Evaluation of the BACTEC® MGIT™ 960 system for recovery of Mycobacterium bovis

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GENERAL INTRODUCTION

Introduction

Bovine tuberculosis is a significant disease caused by *Mycobacterium bovis*. There are numerous clinical signs including enlarged lymph nodes, weakness, anorexia, low grade fever and emaciation associated with this disease. Some animals do not display clinical signs but harbor the organism and then are able to spread the disease to others. A variety of animals including humans are susceptible to this disease. Because of the wide range of animals that are susceptible to tuberculosis, outbreaks of the disease are a concern in the United States. An eradication program is currently in effect to contain the disease when it occurs and its prevent spread to other animals (OIE, 2004).

Rapid detection is important to facilitate the diagnosis of the disease. Although some mycobacteria species can be detected using rapid molecular methods, *M. bovis* is usually diagnosed using culture methods. A combination of liquid culture (BACTEC™ 460 TB system) and solid media are accepted as the “gold standard” for culturing a variety of mycobacteria species (Salfinger et al. 1994; Rose et al. 1995). However the BACTEC™ 460 system has a number of disadvantages such as a risk of exposure to radioactive material, risk of needle stick injury, potential risk of cross contamination, is very labor intensive and lacks computerized data management. The BACTEC™ Mycobacteria Growth Indicator Tube System (BACTEC™ MGIT™ 960 system) is a recently developed, non-radiometric system designed for rapid recovery of mycobacteria from clinical specimens (Becton Dickinson, 2003).

Several studies indicate that the MGIT™ 960 system is a reliable replacement for the BACTEC™ 460 system for recovering *Mycobacterium tuberculosis*. Although some of the growth requirements vary between mycobacteria species, liquid culture media has been very successful for recovering both *M. tuberculosis* and *M. bovis*. The results of these comparative studies show this new culture method is comparable to the radiometric BACTEC™ 460

**Thesis Organization**

This thesis begins with a general introduction and literature review of *Mycobacterium bovis*. This is followed by a paper organized according to the publication format for the Journal of Veterinary Diagnostic Investigation. The paper is followed by general conclusions and acknowledgements. The research paper will be submitted to the Journal of Veterinary Diagnostic Investigation.
LITERATURE REVIEW

Discovery of *Mycobacterium bovis*

Tuberculosis is an infectious communicable disease that has affected humans and animals since ancient times. It is thought to have caused disease before the time of Jesus. Some Bible passages, such as the “wen or scurvy” of cattle mentioned in Leviticus 22:22, are believed to refer to tuberculosis (Collins et al. 1983). Prehistoric skeletal remains from the Neolithic period have also shown evidence of the disease. Tuberculosis can affect many different organs of the body and even the bones. For this reason, it has been referred to by numerous names throughout the centuries, making it difficult to be sure the diseases mentioned in old texts were actually tuberculosis. These names include “Phthisis”, “scrofula”, “the white plague”, “wasting disease” and “consumption”. Consumption is mentioned many times in the Bible and is translated as the modern Hebrew word for tuberculosis. The name tuberculosis emerged in the 19th century to describe diseases resulting in tubercles (Waksman, 1964). Tubercles are described as lesions or nodules attached to organs or bones usually resulting from infection with tubercle bacillus. Currently any disease caused by tubercle bacillus is referred to as tuberculosis, regardless of whether tubercles are involved (Myers, 1969; OIE, 2004).

In ancient times, it was thought that those who did not develop immunity to the disease would die and a general resistance by the population to tuberculosis would develop. It was unknown until a little over a century ago that tuberculosis was transmissible. In 1865, Jean-Antoine Villemin demonstrated that tuberculosis should be classified as a communicable disease along with smallpox, syphilis, scarlet fever and glanders. He, along with other scientists, believed tuberculosis was caused by a virus. They also concluded that phthisis, scrofula and consumption could all be classified as tuberculosis (Waksman, 1964). It wasn’t until 1882 that *Mycobacterium bovis*, the bacterium that causes tuberculosis in humans and animals, was successfully cultured by Robert Koch. Koch simply named it
Tuberkelbacillen meaning bacilli of tuberculosis. The following year it was renamed *Bacterium tuberculosis* by W. Zopf and in 1896 it was assigned to the Genus *Mycobacterium* by K. B. Lehmann and R. Neumann. Koch initially assumed the bacterium causing bovine tuberculosis did not affect humans and the bacterium causing human tuberculosis did not affect bovine species. He discovered his theory was wrong when he attempted to produce a bovine vaccine using human tuberculosis attenuated by aging (Collins et al. 1983; Waksman, 1964). Virulent human tuberculosis was secreted in the milk of the vaccinated cow (Griffith et al. 1913). It was then believed that the tubercle bacilli that caused tuberculosis in birds, humans and bovine animals was one species named *Mycobacterium tuberculosis* (Collins et al. 1983). In 1898, T. Smith determined avian tubercle bacilli were separate from the mammalian tubercle bacilli group. In a series of reports published in 1904-1911 by the Royal Commission of Tuberculosis, it was determined that there are three distinct infectious species of mycobacteria including *M. tuberculosis*, *M. bovis* and *Mycobacterium avium*. Following the reports by the Royal Commission on Tuberculosis, bovine strains were referred to as *M. bovis*, but were not published as such until 1970 (Collins et al. 1983; Karlson et al. 1970).

### Prevalence in the U.S.

The prevalence of bovine tuberculosis was so high in the early part of the 20th century that it caused more loss of livestock than all other diseases combined (USDA, 2005). The severity of this disease led to the establishment of the National Cooperative State/Federal Bovine Tuberculosis Eradication Program in 1917, which involved testing all cattle. A positive reaction to the tuberculin skin test, indicated that the animal had been exposed to one or more types of mycobacteria (Thoen et al. 1988). The entire herd was quarantined and those that reacted to the tuberculin test were slaughtered. Infected premises were cleaned and disinfected after the cattle were taken away. Any animal coming into a clean herd was tested before it was allowed to join the herd. The program was then able to assign every state to a status based on its freedom from tuberculosis in cattle and bison, the effectiveness of the
state’s tuberculosis eradication program and the degree of the state’s compliance with the standards for cattle and bison contained in the Uniform Methods and Rules – Bovine Tuberculosis Eradication regulations. These status designations are the following: Accredited-Free, Modified Accredited Advanced, Modified Accredited, Accredited Preparatory or Nonaccredited (USDA, 2005). This program has resulted in the reduction of the reactor rate from 5% in the early 1900’s to 0.0011% today (Beals, 2004). A surveillance program is currently in use throughout the nation where state and federal meat inspectors check lymph nodes and organs of carcasses in cattle slaughter houses for signs of tuberculosis. When lesions are observed, the tissues are sent to the National Veterinary Services Laboratories in Ames, IA to determine if \(M. \text{bovis}\) is present. Positive samples are reported and the cattle are traced back to the herd where they last came from and those animals are tuberculin tested and slaughtered if positive or quarantined until it is determined that the herd is free of \(M. \text{bovis}\) (USDA, 2005).

Currently every state except Texas, California and Michigan has Accredited-Free Status (Beals, 2004). Texas and California have Modified Accredited Advanced, and in April 2004 Michigan was granted Split State Tuberculosis Status (eleven counties are classified as Modified Accredited and the rest are classified as Modified Accredited Advanced) (USDA, 2005). To reach Accredited Free Status a state must be free of confirmed cases of bovine tuberculosis for five years and must have rules and surveillance programs in place as well as accurate records of cattle transfer (Beals, 2004; USDA, 2005).

