Diagnostic Techniques For Bovine Tuberculosis: An Update

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Abstract: Bovine tuberculosis caused by *Mycobacterium bovis* is a highly infectious zoonotic disease. When transmitted to humans the disease symptoms cannot be distinguished from infection caused by *Mycobacterium tuberculosis the* agent of TB in humans. Transmission of the disease to humans is through direct contact with diseased animals and consumption of unpasteurized milk and milk products. The diagnostic techniques for the detection of bovine tuberculosis includes delayed hypersensitivity tests, microscopic examination using Ziehl–Neelsen stain, culture, gamma-interferon assay, lymphocyte proliferation assay, immunoassay of mycobacterial antigens, enzyme-linked immunosorbent assay (ELISA), high performance liquid chromatography (HPLC) and phage typing. Others include polymerase chain reaction (PCR) test, restriction fragment length polymorphism (RFLP), amplification-based methods which provides more rapid typing techniques most of which depend on PCR-based amplification of *M. tuberculosis* sequences including IS6110-based, 16S-and 23S rRNA-based**,** DR region-based methods and Spoligotyping. Minisatellite-based methods contain variable numbers of tandem repeats (VNTRs) have been demonstrated to be effective and portable methods for typing *M. tuberculosis.* Despite the wide range of techniques which could be used in the diagnosis of bovine tuberculosis, the availability and cost of some of these techniques still posses challenges in the field of diagnosis hence the need for a cheaper and more reliable technique in the diagnosis of bovine tuberculosis.

[Ayi Vandi Kwaghe, Yaqub Ahmed Geidam, Godwin Onyeamaechi Egwu. **Diagnostic Techniques For Bovine Tuberculosis: An Update**. Journal of American Science 2011; 7(11): 204-215]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: Diagnosis, bovine tuberculosis, method, *Mycobacterium bovis*

1. Introduction

Mycobacterium bovis is the causal agent of bovine tuberculosis (TB), it infects approximately 50 million animals all over the world causing economic losses of approximately 3 billion dollars per year (Steele, 1995). The disease is zoonotic, human populations may be infected by direct contact with diseased animals and by the consumption of non-pasteurized milk and its derivatives. In 2003, the World Health Organization (WHO) estimated that approximately 8.8 million persons developed tuberculosis and 1.7 million persons died of this disease (WHO, 2005). In Mexico, 28% of milk is marketed without pasteurization and is used for the preparation of cheese and other dairy derivatives (NOM, 2005) which implies a high risk to public health (Bermudez *et al*., 2010).

Although tuberculosis due to *Mycobacterium bovis* infection has long been recognized as an animal disease, its effect on animal production and human health only became apparent at the turn of the century with the development of livestock industry in Europe and America (de Kantor and Ritacco, 1994). Bovine tuberculosis caused by *Mycobacterium bovis* remains a significant disease of cattle and other species in many countries. This zoonotic disease is highly infectious in humans, and its symptoms are indistinguishable from infection caused by *Mycobacterium tuberculosis* (Biet *et al.*, 2006; De La Rua-Domenech, 2006; Theon *et al.,* 2006). Tuberculosis caused by *M. bovis* has become less important as a public health risk in countries with bovine tuberculosis eradication plans (Theon *et al.,* 2006) but it still poses one of the most important zoonotic threats in developing countries, particularly where *Human immunodeficiency virus* is. Moreover, the notification system for human tuberculosis in most countries does not distinguish cases caused by nonhuman mycobacteria, so the real number of cases might be underestimated (European Commission, 2003).

Immunological responses to *M. bovis* infections in cattle continue to be studied in attempts to develop improved or alternative diagnostic methods. Methods such as skin testing sometimes has practical drawbacks. The gamma interferon test is increasingly being used as a diagnostic blood test for tuberculosis in cattle and is available commercially (OIE ,2009). Molecular techniques such as polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), mycobacterial interspersed repetitive unit- variable numbers of tandem repeat (MIRU-VNTR) and spoligotyping are also used in

the diagnosis of tuberculosis. Choosing a more effective and economical method for tuberculosis diagnosis is essential for the eradication and control of tuberculosis. This review is intended to highlight the various methods used in the diagnosis of bovine tuberculosis and identify the merits and demerits of these methods.

