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MOLECULAR GENETIC TOOLS FOR EPIDEMIOLOGIC STUDIES OF *MYCOBACTERIUM BOVIS* INFECTION

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L'absence de marqueurs permettant de différencier rapidement les souches de M. bovis entrave la découverte de la source d'infection et l'éradication. La confirmation du diagnostic d'infection à M. bovis dépend des cultures sur les lésions suspectes, cette technique est laborieuse et nécessite au moins deux mois. Le diagnostic moléculaire est plus rapide et peu coûteux pour détecter et identifier M. bovis. Pendant ces quatre dernières années, plusieurs essais utilisant les techniques moléculaires en vue d'une identification rapide de M. bovis ont été développées. Ces techniques reposaient sur les anticorps monoclonaux, l'hybridation d'ADN et la PCR suivie par l'utilisation d'une sonde spécifique de M. bovis. L'objectif général de cette étude était de développer la génétique moléculaire comme outil d'identification des souches de M. bovis directement à partir des lésions tuberculeuses. Le but était également de tisser des liens entre la recherche et l'application au diagnostic par les praticiens. Nous avons utilisé notre système de PCR actuel pour diagnostiquer directement la tuberculose bovine en détectant M. bovis dans les lésions tuberculeuses. Nous avons adapté « Sjobring PCR » qui a donné de bons résultats lors de son application expérimentale sur des tissus. Nous avons aussi développé l'extraction d'ADN de M. bovis à partir de tissus sains mais associés expérimentalement avec des cellules de M. bovis. Nous avons pu retrouver l'ADN de M. bovis dans ces tissus à un niveau minimum de 10 cellules/mg de tissu.

INTRODUCTION

Mycobacterium bovis is the causative agent of bovine tuberculosis. The pathogen causes losses in production of beef and dairy cattle and economic losses due to import and export restrictions in the cattle industry. *M. bovis* is also transmissible to humans. People working with animals or animal products in the dairy industry and in slaughter houses are especially at risk. Bovine tuberculosis remains a major problem in the livestock industry despite an extensive eradication program in the USA that began in 1917. The lack of a quick, accurate method for identification of infected animals, and an absence of markers to rapidly differentiate *M. bovis* strains have hampered efforts for total eradication of bovine tuberculosis. Current confirmation of *M. bovis* diagnosis is accomplished by culture of suspected lesions. This process is laborious and time consuming, usually requiring at least 2 months from the time of initiation of cultures until final identification is complete. Diagnosis based on molecular techniques such as Polymerase Chain Reaction (PCR) can provide quicker, less expensive and less labor intensive tools for *M. bovis* epidemiology.

During the last four years, molecular techniques have been developed for both diagnosis and epidemiological investigation of *M. bovis* infection in animal populations. Molecular methods for the rapid identification of *M. bovis* have concentrated on DNA hybridization, *M. bovis*-specific monoclonal antibodies (10), and by PCR followed by use of *M. bovis*-specific probe on the amplicon (1). Outside of the United States, progress in genetic fingerprinting of *M. bovis* for epidemiological studies has focused on restriction enzyme analysis (REA) of whole *M. bovis* genomic DNA, and restriction fragment length polymorphism (RFLP) analysis with insertion sequence probes. REA has been very useful in tracing the origin of *M. bovis* infection, and does not appear to be influenced by the host species (7, 5). RFLP analysis with a probe specific for the insertion sequence (IS) 1081 revealed very little variation among 160 *M. bovis* strains representing 95 REA genotypes, and a probe specific for IS6110 identified only 15 variants among the 95 REA types (2). While the above mentioned techniques (REA and RFLP) are sensitive detection tools, they are limited in their clinical and epidemiological application. All of these techniques require working with isolates grown to high titer. This is a very slow and labor intensive process. We emphasize that these techniques cannot be used in the direct diagnosis of *M. bovis* in suspected lesions. Quick and reliable molecular techniques are needed to identify and differentiate among *M. bovis* strains. Furthermore, there is only a limited molecular program for investigating bovine tuberculosis outbreaks in the United States. Our ability to accurately and quickly define points of origin of new outbreaks or chronic infections in the USA are limited due to the lack of genetic molecular applications. The purpose of this presentation is to describe our on going study to utilize PCR to detect *M. bovis* directly from formalin treated infected tissues and/or tuberculosis suspected lesions. Laboratory procedures and preliminarily findings are presented.