**Transmission**

The primary route of transmission in cattle is via the respiratory tract, thus animals in confined areas are at an increased risk of infection. Other modes of transmission include congenital and sexual transmission, and milk consumption (Collins et al. 1983; Thoen, 1995). It is also possible to spread the organism via saliva or other bodily fluids that contaminate a watering place shared by infected and non-infected animals. Many types of animals besides
cattle can become infected with *M. bovis*. These animals include badgers, nonhuman primates, opossums, swine, bison, deer, elk, llamas, ferrets, bear, raccoons, coyotes, dogs, cats and some exotic animals (Thoen, 1984; Whipple, 2000).

**Pathogenesis**

The exact pathogenesis of tuberculosis in cattle is unclear as several components are involved in the evolution from initial infection to establishment of the disease. Inhalation of the organism into the lungs is understandably where infection begins. From there it is carried to the small air passages triggering a delayed type hypersensitivity (DTH) response in previously exposed individuals (Thoen et al. 1988; Thoen, 1995). A DTH response is mediated by T-cell lymphocytes which become activated by macrophages infected with the organisms. These T-cells respond by releasing cytokines (secreted substances) into the infected area (Thoen, 1986). These cytokines attract mononuclear cells (monocytes) in the blood to the sites where the organism is present. As soon as the monocytes arrive at the infected area they are activated and become macrophages. These macrophages carry the organism to the lymph nodes and parenchyma of the lungs. Once there, the macrophage ingests and attempts to kill the organism by fusion of the phagosome and lysosome. This fusion results in the release of hydrolytic enzymes which usually destroy the organism (Rook et al. 1983).

Virulent tubercle bacilli can resist killing by activated macrophages through a combination of toxic lipids and other factors that disrupt phagolysosome formation, cause release of hydrolytic enzymes from unfused lysosomes, or inactivation of lysosomal enzymes. The bacilli may also escape from the phagolysosome into the cytoplasm via vacuoles. Some of the killing mechanisms of the phagocytes, such as reactive oxygen radicals, may be useless against virulent tubercle bacilli. The organisms can even be protected against bactericidal components in the serum when ingested by the phagocytes.
When inside the phagocytes, the virulent tubercle bacilli are able to multiply and escape to infect more phagocytes (Thoen et al. 1988; Thoen, 1995; Tizard, 1992).

Accumulation of large numbers of phagocytes containing the organism results in formation of a granuloma or tubercle. Granulomas are a solid grouping of inflammatory cells that come together in an attempt to wall off the bacteria and prevent the organism from spreading to other areas of the body. The cell mediated immune response begins about 10 to 14 days after infection or around the time the granuloma starts to develop. T-cells release cytokines that attract, immobilize and activate more mononuclear cells to the site of the granuloma. Accumulation of additional mononuclear cells can result in cellular hypersensitivity. This can lead to caseous necrosis caused by cell death and tissue destruction (Thoen, 1995; Tizard, 1992).

The cell mediated immune response causes the release of strong enzymes that can break down lipids and proteins in the surrounding tissue cells. Tissue cavities may form in the infected area due to cellular degradation. Sometimes these tissue cavities can rupture and release the organism into the bronchi of the lungs. Transmission of the organism to other animals can result when the tubercle bacilli are aerosolized from the bronchi through respiration and sneezing. Granulomas may develop in many different lymph nodes including those draining the lung, liver, spleen, kidney and uterus. The thoracic lymph nodes are most frequently infected because activated mononuclear cells containing the organisms travel through the lymphatic vessels and are carried to the bronchial or mediastinal thoracic lymph nodes (Thoen et al. 1981; Tizard, 1992).

The virulence factors of \textit{M. bovis} contribute to its ability to cause disease. Lipids within the cell wall comprise a group of virulence factors. One of these lipids is the cord factor, a glycolipid that induces swelling of mitochondria in the liver cells. This swelling results in reduction of respiratory and phosphorylative activity. Liver cells are also disrupted by the cord factor through collapse of the rough endoplasmic reticulum and disconnection of
the ribosomes in these cells. Sulfolipids in the cell wall are also included in the group of lipid virulence factors. These sulfolipids are important in establishment of infection by preventing phagolysosome formation in activated macrophages and reducing phosphorylative oxidation in mitochondria. \textit{M. bovis} also has virulence factors in the form of secreted proteins. These proteins are part of the antigen 85 complex which binds fibronectin and can prevent it from binding to the bacterial cell. This can lead to a reduction in phagocytosis because fibronectin is thought to play a role in complement fixation (Thoen, 1995).

Virulent mycobacteria can also produce heat shock proteins such as superoxide dismutase. This protein may reduce the effect of toxic oxygen radicals in macrophages but this is unclear because the effects of heat shock proteins are still under investigation. The overall progression of tuberculosis in the host is dependent on the interaction between the host’s immune responses and the virulence of the bacteria for that particular host (Thoen, 1995).

**Clinical Signs**

Because of the slow multiplication rate of \textit{M. bovis} (about once every 20 hours), the disease progression is very slow and may take months to develop (Thoen, 1984). For this reason the disease is chronic and rarely evident until it reaches an advanced stage. The extent of disease progression and location of infection determine what clinical signs will be present. Swollen lymph nodes are evidence of infection, but this symptom is not limited to mycobacterial infections. Visible signs of infection with \textit{M. bovis} include weakness, anorexia, dyspnea, low grade fever and emaciation. The most common clinical indication of tuberculosis is chronic wasting which is independent of the nutritional quality of the daily feed (Thoen, 1984; Thoen, 1995).

**Diagnosis**

\textit{Skin Test}. Skin tests are frequently administered to animals suspected of a tuberculosis infection. The antigen for the test is a purified protein derivative (PPD) made
from tubercle bacilli, *M. bovis*. The PPD is inoculated intradermally in the caudal folds at the base of the tail (intradermal injection is 1mg/ml) or in the skin around the cervical region (cervical PPD is 2mg/ml) (USDA, 2005). Infected animals react to the skin test with a delayed type hypersensitivity (DTH) response. If swelling occurs around the injection site 72 hours following the injection, it is considered to be a positive test result. This DTH is a local inflammatory reaction that can indicate an animal may be infected with *M. bovis*. This immune response can also occur when an animal is tested multiple times and develops an allergy to the PPD (Thoen, 1995; USDA, 2005). Allergic reactions also occur when animals have been sensitized with *M. bovis* BCG vaccine (Cole, 2005).

When testing cattle, 1mg/ml of PPD tuberculin is used for an intradermal tuberculin skin test. *M. bovis* and *M. tuberculosis* can be used interchangeably for the tuberculin test because animals infected with mammalian tuberculosis react equally to PPD prepared from either organism. Most countries now use *M. bovis* production strain AN5 to prepare their PPD for cattle (OIE, 2004). Purified protein derivative tuberculins can be used to differentiate between infections with mammalian tuberculosis and other mycobacteria using a comparative cervical test. This test consists of injection of *M. avium* PPD at 0.4mg/ml and *M. bovis* PPD at 1mg/ml tuberculin on separate locations around the cervical area. The swelling that results after 72 hours is measured and can indicate which mycobacteria species may be causing the disease (OIE, 2004; Thoen, 1995; USDA, 2005).