2. Diagnostic Methods

Delayed hypersensitivity test

This test is the standard method for detection of bovine tuberculosis. It involves measuring skin thickness, injecting bovine tuberculin intradermally into the measured area and measuring any subsequent swelling at the site of injection 72 hours later. The comparative intradermal tuberculin test with bovine and avian tuberculin is used mainly to differentiate between animals infected with M. bovis and those sensitized to tuberculin due to exposure to other *Mycobacteria* or related genera (OIE, 2009). Due to their higher specificity and easier standardization, purified protein derivative (PPD) products have replaced heat-concentrated synthetic medium tuberculins (OIE, 2009).

The Single Intradermal Test

The caudal fold test as described by Wiggins and Essey (undated) involves injecting a dose of 0.1 ml bovine tuberculin, purified protein derivative (PPD) is injected intradermally at the centre of the caudal fold approximately 7cm distal to the base of the tail. The use of disposable tuberculin syringes is recommended. Reading of the test is by palpation of the injection site at 72hours (± 6 hours) post injection. Animals may be classified as negative, suspect or reactor. Cattle are classified as negative when there is no detectable response at the injection site. All responses at the injection site result in an animal being classified as "suspect" or "reactor". Suspect may be slaughtered under permit or retested using the Single Intradermal Comparative Tuberculin Test (SICTT) at either less than 10days (short-interval-re-test) or more than 60days following the commencement of the caudal fold test (Monaghan *et al.*,1994).

Microscopic examination

Demonstration of *Mycobacterium bovis* can be done microscopically on direct smears from clinical samples and on prepared tissue materials. The acid fastness of *M. bovis* is normally demonstrated with the classic Ziehl–Neelsen stain, but a fluorescent acid-fast stain may also be used. Immunoperoxidase techniques may also give satisfactory results. The presumptive diagnosis of mycobacteriosis can be made if the tissue has characteristic histological lesions (caseous necrosis, mineralization, epithelioid cells, multinucleated giant cells and macrophages) [OIE, 2009].

Culture

To process specimens for culture, the tissue is first homogenized using a mortar and pestle, stomacher or blender, followed by decontamination with either detergent (such as 0.375–0.75% hexadecylpyridiniumchloride [HPC]), an alkali (2–4% sodium hydroxide) or an acid (5% oxalic acid). The alkali or acid mixture is shaken for 10–15 minutes at room temperature and then neutralized. Neutralization is not required when using HPC (OIE, 2009). The suspension is centrifuged, the supernatant is discarded, and the sediment is used for culture and microscopic examination. For primary isolation, the sediment is usually inoculated on to a set of solid egg-based media, such as Lowenstein–Jensen, Kirchner, Coletsos base or Stonebrinks; these media should contain either pyruvate or private and glycerol. An agar-based medium such as Middlebrook media (7H9, 7H10, and 7H11) or blood based agar medium (Cousins *et al*., 1989; Moore *et al.*, 2006) may also be used. Cultures are incubated for a minimum of 8 weeks (and preferably for 10–12 weeks) at 37°C with or without CO2. The media should be in tightly closed tubes to avoid desiccation. Slopes are examined for macroscopic growth at intervals during the incubation period. When growth is visible, smears are prepared and stained by the Ziehl–Neelsen technique. Growth of *M. bovis* generally occurs within 3–6 weeks of incubation depending on the media used (OIE, 2009). A culture of the acid fast bacilli (AFB) can distinguish the various forms of *Mycobacteria*, although results from this may take four to eight weeks for a conclusive result. New automated systems that are faster include the MB/BacT, BACTEC 9000, and the Mycobacterial Growth Indicator Tube (MGIT), (Drobniewski *et al.*, 2003). The Microscopic Observation Drug Susceptibility assay culture may be a faster and more accurate method (Moore *et al.*, 2006).

Blood-Based Laboratory Tests

Diagnostic blood tests such as the gamma interferon assay, which uses an enzyme-linked immunosorbent assay (ELISA) as the detection method for interferon, the lymphocyte proliferation assay, which detects cell-mediated immune responses, and the indirect ELISA, which detects

antibody responses are available. The logistics and laboratory execution of some of these tests may be a limiting factor. The use of blood-based assays can be advantageous (OIE, 2009).

