingerprinting Texas deer isolates and surveying herds involving multiple IS6110 copy isolates would provide the necessary epidemiological information to establish an association between a particular RFLP type and severity of the lesions. RFLP types were geographically displayed by Global Positioning System coordinates to facilitate understanding the epidemiological basis for the distribution of distinct RFLP types.

## Summary

Seventy-nine *M. bovis* isolates recovered from Mexican and Texas cattle were categorized into 16 and 25 distinct types based on IS6110 and DR fingerprint patterns, respectively. By using a combination of both fingerprint patterns, 30 distinct RFLP types were defined. Fifty-eight of seventy-nine isolates (73%) were distributed among nine clusters. Clustered isolates were identified within herds, as well as in geographically disperse herds in Texas and Mexico. This observation is consistent with active transmission within herds and

among herds, presumably as a result of active or historical cattle movement. The majority of bovine isolates exhibit a single copy of IS6110 (64 of 79). Interestingly, in contrast to previous studies, a high percentage of bovine isolates (15 of 79) exhibited multiple IS6110 copies (2-5) distributed among eleven different RFLP types. It is speculated that transmission from non-cattle sources may be responsible. Continued fingerprinting of isolates originating from non-bovine sources and herd surveys is expected to provide useful information regarding the epidemiology of tuberculosis in this region.