**Pathology.** A presumptive diagnosis can be made by veterinarians and meat inspectors during necropsy or at the slaughter houses. Tuberculosis lesions are often yellowish, caseous necrotic areas on white to light gray fibrous tissue. Some lesions are dry, others are liquid filled and some contain calcification. Mycobacteriologic examinations are performed on tissues or sputum samples (Smith, 1990; Thoen, 1995). Histopathologic examinations can be completed within 1-2 days while bacterial examinations take 4-8 weeks or longer (Miller et al. 2002).
The National Veterinary Services Laboratories (NVSL) is the national reference laboratory for *M. bovis* (NVSL, 1992; USDA, 2005). At the NVSL, most diagnostic samples are submitted from slaughter plants and contain a lesion which is divided into two parts for testing. One portion of the lesion is sent to histopathology while the other is sent to the bacteriology laboratory. Tissues are fixed in 10% buffered formalin and are sent to the histopathology laboratory. Lesions are selected and thin portions are placed into a cassette. The cassette is demineralized, dehydrated and made into a paraffin block. Sections from the paraffin block are stained using hematoxylin and eosin stains for microscopic examination. *M. bovis* lesions usually appear with a center of caseous necrosis surrounded by epithelioid cells, lymphocytes and giant cells. Fibrous connective tissue encapsulates the necrotic area which attempts to limit the spread of the infection. A separate section cut from the paraffin block is mounted on a glass slide and stained using the Ziehl-Neelsen acid-fast method to detect acid-fast bacilli. The observation of a typical *M. bovis* lesion results in a diagnosis consistent with a mycobacteriosis infection. This diagnosis is reported as “mycobacteriosis compatible”. Once this diagnosis has been made PCR analysis is performed on the sample using primers for IS6110 to identify *M. tuberculosis* complex species (Miller et al. 2002; NVSL, 1992).

Isolation

Pieces of tissue with lesions are also submitted to the bacteriology laboratory in saturated aqueous sodium borate (NVSL, 1992). Many samples are contaminated by other bacteria that grow more rapidly than mycobacteria; therefore, treatment of the samples is necessary. There is no reagent that will destroy all contaminating bacteria and fungi without reducing the growth of mycobacteria (Collins et al. 2004; Thoen, 1995).

Culture Media. The gold standard for isolation of *M. bovis* includes growth on Middlebrook 7H10 with sodium pyruvate, Middlebrook 7H11 with sodium pyruvate, as well as a liquid culture medium (usually BACTEC™ 460 medium). This combination of culture
media has been used for many years and has been proven to be an effective method of isolating mycobacteria (NVSL, 1992).

**Solid media.** Various types of solid media have been used throughout the years. Most types are variations of those described by Löwenstein and modified by Jensen, including BBL Mycobactosel Löwenstein-Jensen (LJ) media. These solid media contain coagulated egg base, phosphate buffer, magnesium salts and asparagines or other amino acids and starch (Difco, 1998). The original media used to grow *M. bovis* contained glycerol. Glycerol was later found to promote growth of *M. tuberculosis* but inhibit growth of *M. bovis* (Thoen, 1995). Sodium pyruvate was more conducive to growing *M. bovis* and eventually replaced glycerol. Solid media prepared according to Middlebrook & Cohn has an agar base instead of an egg base. Two types of Middlebrook media commonly used for mycobacteria isolation are Middlebrook 7H10 and Middlebrook 7H11. Both 7H10 and 7H11 solid media are available with 2% glycerol or 0.2% pyruvic acid. These agar-based media are recommended for primary isolation because colonies may be visible after 10-12 days since the media is transparent. Middlebrook 7H10 media contains malachite green which is beneficial for primary isolation. L-asparagine or potassium aspartate is also added to the 7H10 media to aid in niacin production for biochemical testing. Middlebrook 7H11 media is a modified version of 7H10 media with enzymatic hydrolysate of casein added to improve in recovery of fastidious organisms (Thoen, 1995).

Mycobacteria require a 4-8 week incubation period or longer at 37 ± 2°C. Humidity control can be used to prevent the cultures from drying out during the long incubation period. Mycobacteria are aerobic but grow best in the presence of 10% CO₂ (NVSL, 1992).

**Liquid Systems.** Liquid media have proven to be beneficial in isolating *M. bovis*. Many laboratories have used the BACTEC™ 460 system for the growth of *M. tuberculosis* complex (Cruciani et al. 2004; Hanna et al. 1999; Huang et al. 2001; Rohner et al. 2000; Somoskovi et al. 2000). This system includes the BACTEC™ 460 12B medium, BACTEC™
460 radiometric instrument, PANTA™ antimicrobial mixture and reconstituting fluid. The BACTEC™ 460 medium contains a $^{14}\text{C}$ substrate that is metabolically converted to radio-labeled by-products by \textit{M. bovis}. These radioactive by-products are then detected by the BACTEC™ 460 instrument through needles inserted into the septum of each vial to determine the growth rate. Growth rate and quantity of mycobacteria in the BACTEC™ 460 vials are directly related to the rate and amount of $^{14}\text{C}$ that is released by the bacteria. This radioactive culture method has been valuable for culturing \textit{M. bovis} since it grows easily in the rich BACTEC™ 460 7H12 broth. As long as contaminating bacteria are not present, mycobacteria are able to grow and spread throughout the liquid medium. This is a more favorable environment for replication than solid media because on solid media the bacteria are limited to the area on which they were inoculated (Isenberg, 2004; Siddiqi, 1996).

Some disadvantages of the BACTEC™ 460 system include the following: time to detection of mycobacteria is dependent on the number of organisms, specimen quality, collection method and processing method; the system is labor intensive since the vials must be physically placed in the instrument one to two times a week and the results must be manually recorded by a technician; inoculation on solid media is required to determine colony morphology and pigmentation. Another disadvantage is that the BACTEC™ 460 instrument does not distinguish between mycobacteria and contaminating bacteria which may then result in an indication that a vial is positive when bacterial growth is due to contaminants. Mixed mycobacteria cultures can also be a problem because fast growing mycobacteria are detected earlier than those with slow growth properties. This may prevent the slower growing pathogens from being detected. It is necessary to subculture the positive vials onto solid media to separate mixed mycobacteria cultures (Siddiqi, 1996).

The BACTEC™ 460 system which is currently used in many laboratories, is a radiometric liquid culture method (Siddiqi, 1996). Radioactivity must be monitored in each laboratory monthly and a supervisor is required to be licensed by the Radiological Safety
Staff (RSS). Records of radioactivity readings and the amount of radioactive material stored in the lab must be properly maintained for review by the RSS. Ordering and disposal of radioactive material is time consuming and tedious. Technician time is also a significant factor since it engages one technician up to three days each week.

The MGIT™ 960 liquid growth system has been successfully used to grow *M. tuberculosis* complex. Some laboratories interested in the recovery of *M. tuberculosis* have conducted studies comparing the MGIT™ 960 system with their current culture methods and have found it to be comparable (Cruciani et al. 2004; Hanna et al. 1999; Huang et al. 2001; Rohner et al. 2000; Somoskovi et al. 2000).

**Identification**

**Direct Microscopic Examination.** Microscopic examination is one of the most rapid and inexpensive methods of initial diagnosis of mycobacterial infections. Mycobacteria are difficult to stain with traditional gram stains as the dyes do not permeate the mycobacterial cell walls. The cell walls can appear to be invisible, have clear zones, or appear as beaded gram-positive rods when the gram stain is used. Large amounts of lipid (mycolic acid) in the cell wall prevent the stain from entering and remaining in the cell wall. When growth appears on solid media or liquid media, smears are made to determine the colony morphology of the bacteria. Mycobacteria are stained using a carbol fuchsin stain (Ziehl-Neelsen or Kinyoun) or a fluorochrome stain (auramine O or auromine rhodamine). When using the Ziehl-Neelsen acid-fast stain, *M. bovis* appears as a slender acid-fast positive rod about 1 to 10 µm long. Usually the cells look slightly bent and in liquid medium *M. bovis* exhibits serpentine cording (Isenberg, 2004).