Gamma-interferon assay (the alternative test for international trade)

In this test, the release of a lymphokine gamma interferon (IFN-γ) is measured in a whole-blood culture system. The assay is based on the release of IFN-γ from sensitized lymphocytes during a 16–24-hour incubation period with specific antigen (PPD-tuberculin) (Wood *et* al., 1990). The test makes use of the comparison of IFN-γ production following stimulation with avian and bovine PPD. The detection of bovine IFN-γ is carried out with a sandwich ELISA that uses two monoclonal antibodies to bovine gamma-interferon. It is recommended that the blood samples be transported to the laboratory and the assay set up as soon as practical, but not later than the day after blood collection (Ryan *et al*., 2000; Coad *et al*., 2007). In some areas, especially where 'nonspecificity' is prevalent, some concerns about the accuracy have been expressed. However, because of the IFN-γ test capability of detecting early infections, the use of both tests in parallel allows detection of a greater number of infected animals before they become a source of infection for other animals as well as a source of contamination of the environment (Gormley *et al.,* 2006). The use of defined mycobacterial antigens such as ESAT 6 and CFP-10 shows promise for improved specificity (Buddle *et al*., 2001).

Lymphocyte proliferation assay

This type of *in-vitro* assay compares the reactivity of peripheral blood lymphocytes to tuberculin PPD from *Mycobacterium bovis* (PPD-B) and a PPD from *Mycobacterium avium* (PPD-A). The assay can be performed on whole blood (Buddle *et al*., 2001) or purified lymphocytes from peripheral blood samples (Griffin *et al.*, 1994). These tests endeavour to increase the specificity of the assay by removing the response of lymphocytes to 'nonspecific' or cross-reactive antigens associated with non-pathogenic species of mycobacteria to which the animal may have been exposed. Results are usually analyzed as the value obtained in response to PPD-B minus the value obtained in response to PPD-A. The B–A value must then be above a cut-off point that can be altered in order to maximize either specificity or sensitivity of the diagnosis. The assay has scientific value, but is not used for routine diagnosis because the test is time-consuming and the logistics and laboratory execution are complicated (it requires long incubation period and the use of radio-active nucleotides). As with the IFN-γ test, the lymphocyte proliferation assay should be performed shortly after blood is collected. The test is relatively expensive and has not yet been subject to inter-laboratory comparisons (OIE, 2009).

Immunoassay of mycobacterial antigens

Enzyme-linked immunosorbent assay (ELISA) and radio-immunoassay are still in the development phase though they offer rapid species-specific identification (Daniel, 1989). Monoclonal antibodies may be useful to confer specificity for individual epitopes in these assays. Dot blot immunoassays are capable of recognizing species-specific catalyses (Science Forum, 2000).

Enzyme-linked lmmunosorbent Assay (ELISA)

ELISA appears to be the most suitable of the antibody-detection tests and can be a complement, rather than an alternative, to tests based on cellular immunity. An advantage of ELISA is its simplicity and cheap technique which many studies have shown good results with a high sensitivity and specificity especially for antigen detection in cerebrospinal fluid (CSF). However, other studies have shown that it is not more specific than other serodiagnostic methods (Azra and Yasmeen, 2001; OIE, 2009). The sensitivity of the test is limited mostly because of the late and irregular development of the humoral immune response in cattle during the course of the disease. Specificity is also poor in cattle when complex antigens such as tuberculin or *M. bovis* culture filtrates are used. However, a comparison of antibody levels to PPD-B and PPD-A has been shown to be useful in increasing specificity in the ELISA (Griffin *et al*., 1993). It has now become apparent that antigen is not as specie specific as was originally believed and contains non-specific and specific epitopes (Talib *et al.,* 1993) .

Detection of Biological Compounds

New techniques have been developed to detect specific components produced either by the mycobacteria or by the diseased host in response to infection. Adenosine deaminase enzyme(ADA) is a host enzyme produced by activated T cells. This has been shown to increase in active tuberculosis (Azra and Yasmeen, 2001).