MATERIALS AND METHODS

Sjobring procedure (9) was used in the preparation of the *M. bovis*-specific primer sequences. This PCR amplifies a 419 bp segment of the protein antigen b (Pab) coding region of *M. bovis*. Although this PCR may sometimes amplify a 490 bp product in *M. avium* and *M. paratuberculosis*, they are easily distinguished from *M. bovis*, due to the distinct size difference. A 419 bp segment of Pab in *M. tuberculosis* is also amplified, but due to the fact that *M. tuberculosis* is rarely a cause of tuberculosis in ungulates, this lack of differentiation of *M. bovis* and *M. tuberculosis* is not perceived to be a problem. This technique has been 100% specific and sensitive in detecting cultures of *M.*

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bovis, and differentiating those cultures from *M. avium* and *M. paratuberculosis*. Following identification of *M. bovis* as indicated above, we can differentiate *M. bovis* from *M. tuberculosis* by utilizing primers which yield a 396 bp product for *M. tuberculosis* but no product for *M. bovis* (3). If we receive lesions without confirmation of the presence of *M. bovis*, then both *M. bovis* and *M. tuberculosis* PCR will be performed. Tissue samples from the current active USDA - surveillance system were obtained from the National Veterinary Service Laboratory (NVSL). For bio-safety reasons, only inactivated tissue samples that are treated with 10% formalin (or other procedures for inactivation) are accepted in our laboratories. We have optimized the methods of Sjobring and others (9,4) with the some specific modifications that are available from the senior author upon request. Template (amplicon from Sjobring PCR) is added to the reaction mixture, placed into a thermocycler (MJ Research Minicycler). Trials using the above PCR's for detection of *M. bovis* in both experimentally spiked and naturally infected formalin fixed tissues are in preliminary stages. At this point, the effect of various fixation times on PCR efficiency (specificity and sensitivity) are under studied.

RESULTS AND DISCUSSION

We compared several tissue processing methods to obtain maximum sensitivity. The problems associated with performing PCR on tissues were overcome with the use of nested PCRs, conducted by reamplification of Sjobring PCR products with a second set of primers that target complementary sequences interior to the original amplified product. The potential advantages of nested PCR are significant: The sensitivity can be very high, exhibiting the potential to detect single copies of the target and eliminating the need for detection with probes; the specificity of the first PCR product can be verified, and due to the dilution of inhibitors that might have reduced efficiency in the first PCR performance can be improved (8). We feel that these considerations render the nested PCR a prime candidate for increasing sensitivity for detection of *M. bovis* from tissue. Miyazaki, et al. (6) reported the development of a nested PCR system that aids in the detection of *M. tuberculosis* (and *M. bovis*). The use of this nested PCR increased sensitivity 100 fold, and is able to detect 0.1 - 1 cultured cells. Neither *M. avium* nor *M. paratuberculosis* amplify with the nested PCR. We have also adapted this PCR in detecting *M. bovis* from experimentally spiked tissues. By utilizing the same tissue processing methods and nested PCR we are able to detect 10^3 - 10^4 cells/g in experimentally spiked tissue, and the need to perform a series of dilutions is unnecessary. Preliminary studies have assessed the feasibility of detection of M. bovis from formalin fixed tissues. We performed extractions of M. bovis DNA from uninfected formalin fixed tissues that were experimentally spiked with M. bovis cells. Methodology employed thus far involves a 24 hour fixation in formalin, removal of formalin via evaporation or a diffusion procedure, and extraction of DNA. We have amplified M. bovis DNA from these tissues (10 cells/mg tissue). Next, the same procedure was performed using formalin fixed tissue experimentally infected with formalin fixed M. bovis cells. In this case, we were able to amplify DNA from 10^2 cells. An inherent problem associated with using nested PCR procedures is the risk for contamination by aerosolized amplified DNA when transferring amplification products to nested PCR reaction tubes. In order to decrease the risk of false positive interpretations we include several blank reactions at various intervals of primary and nested PCR's. Once optimal sensitivity and specificity have been ascertained, we will attempt to detect M. bovis DNA from several known naturally infected and uninfected formalin fixed tissues. Although our exact methodology as such is still being explored and developed, we have indicating already succeeded in detecting M. bovis DNA from naturally infected formalin fixed tissue that our approach is feasible and clinically applicable.

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