The Ziehl-Neelsen acid-fast stain is the most commonly used carbol fuchsin stain (Isenberg, 2004; NVSL, 1992; Thoen, 1995). The mycolic acids retain the carbol fuchsin stain that is applied first. Phenol in the carbol fuchsin stain facilitates diffusion of the stain into the cells. Applying heat during the staining procedure also promotes uptake of the stain.
The mycolic acids form stable complexes with the fuchsin dye which prevents decolorization when 3% acid alcohol is applied. The resistance of the cells to decolorization is essential for the bacteria to be termed acid-fast. Other cells that may be present on the slide are easily decolorized by the acid alcohol. Brilliant Green is often used as a counter-stain for the Ziehl-Neelsen acid-fast method. Mycobacteria are observed using a 100 x oil immersion objective. The mycobacteria are red (acid-fast positive) and non-acid-fast bacteria are green (Isenberg, 2004; NVSL, 1992; Thoen, 1995).

Acid-fast staining does not distinguish *M. tuberculosis* or *M. bovis* from non-tuberculosis mycobacteria. Non-mycobacteria acid-fast organisms include *Nocardia, Rhodococcus, Legionella micdadei, Cryptosporidium* species and *Isospora* species (Isenberg, 2004). Acid-fast stains are not sensitive and rapidly growing mycobacteria are variably acid-fast. The overall sensitivity of the Ziehl-Neelsen method ranges from 50% to 80% (Murray et al. 2003). Despite the variable sensitivity rate, this method is used to determine colony morphology for initial bacteria identification. Microscopic examination cannot be used to precisely identify mycobacterial species therefore biochemical tests and nucleic acid probes are necessary.

**Biochemical Tests.** Niacin, nitrate and pyrazinamidase (PZA) biochemical tests are used in addition to colony morphology and acid-fast staining for confirming the identification of mycobacteria. Niacin and nitrate biochemical tests are used to distinguish *M. bovis* from *M. tuberculosis*. The niacin accumulation test is used because all actively growing mycobacteria produce nicotinic acid. Reagents are added to these cultures and a niacin strip is inserted to detect niacin production (Isenberg, 2004; NVSL, 1992). *M. tuberculosis* is one of the few mycobacteria species that does not further metabolize nicotinic acid. While the niacin test is used to distinguish *M. tuberculosis* from *M. bovis*, it should not be used alone as the sole determining test. *M. bovis* is classified as niacin negative but 4% of *M. bovis* isolates are niacin positive (Isenberg, 2004).
Nitrate reduction biochemical tests should be used in conjunction with niacin accumulation tests. *M. tuberculosis* and *M. bovis* differ in their ability to reduce nitrate. *M. tuberculosis* is able to reduce nitrates while *M. bovis* is unable to reduce nitrates. This can be detected by growing the bacteria in nitrate substrate broth and adding reagents to test for a color change indicating positive results (Isenberg, 2004).

The PZA biochemical test can also be used to differentiate *M. tuberculosis* and *M. bovis*. Some mycobacteria have the pyrazinamidase enzyme that deaminates PZA to pyrazinoic acid and ammonia. These can be detected by addition of ferrous ammonia sulfate. *M. bovis* is negative for the enzyme and does not deaminate PZA. This test was not used in this study because niacin and nitrate tests were sufficient for differentiation. Nucleic acid probes are able to assign the isolate to the *M. tuberculosis* complex and *M. bovis* is the only mycobacteria species from this group that primarily infects cattle. Since *M. bovis* is negative for nitrate reduction and niacin production, but *M. tuberculosis* is positive for both tests, these are the only biochemical tests needed to determine the species level (Isenberg, 2004).

**Molecular Techniques**

The Gen-Probe® AccuProbe system is a nucleic acid hybridization assay. This assay depends on the ability of complementary nucleic acid strands of the DNA probe and the ribosomal RNA from the organism to join together to form double stranded complexes. This method is used on acid-fast positive cultures to determine whether the organism is included in the *M. tuberculosis* complex. The *M. tuberculosis* complex includes *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *Mycobacterium africanum*, *Mycobacterium microti* and *Mycobacterium canetti* (Isenberg, 2004; Richter et al. 2004).

Advantages of the AccuProbe system include rapid turn around time for classification of the *M. tuberculosis* complex. The mycobacteria do not need to be viable, since the test only requires a specific RNA sequence from the organism. The AccuProbe system is a valuable tool to identify mycobacteria species in diagnostic laboratories. One of the major
disadvantages is that growth of mycobacteria on solid or liquid culture media is required for this test because it cannot detect the organism in clinical samples. The AccuProbe system should not be used as the sole system of identification. Other laboratory methods are needed to correctly identify the type of mycobacteria present in the culture. The *M. tuberculosis* complex test kit cannot distinguish between different species within the *M. tuberculosis* complex. It is best to rerun the test when the results are around 30,000 RLU’s (relative light units) because these results are considered weak positives. Contaminating bacteria can occasionally affect the AccuProbe results. Results need to be noticeably greater than 30,000 RLU’s to ensure the cultures are true positives (Alcaide et al. 2000; Somoskovi et al. 2003).

Spoligotyping is another DNA based method that utilizes the variation in spacer sequences in the DNA to identify mycobacteria species. This method focuses on the DNA polymorphism located in the DR locus. Multiple copies of a specific sequence interspersed with unique spacer sequences are found in this locus which are easy to identify using spacer oligotyping (spoligotyping) (Riley, 2004).

Mycobacterial diseases are difficult to diagnose and multiple tests are required to identify the species level. This is a problem for bovine tuberculosis because *M. bovis* has a slow replication rate which can delay diagnosis of the disease. Culture methods can impact the time to diagnosis because it can take up to 8 weeks to recover an isolate from a sample. Species identification tests can require days to weeks for final results. A rapid culture method would be beneficial to reduce the time to diagnosis by quickly recovering isolates which can then be identified more quickly by molecular methods.
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EVALUATION OF THE BACTEC™ MGIT™ 960 SYSTEM FOR RECOVERY OF MYCOBACTERIUM BOVIS


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Abstract

The BACTEC™ MGIT™ 960 system was evaluated to determine how it compares to the BACTEC™ 460 radiometric system for recovery of Mycobacterium bovis from tissue samples. Five hundred and six bovine lymph node samples were collected from 21 abattoirs in the United States and three in Mexico between November 2003 and September 2004. Processed samples were inoculated into a MGIT™ tube, BACTEC™ 460 vial, Middlebrook 7H10 and Middlebrook 7H11 solid media. The MGIT™ tubes were inserted into the MGIT™ 960 instrument for incubation and testing. The BACTEC™ 460 vials and MGIT™ tubes were incubated for six weeks (or until positive). Solid media tubes were incubated up to 8 weeks. Ziehl-Neelsen slides were prepared for each type of media to check for contaminants and confirm growth of acid-fast positive rods. Samples containing acid-fast rods were confirmed as members of the M. tuberculosis complex by a nucleic acid assay. Niacin and nitrate biochemical tests were used to distinguish M. bovis from M. tuberculosis isolates.

The study shows the MGIT™ 960 system had a higher recovery rate of M. bovis than the BACTEC™ 460 and solid media systems. In addition the MGIT™ 960 system had the lowest mean time to detection. The BACTEC™ 460 and MGIT™ 960 systems had similar contamination rates. These results indicate the MGIT™ 960 system is better than the BACTEC™ 460 system for recovering M. bovis from tissue samples.