High Performance Liquid Chromatography (HPLC)

This tool is used to detect species specific

mycolic acids produced by *Mycobacteria* which contain these unique fatty acids(Butler *et al.,* 1986; Thibert and Lapierre, 1993), each species has its own unique mycolic acid pattern. This technique is rapid and reproducible and enables species to be identified within 2 to 18 hours. The disadvantage is the expensive equipment and software (Azra and Yasmeen, 2001).

Non-DNA typing method (phage typing)

Before the advent of molecular techniques, phage typing was the most widely used method in differentiation of strains of TB complex and *Mycobacterium tuberculosis* strain (Bates and Fitzhugh, 1967). This technique is useful in typing *M. tuberculosis* strains from out breaks (Snider *et al.*, 1984) and laboratory cross examination (Jones, 1988). Members of the *M. tuberculosis* complex have been differentiated through the evaluation of biochemical features and their different susceptibility to antibiotics (Collins *et al.*, 1982). Due to limited number of possible patterns, this method is only useful for tracing spread of strains with usual characteristics (Collins *et al*., 1982). The disadvantage of this method is that it is cumbersome and lacked sensitivity because of the limited number of *Mycobacterium* phage types available (Snider *et al*., 1984).

Genotyping

Nucleic Acid Probes

Nucleic acid probes have gained Increased acceptance for the diagnosis of tuberculosis(Gisenbach *et al.,* 1988; Ellner *et al.*, 1988; Patel *et al.*, 1989).These probes can be used to identify isolates growing on conventional solid culture media, broth cultures or radiometeric media for the detection of *Mycobacterium tuberculosis* complex. A detection time of as less as two hours is needed after the sample has been cultured (Azra and Yasmeen, 2001). Nucleic acid hybridization is a powerful, rapid, accurate and widely used technique which exploits the ability of complementary sequence of DNA or RNA to pair with each other to form a duplex. The probes should be complementary to the amplified sequence. In situ hybridization is used to detect and locate specific DNA or RNA segments in tissues or on chromosomes by making use of radioactive or fluorescent DNA/RNA probes complementary to the required sequence (Azra andYasmeen, 2001). However, in paucibacillary states like tuberculous meningitis and pleural effusion, the number of bacilli is too low to be picked up by this technology. The most commonly used DNA target for amplification is a 36 base pair repeat sequence from the *M. tuberculosis* genome (Dewit *et al.*, 1990). Nucleic acid probes form a useful adjunct to cultures for confirmation of the diagnosis (Azra and Yasmeen, 2001).

Polymerase Chain Reaction (PCR)

PCR has been widely evaluated for the detection of *M. tuberculosis* complex in clinical samples (mainly sputum) in human patients and has recently been used for the diagnosis of tuberculosis in animals (OIE, 2009). A number of commercially available kits and various 'in-house' methods have been evaluated for the detection of the *M. tuberculosis* complex in fresh and fixed tissues. Various primers have been used. Amplification products have been analyzed by hybridization with probes or by gel electrophoresis. Commercial kits and the in-house methods, in fresh, frozen or boric acid-preserved tissues, have shown variable and less than satisfactory results in interlaboratory comparisons (Naranjo *et al*., 2008). False-positive and false negative results, particularly in specimens containing low numbers of bacilli, have reduced the reliability of this test. Variability in results has been attributed to the low copy number of the target sequence per bacillus combined with a low number of bacilli. Variability has also been attributed to decontamination methods, DNA extraction procedures, techniques for the elimination of polymerase enzyme inhibitors, internal and external controls and procedures for the prevention of cross-contamination. Improvement in the reliability of PCR as a practical test for the detection of *M. tuberculosis* complex in fresh clinical specimens will require the development of standardized and robust procedures. Cross contamination is the greatest problem with this type of application and this is why proper controls have to be set up with each amplification. However, PCR is now being used on a routine basis in some laboratories to detect the *M. tuberculosis* group in paraffin embedded tissues (Miller *et al*., 1997; Miller *et al*., 2002). Although direct PCR can produce a rapid result, it is recommended that culture be used in parallel to confirm a viable *M. bovis* infection (OIE, 2009).