USDA, APHIS, VS, NVSL, Mycobacteria & Brucella Section (Hines, Payeur)
Iowa State University; Veterinary Diagnostics and Production Animal Medicine (Hoffman)
Introduction

Bovine tuberculosis is a chronic disease that primarily affects cattle. Clinical signs of this disease include swollen lymph nodes, weakness, anorexia, dyspnea, low grade fever and emaciation. These symptoms can take weeks to months to develop and some animals do not develop any visible symptoms. This disease can affect a variety of other animals such as coyotes, raccoons, elk and deer. These wild animals may spread the disease from one cattle herd to another making an eradication program necessary.\(^{16,17}\)

*Mycobacterium bovis* is an acid-fast bacilli that is a member of the *M. tuberculosis* complex. It is transmitted primarily through inhalation and can form granulomas in secondary lymphoid organs. It is relatively difficult to culture because of its very slow replication rate and fastidious nature. In addition a long incubation period is required on solid culture media making rapid diagnosis difficult.\(^{2,17}\)

Culture is the primary method used to detect and confirm *M. bovis* infection. Isolation of *M. bovis* from tissue lesions of animals suspected of having tuberculosis is routinely performed using BACTEC™ 12B radiometric media\(^8\) and solid media (Middlebrook 7H10 or Middlebrook 7H11). A combination of liquid and solid media is considered the “gold standard” for culturing mycobacteria.\(^{11}\) However, the BACTEC™ 460 culture method is labor intensive and poses a risk of exposure to radiation. The BACTEC™ Mycobacteria Growth Indicator Tube System\(^b\) (BACTEC™ MGIT™ 960 system) is a recently developed, fully automated, continuous monitoring, non-radiometric instrument designed for rapid growth of acid-fast bacilli from clinical specimens.\(^1\)

The purpose of this study was to compare the recovery rate of *M. bovis* using three culture methods including the BACTEC™ MGIT™ 960 system, the BACTEC™ 460 system and Middlebrook 7H10 and Middlebrook 7H11 solid media. The results of this study will determine if the MGIT™ 960 system can be recommended as an alternative to the BACTEC™ 460 radiometric system for diagnosis of *M. bovis* infection.
Materials and Methods

Samples. Five hundred and six bovine lymph node samples were processed and screened by histopathology examination.\textsuperscript{10,11} Four hundred and sixty eight samples were from Mexican abattoirs and 38 were from the United States. Histopathology results and individual diagnostic test results are displayed in Tables 1 through 6 in the appendix.

Processing. All samples were processed according to the Laboratory Methods in Veterinary Mycobacteriology for the Isolation and Identification of Mycobacteria manual.\textsuperscript{11} The tissue samples were decontaminated by soaking in 10\% bleach solution up to 30 minutes. They were then placed in mason pint size blender jars containing nutrient broth with 0.4\% phenol red. The tissues were liquefied using a standard blender\textsuperscript{e} and then treated with 0.5N sodium hydroxide. The reaction was terminated by the addition of 6N hydrochloric acid which lowered the pH of the tissue solutions to an acidic range. The tissue suspensions were brought back to neutral pH by the addition of 1N sodium hydroxide. The tissue suspensions were then centrifuged in a refrigerated centrifuge\textsuperscript{d} at 6000 x g.

Isolation. Half of the supernatant was discarded and the pellet was swabbed onto the appropriate solid media tubes.\textsuperscript{11} Solid media used in this study were Middlebrook 7H10 and Middlebrook 7H11 both containing sodium pyruvate. The solid media tubes were incubated at 37 ± 2°C in a CO\textsubscript{2} incubator and examined once each week until colonies were observed or until an incubation period of 8 weeks was complete, at which time tubes with no growth were discarded. Each tissue suspension (0.5 ml) was also inoculated into BACTEC\textsuperscript{TM} 460 liquid culture vials and MGIT\textsuperscript{TM} 960 liquid culture tubes.\textsuperscript{1,14} This procedure was performed for all samples throughout the project.

BACTEC\textsuperscript{TM} 460 System\textsuperscript{p}. Each BACTEC\textsuperscript{TM} 460 20 ml culture vial contained 4 ml of 7H9 broth base, casein hydrolysate, bovine serum albumin, catalase and \textsuperscript{14}C labeled palmitic acid. Prior to inoculation, the BACTEC\textsuperscript{TM} 460 vials were supplemented with 0.2 ml of PANTA\textsuperscript{TM} which contained a lyophilized antimicrobial mixture of amphotericin B, azlocillin,
nalidixic acid, polymyxin B and trimethoprim. The fluid used to reconstitute the lyophilized PANTA™ mixture was an aqueous solution of polyxyylene stearate which augmented the growth of mycobacteria. A prior study in the Mycobacteria and Brucella section (by Janet Marquardt, personal communication) determined that the addition of erythromycin to the PANTA™ mixture reduced contaminating bacteria in the BACTEC™ 460 vials. For this reason, 32 µg/ml of erythromycin was added to the BACTEC™ reconstituting fluid to supplement the PANTA™ antibiotic mixture for the BACTEC™ 460 vials.

The BACTEC™ 460 vials were incubated at 37 ± 2°C and monitored using the BACTEC™ 460 TB instrument. The samples in the BACTEC™ 12B media were monitored twice a week for the first four weeks and once a week for the remaining two weeks for a total incubation period of six weeks. The instrument detected the amount of 14C in each vial which was quantified on a scale of 0-999 referred to as the Growth Index (GI). A GI reading of 300 or more indicated a positive vial and a smear was stained to detect acid-fast bacilli by the Ziehl-Neelsen method. Smears were also made from vials that were negative at the end of six weeks.

**BACTEC™ MGIT™ 960 system**. The MGIT™ 960 system includes MGIT™ 960 growth indicator tubes, BACTEC™ MGIT™ 960 instrument and MGIT™ 960 supplement kit. Each BACTEC™ MGIT™ 960 culture tube contains 7 ml of modified Middlebrook 7H9 broth base with casein peptone and a fluorescent compound embedded in silicone on the bottom of the tube. The supplement kit contains BBL™ MGIT™ PANT A™ which is the same lyophilized antibiotic mixture as the PANTA™ antibiotic mixture for the BACTEC™ 460 vials. The PANTA™ kit includes a growth supplement containing bovine serum albumin with glucose, catalase, oleic acid and polyoxyethylene stearate used to reconstitute the lyophilized PANTA™ antimicrobial mixture. The MGIT™ 960 liquid culture tubes were supplemented with 0.8 ml of the reconstituted BBL™ MGIT™ PANTA™, to reduce growth of contaminating
bacteria and supplement the mycobacteria. Erythromycin was not added to the PANTA antimicrobial mixture for the MGIT™ 960 tubes.

The MGIT™ 960 tubes were incubated at 37 ± 2°C in the BACTEC™ MGIT™ 960 instrument. Oxygen dissolved in the media quenched the fluorescence in the bottom of the tube until growth of bacteria used up the oxygen and fluorescence was emitted. The instrument automatically monitored the fluorescence within each indicator tube every hour to determine the status of the culture. When enough bacteria had grown in the indicator tube to allow for fluorescence, the instrument signaled positive on the outside of the drawer and the tube was removed. If the tube did not signal positive by the end of the 42 day incubation, the instrument signaled negative and the tube was discarded if it was acid-fast negative.

Organism identification. Organism identification was confirmed by a combination of acid-fast staining, nucleic acid probes and biochemical tests. MGIT™ 960 tubes were considered positive on the day the instrument signaled positive. BACTEC™ 460 vials were considered positive on the day the vial first reached a GI reading of 300 or higher. Solid media tubes were considered positive on the day when colonies were first observed. Smears were made of positive BACTEC™ 460 vials and MGIT™ 960 tubes as well as colonies observed on solid media. The smears were stained for acid-fast bacteria by the Ziehl-Neelsen method and examined microscopically. Smears were made again at six weeks for MGIT™ 960 tubes that signaled positive but were initially acid-fast negative and also for tubes that signaled negative on the instrument. Smears were also made again at six weeks for the BACTEC™ 460 vials that had a GI of at least 300 but were initially acid-fast negative as well as vials that never reached a GI of 300 by six weeks. If acid-fast bacilli were seen in these tubes or vials at six weeks the positive date was considered to be the date on which the six week acid-fast smear was read. Nucleic acid probes and biochemical tests were then performed in order to identify the organism. Solid media tubes that were negative for growth by the end of the eight week incubation period were discarded.
Contaminated BACTEC™ 460 vials and MGIT™ 960 tubes were considered to be those that signaled positive and bacterial or fungal growth was observed, but acid-fast bacilli were not present after all smears were complete at the end of six weeks. Solid media tubes were considered contaminated if pure colonies of mycobacteria were unable to be isolated from the tubes.

Molecular Techniques. Nucleic acid probes\(^6\) were used on each culture medium containing acid-fast bacilli to confirm that the organism was in the \(M.\ tuberculosis\) complex. The nucleic acid probe system consisted of a single-stranded DNA probe with a chemiluminescent label which was complementary to the ribosomal RNA (r-RNA) of the target organism. A lysing reagent along with sonication was used to release the r-RNA from the organism. The released r-RNA combined with the labeled DNA probe to form a stable DNA:RNA hybrid. A selection reagent was added to differentiate between the hybridized and non-hybridized probe. The labeled DNA:RNA hybrids were measured by the luminometer\(^6\). A positive result was indicated by a signal greater than or equal to 30,000 RLU (relative light units). A negative result was a signal less than 30,000 RLU.\(^8\)

Biochemical Tests. Samples that were determined to be \(M.\ tuberculosis\) complex positive were inoculated onto a fresh Middlebrook 7H10 media tube for biochemical testing. The niacin accumulation biochemical test was used to distinguish between \(M. bovis\) and \(M. tuberculosis\) within the \(M. tuberculosis\) complex. \(M. bovis\) is negative for niacin accumulation while \(M. tuberculosis\) is positive. The media surface was stabbed several times and sterile water or saline was added for niacin extraction. The extract was removed and a niacin strip was inserted and incubated at room temperature. Test results were then observed by placing the liquid against a white background. Positive results were determined when the liquid appeared yellow while negative results were indicated by the liquid remaining colorless.\(^9\)
Nitrate reduction was also used to confirm the identification of the organism. *M. bovis* is unable to reduce nitrates while *M. tuberculosis* reduces nitrates. An actively growing culture (2-3 weeks old) was inoculated into the nitrate substrate broth. This was incubated and a solution of concentrated hydrochloric acid and sterile water was added along with sulfanilamide and β-N-(1-naphthyl)ethylenediamine dihydrochloride. When the substrate broth turned from a pink to a red color, the organism was considered positive for nitrate reduction. Negative results were indicated by no color change in the substrate broth. 8

Statistical Methods. Statistical analyses were performed comparing recovery rate, mean time to detection and pair-wise comparisons of the MGIT™ 960 system to the BACTEC™ 460 system and solid media tubes. Cochran’s Q-test was used to generate a Chi-square statistic for analyzing recovery rates of each media type. Mean time to detection was compared using an analysis of variance to analyze the data for each culture system. The least significant difference (LSD) method was also used to do a pair-wise comparison of the mean time to detection for the response of each system. 13

Results

A comparison of *M. bovis* recovery in each media system is shown in Table 1. One hundred and twenty nine *M. bovis* isolates were recovered from the 506 bovine lymph node samples in this study. A majority of the isolates (83) were recovered in all three media systems. Only 13 isolates, recovered in both the MGIT™ 960 and BACTEC™ 460 systems, were not recovered on solid media. Ten isolates were recovered using the MGIT™ 960 or solid media but were negative in the BACTEC™ 460 system. Sixteen isolates were recovered in the MGIT™ 960 system but were not detected by the other two systems. There were two instances where the MGIT™ 960 did not detect isolates that were recovered using the other two systems. Four isolates were recovered using the BACTEC™ 460 system, but were undetected by the other two systems. One isolate was recovered on solid media, but was not detected by either of the liquid culture systems.
Table 1. Recovery of *M. bovis* in the 3 media systems.

<table>
<thead>
<tr>
<th>BACTEC™ 960</th>
<th>MGIT™ 960</th>
<th>BACTEC™ 460</th>
<th>Solid Media</th>
<th># of Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 shows the individual recovery rates for each system. The MGIT™ 960 system had the best rate of recovery at 94.5%. The BACTEC™ 460 system recovered 79.1% while 74.4% were recovered using Middlebrook 7H10 & Middlebrook 7H11 solid media.

Table 2. Percent positive samples by each media system.

<table>
<thead>
<tr>
<th>System</th>
<th>Organisms recovered by system / Total positive samples</th>
<th>Percent Recovery by system</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGIT™ 960</td>
<td>122/129</td>
<td>94.5%</td>
</tr>
<tr>
<td>BACTEC™ 460</td>
<td>102/129</td>
<td>79.1%</td>
</tr>
<tr>
<td>Solid Media</td>
<td>96/129</td>
<td>74.4%</td>
</tr>
</tbody>
</table>

The MGIT™ 960 system recovered the most *M. bovis* isolates of the three systems. Table 3 displays the differences in recovery between the systems when looking at pair-wise comparisons. The MGIT™ 960 system had the best individual recovery rate. When compared to BACTEC™ 460 system, there was a statistically significant difference of 14.8% in the recovery rates. When the MGIT™ 960 system was compared to the Middlebrook 7H10 & Middlebrook 7H11 solid media, the pair-wise differences between the two was 19.5% which
was also a statistically significant difference. There was no significance difference between
the solid media and the BACTEC™ 460 system with only 4.7% difference in recovery rates.

**Table 3.** Pair-wise comparisons of three media systems for differences in recovery rate of *M. bovis*.

<table>
<thead>
<tr>
<th>Systems Compared</th>
<th>Percent Differences</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGIT™ 960 vs. BACTEC™ 460</td>
<td>14.8%</td>
<td>P&lt;0.01*</td>
</tr>
<tr>
<td>MGIT™ 960 vs. Solid</td>
<td>19.5%</td>
<td>P&lt;0.01*</td>
</tr>
<tr>
<td>BACTEC™ 460 vs. Solid</td>
<td>4.7%</td>
<td>P&gt;0.25</td>
</tr>
</tbody>
</table>

* Statistical significant difference between the two media systems.

The mean time to detection is of major interest in this study because of the rapid
recovery rate of the MGIT™ 960 system compared to BACTEC™ 460 and solid media. The
mean time to detection for each system is displayed in Table 4. The MGIT™ 960 system had
a significantly lower mean time to detection than the other two systems. Recovery of the
organism required only 15.8 days (SE 0.8). Recovery using the BACTEC™ 460 system
required 28.2 days (SE 1.0) to detect the bacteria, while the Middlebrook 7H10 &
Middlebrook 7H11 solid media required an average of 43.4 days (SE 1.0) for colonies to
appear on the media.

**Table 4.** Mean time to recovery of *M. bovis* for each media system and the standard error of
each mean.

<table>
<thead>
<tr>
<th>Media System</th>
<th>Days to detection</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGIT™ 960</td>
<td>15.8</td>
<td>0.8*</td>
</tr>
<tr>
<td>BACTEC™ 460</td>
<td>28.2</td>
<td>1.0*</td>
</tr>
<tr>
<td>Solid Media</td>
<td>43.4</td>
<td>1.0*</td>
</tr>
</tbody>
</table>

* Statistical significant difference
The contamination rate of each media system is displayed in Table 5. The contamination rate of the MGIT™ 960 system was 18.9% which was slightly higher than the BACTEC™ 460 system (11.8%). Solid media had a very high contamination rate with half of the tubes showing contamination (50.2%). Contamination was caused by both bacteria and fungus, but these organisms were not identified.

**Table 5.** Contamination rate for each media system.

<table>
<thead>
<tr>
<th>Media System</th>
<th>Percent Contamination</th>
<th># Contaminated / Total Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGIT™ 960</td>
<td>18.9%</td>
<td>35/185</td>
</tr>
<tr>
<td>BACTEC™ 460</td>
<td>11.8%</td>
<td>16/136</td>
</tr>
<tr>
<td>Solid media</td>
<td>50.2%</td>
<td>110/219</td>
</tr>
</tbody>
</table>

**Discussion**

It is important that mycobacteria infections be diagnosed quickly. Preventing the spread to other herds or individual animals is necessary to prevent major outbreaks and control tuberculosis. Traditional culture methods may require eight weeks or longer for recovery of mycobacteria. The current gold standard used for isolation of *M. bovis* is the BACTEC™ 460 liquid culture medium in combination with solid media. This media combination has been an effective method of isolating mycobacteria in the Diagnostic Bacteriology Laboratory of the NVSL for many years.  

The results of this study indicate that the recovery rates using either liquid culture medium was better than that of the solid media. The MGIT™ 960 system was more successful in recovery of *M. bovis* isolates than the BACTEC™ 460 system. The MGIT™ 960 media is very rich in nutrients which may be why it was able to recover more isolates than the BACTEC™ 460 system. It is a liquid media which may be the reason why it recovered more isolates than the solid media. Bacteria are able to grow and spread throughout liquid media...
which makes it a better culture medium. Solid media has the lowest recovery rate because the bacteria are only able to utilize the nutrients in the vicinity of the colony.

The MGIT™ 960 system and the BACTEC™ 460 system had comparable contamination rates with the BACTEC™ 460 system having a slightly lower contamination rate. Contamination of the solid media was a large problem since the rate of contamination was slightly over 50%. The high contamination rate may be due to the lack of antibiotics in the solid media. An antibiotic cocktail was added to both types of liquid media but no antibiotics were added to the solid media. Erythromycin antibiotic and CO₂ were added to the BACTEC™ 460 vials but were not added to the MGIT™ 960 tubes which may account for the slightly higher contamination rate for the MGIT™ 960 system. Adding erythromycin to the PANTA™ antimicrobial mixture for the MGIT™ 960 tubes may reduce the contamination rate to somewhere closer to the contamination rate of the BACTEC™ 460 system.

Studies were performed recently comparing the MGIT™ 960 system to the BACTEC™ 460 TB system as well as different types of solid media for detecting mycobacteria including M. tuberculosis. The detection rates for the MGIT™ 960 system were comparable to the BACTEC™ 460 system. Mean time to detection and contamination rates of the MGIT™ 960 system were similar to those of the BACTEC™ 460 system. The solid media used in the other comparison studies included Middlebrook 7H10, Middlebrook 7H11 and LJ medium. The detection rates of the solid media used in these studies were lower than the detection rates of both liquid media systems. The mean time to detection was higher than both liquid media systems, but contamination rates were similar to those of the liquid media systems.

The similarity between the results for the MGIT™ 960 system and the BACTEC™ 460 system led to the conclusion that the MGIT™ 960 system was comparable to the BACTEC™ 460 system when recovering M. tuberculosis. The success of these comparison studies for recovering M. tuberculosis in human samples led to the interest in determining if
the MGIT™ 960 system could be as successful for recovery of *M. bovis* from bovine tissue samples. The results of this study indicate that biosafety level III diagnostic laboratories could utilize this new liquid media system for recovering *M. bovis*. Because of the decreased time required for recovering the organism, positive animals can be identified quickly and potential outbreaks averted by taking steps to stop the spread of the disease.

Research laboratories can also utilize this system for recovering the organism more rapidly from challenged animals. This may decrease the duration of their studies as well as dollars expended for experimental purposes. Livestock producers across the country will also be positively impacted because the MGIT™ 960 system will decrease the time between sample submission and diagnosis so appropriate interventions can be instituted to prevent disease transmission. This can help reduce financial costs and loss of livestock suffered by producers.

The MGIT™ 960 instrument requires less laboratory space and has the advantage of being connected to a printer for data management. Cost of the MGIT™ 960 system is slightly higher since each tube is about $4.15 while the BACTEC™ 460 vials are only $3.00. The MGIT™ 960 system is a non-radiometric method while the BACTEC™ 460 system relies on radioactive material. Reduction in technician time is the most important advantage of this system since it is completely automated while, the BACTEC™ 460 system is only semi-automated which can be very time consuming. Other benefits of this procedure are increased sensitivity of the liquid culture media, decreased time required for growth as well as time required for reporting out *M. bovis* positive cases.⁸

There are some disadvantages with the MGIT™ 960 method. The time for the detection of positive tubes is dependent on the number of organisms, specimen quality, collection method and processing methodology. Inoculation on solid media is required to determine colony morphology and pigmentation. Both liquid media systems can give false positive results if contaminating bacteria are present, but the MGIT™ 960 system seems to
have a slightly higher incidence of false positive results because of the richness of the medium. Contamination was not observed to be a significant problem for either liquid media systems in this study. Mixed mycobacteria cultures can also be a problem because fast growing mycobacteria are detected earlier than slow growers. It is necessary to subculture the positive tubes onto solid media to separate mixed mycobacteria cultures. The MGIT™ PANTA™ mixture can also inhibit *M. bovis* when used at too high a concentration, but PANTA™ is necessary to reduce the contaminating bacteria in the culture.¹

Although histopathology examination can provide an accurate diagnosis of an infection caused by mycobacteria, it is necessary to grow the bacterium in order to determine the species and strain differentiation. Another advantage of growing the organism is to use it for epidemiological studies. Restriction fragment length polymorphism analysis (RFLP) and spoligotyping can compare genetic similarities between isolates which can help determine how and where the organism has spread. These procedures can be used to help trace-back an infected animal using the isolate recovered in the laboratory. By comparing the genetic similarities between isolates, it is possible to determine whether two animals were infected by the same bacterial strain.⁵ This information can be used in conjunction with the sale history of the animals to determine the location where they were exposed and other animals that may have been exposed. Spoligotyping was not utilized in this project because this new technology has not been incorporated into our laboratory at this point in time.

**Acknowledgements**

The technicians and students in the Mycobacteria & Brucella section of the NVSL have been instrumental in performing laboratory techniques. Patrick Camp and Lisa Ankerstjerne helped process samples and read slides. John Fevold taught me how to perform nucleic acid probes and completed tests on many of the samples. The pathologists in the diagnostic laboratory at the NVSL provided histopathology examination and PCR results for
all of the samples. Thanks also to Harold Ridpath for providing help with statistical analysis and data organization.

**Sources and Manufacturers**

a. BACTEC™ 460 radiometric system. Becton Dickinson and Company, Sparks, MD.
b. BACTEC™ MGIT™ 960 system. Becton Dickinson and Company, Sparks, MD.
d. Sorvall® RC3BP. Kendro Laboratory Products, Newtown, CT.
e. AccuProbe. Gen-Probe®, San Diego, CA.

**References**


GENERAL CONCLUSIONS

The work presented here represents experimentation to gain additional information about the application of the MGIT™ 960 system in a diagnostic environment. The results indicate that the MGIT™ 960 system is a reliable liquid culture system for recovery of *M. bovis*. Rates of recovery with both liquid culture systems are comparable but the mean time to detection favors the MGIT™ 960 system. When using a combination of Middlebrook 7H10 & Middlebrook 7H11 solid media and the MGIT™ 960 system, the recovery rate of *M. bovis* increased. Based upon these findings the MGIT™ 960 system can be considered a cultural equivalent to the BACTEC™ 460 system for recovery of *M. bovis*.

Future studies involving the MGIT™ 960 system may include antibiotic susceptibility testing, identification of contaminating bacteria, PANTA™ antibiotic studies as well as an evaluation of the system for growing more diverse mycobacteria species. A study involving antibiotic susceptibility testing on *M. bovis* isolates may be beneficial to determine whether this automated liquid media system could reduce technician time involved in antibiotic susceptibility testing for determining the antibiotic resistance profiles of *M. bovis* isolates and aid in the establishment of an antibiotic regime that would be successful in treating infected humans or animals.

A study designed to identify the most common bacterial contaminants of the MGIT™ 960 liquid media would provide valuable information for diagnostic laboratories interested in using this media system. This project could be followed up with a study involving the MGIT™ 960 PANTA antibiotic mixture. By identifying the contaminating bacteria, adjustments can be made to the PANTA antibiotic mixture to reduce bacterial contaminants. Finally an evaluation of the system for recovering more diverse mycobacterial species would be beneficial for zoos, animal habitats and private pet owners. Rapid recovery of organisms in diagnostic samples from various animal species could prevent disease transmission to
humans and other animals. This can also lead to faster administration of the correct antibiotics required for an infection.

Histopathology examination for this study was less sensitive than the MGIT™ 960 system for \textit{M. bovis} diagnosis (Tables 2-5, Appendix). One hundred twenty nine \textit{M. bovis} isolates were recovered with the MGIT™ 960 system while histopathology examination indicated that there were 117 compatible for mycobacteriosis. Of these 117 only 99 were PCR positive for \textit{M. tuberculosis} complex. A number of theories can be applied to explain the differences in sensitivity. It is possible that the pathology laboratory did not receive the same lesion as the bacteriology laboratory. Human error during analysis of the samples can also account for these differences. It is possible there were no bacteria present in the lesion analyzed by the pathology laboratory. Sometimes there are very few organisms present in the sample and they are very difficult to locate. Results of PCR examination may not always match histopathology results. A sample may not be positive for \textit{M. tuberculosis} complex even though it has been determined to be compatible for mycobacteriosis because formalin is known to degrade DNA.\textsuperscript{10} This may explain the differences between those compatible for mycobacteriosis and those found to be positive for \textit{M. tuberculosis} complex by PCR examination (Table 4, Appendix).

In conclusion, the high recovery rate and rapid nature of the MGIT™ 960 system make it a better liquid media system than the BACTEC™ 460 system. Other benefits of the MGIT™ 960 system are elimination of radioactive material, reduction in technician time, sensitivity of the liquid culture media and time required for reporting out \textit{M. bovis} positive cases can be reduced. These studies further demonstrate that disease diagnosis was more successful when using a combination of histopathology examination and culture methods (both liquid and solid media). Histopathology examination was shown to be less sensitive than the MGIT™ 960 system but can contribute to diagnostic investigation by providing an initial diagnosis. The MGIT™ 960 system was shown to greatly improve the overall recovery
of *M. bovis* from tissue samples. This method performed better than the gold standard of solid media and BACTEC™ 460 system. Based on these findings the MGIT™ 960 system can replace the BACTEC™ 460 system as a successful liquid culture method.
## APPENDIX

### Table 1. Individual test results for each media system.

<table>
<thead>
<tr>
<th>Media System</th>
<th>Bacterial Growth (+)</th>
<th>Acid-Fast (+)</th>
<th>Acid-Fast (-)</th>
<th>Probe (+) M. tb Complex</th>
<th>M. bovis (+) no ID</th>
<th>Acid Fast (-) M. bovis isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGIT™ 960</td>
<td>185</td>
<td>150</td>
<td>35</td>
<td>126</td>
<td>122</td>
<td>0</td>
</tr>
<tr>
<td>BACTEC™ 460</td>
<td>136</td>
<td>120</td>
<td>16</td>
<td>102</td>
<td>102</td>
<td>1</td>
</tr>
<tr>
<td>Solid Media</td>
<td>219</td>
<td>109</td>
<td>110</td>
<td>96</td>
<td>96</td>
<td>2</td>
</tr>
</tbody>
</table>

### Table 2. Histopathology results (mycobacteriosis compatible) vs. M. bovis positive samples.

<table>
<thead>
<tr>
<th>Total # Samples</th>
<th>Histopathology Positive</th>
<th>Culture Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>506</td>
<td>117</td>
<td>129</td>
</tr>
</tbody>
</table>
Table 3. Comparison of histopathology PCR and 3 media culture systems.

<table>
<thead>
<tr>
<th>Histopathology</th>
<th>MGIT™ 960</th>
<th>BACTEC™ 460</th>
<th>Solid Media</th>
<th># Mycobacteriosis Compatible Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR (+) <em>M. tb</em> complex</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>Pos</td>
<td>Pos</td>
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<tr>
<td>Neg</td>
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<td>Neg</td>
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<td>11</td>
</tr>
</tbody>
</table>
Table 4. Percent samples positive for *M. bovis* by each media system and PCR analysis on formalin fixed tissue.

<table>
<thead>
<tr>
<th>System</th>
<th>Percent Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR (+) <em>M. tb</em> complex</td>
<td>84.6% (99/117)</td>
</tr>
<tr>
<td>MGIT™ 960</td>
<td>82.1% (96/117)</td>
</tr>
<tr>
<td>BACTEC™ 460</td>
<td>74.4% (87/117)</td>
</tr>
<tr>
<td>Solid Media</td>
<td>66.7% (78/117)</td>
</tr>
</tbody>
</table>

Table 5. Histopathology PCR results for *M. tuberculosis* complex vs. *M. bovis* isolates recovered by MGIT™ 960 system.

<table>
<thead>
<tr>
<th>PCR (+) &amp; <em>M. bovis</em> (+)</th>
<th>PCR (-) &amp; <em>M. bovis</em> (+)</th>
<th>PCR (+) &amp; <em>M. bovis</em> (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>96</td>
<td>36</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 6. Miscellaneous mycobacteria recovered in 3 media systems.

<table>
<thead>
<tr>
<th>Mycobacteria species</th>
<th>MGIT™ 960</th>
<th>BACTEC™ 460</th>
<th>Solid Media</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em> complex</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>M. avium</em> complex</td>
<td>7</td>
<td>3</td>
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<tr>
<td><em>M. terrae</em></td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><em>M. lentiflavium</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>M. kansasii</em></td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>M. abscessus</em></td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><em>M. pulvers</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>M. scrofulaceum</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>M. avium</em> subsp. Paratuberculosis</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M. species</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

* Miscellaneous species were identified by sequencing

Table 7. Media, reagents, and dyes supplied by the media kitchen at the NVSL in Ames, IA.

<table>
<thead>
<tr>
<th>Culture Media</th>
<th>Niacin Test</th>
<th>Nitrate Test</th>
<th>Ziehl-Neelsen Stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Middlebrook 7H10</td>
<td>10% Cyanogen Bromide</td>
<td>Nitrate Reduction</td>
<td>Carbol Fuchsin Dye</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td></td>
</tr>
<tr>
<td>Middlebrook 7H11</td>
<td>Ethyl Aniline</td>
<td>Nitrate Reduction</td>
<td>Acid Alcohol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reagent 1</td>
<td></td>
</tr>
<tr>
<td>Nutrient Broth with</td>
<td></td>
<td>Nitrate Reduction</td>
<td>Brilliant Green Dye</td>
</tr>
<tr>
<td>0.4% Phenol Red</td>
<td></td>
<td>Reagent 2</td>
<td></td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

Many people provided support and guidance for the successful completion of my Master of Science degree. My committee has been easy to work with and very encouraging. Thanks to Dr. Payeur for helping to design my research project and for her guidance along the way. Also special thanks to Dr. Hoffman for her advice and commitment to helping me achieve my goals. I would also like to recognize the people in the Mycobacteria and Brucella Section of the National Veterinary Services Laboratories for their help with my research project. Thanks to my family for supporting me throughout my college career.