2.9.3 Restriction fragment length polymorphism (RFLP)

Differentiation of strains of *M. tuberculosis* complex using nucleic acid-based technology is based on strain specific differences and frequencies of certain DNA sequences in chromosomal DNA. This is usually demonstrated by digestion of the genomic DNA with specific restriction enzymes

and analysis of the generated patterns after separation of the DNA fragments on agar-rose gel: restriction fragment length polymorphism (RFLP), (Collins and Lisle, 1984; Patel *et al.*, 1996). This kind of analysis is technically possible and no hybridization step with defined probes is needed. However, interpretation of the results is difficult because the large number of fragments generates a complex pattern and only a small number of different RFLP types are observed.

This method has been used in investigation of outbreaks of tuberculosis and in epidemiological studies to distinguish between endogenous reinfection and reactivation (Harries, 1990). The time required for *mycobacterium* IS6110- based RFLP analysis is 6 to 7 days, IS6110 (insertion sequence) is a sequence on the genome that allows the insertion of a piece of DNA (Azra and Yasmeen, 2001).

2.9.4 Pulsed field gel electrophoresis (PFGE)

Pulsed field gel electrophoresis (PFGE) has been designed to simplify RFLP. The method uses a less frequently cutting enzyme that generates high molecular weight fragments and allows separation of these fragments under special condition in PFGE. The main limitation of the technique is that the small polymorphism characteristics for different strains will not always produce sufficient discrimination (Varnerot *et al.*, 1992; Zhang *et al.*, 1992).

2.9.5 RFLP with hybridization

DNA polymorphism can also be demonstrated through hybridization of digested nucleic acids with genomic DNA or cloned fragments. Total DNA can be used as the probe but the use of the complete genome as a probe usually results in considerable background and affects the interpretation of the of the results. Some study groups have used cloned repetitive DNA from *M. tuberculosis* as probes (Eisenach *et al.*, 1986; 1988) and one of them appeared to differentiate all strains of *M. tuberculosis* analyzed (Zainuddin and Dale, 1989).

2.9.6 Methods based on repetitive elements (insertion sequences)

Repetitive elements and insertion sequences are frequently used as target sequences for differentiation between mycobacterial strains. Five repetitive DNA elements are useful in strain differentiation of *M. tuberculosis* complex (Dale, 1995; Poulet and Cole, 1995). For use of repetitive sequences in epidemiological studies, different strains must be present as shown in the table below:

Table 1: showing different strains used in repetitive sequences.

Repeated sequence (IS)	Host range	Copy number	polymorphism
1S611O (IS986, IS987)	*M. tuberculosis*	0-20	High
	M. africanum	0-20	High
	M. bovis	1-20	High
	M.bovis-BCG	1-2	*None*
IS1081	*M. tuberculosis*	5-6	Low
	M. africanum	5-6	Low
	M. bovis	5-6	Low
	M. bovis-BCG	5-6	Low
DR cluster	*M. tuberculosis*	1	High
	M. africanum	1	High
	M. bovis	1	High
	M. bovis-BCG	1	High

Modified from Poulet and Cole, 1995

IS6110: Is the element most widely used as a probe for RFLP. It is an insertion sequence belonging to the enterobacterial IS3 family (*McAdam et al.,* 1990). This sequence hybridized with a plasmid isolated from (Zainuddin and Dale, 1989) and, depending on the organism in which it was characterized, is called IS6110 or IS986 in *M. tuberculosis* (as a description of IS6110 was published first and it is the preferred name in *M. tuberculosis*) or IS987 in *M. bovis*-BCG (Eisenach *et al.*, 1990; Hermans *et al.*, 1990b; Thierry *et al.*, 1990). IS6110 is a 1361 bp long sequence that was detected in members of the *M. tuberculosis* complex and differences of only a few nucleotides have been detected between the sequenced copies. The number of IS6110 copies present in the genome is species- and strain -dependent. Most strains of *M. tuberculosis* carry between eight to 15 copies in different positions of the genome although single copy strains are common. This sequence is characterized by presence of inverted repeats (direct repeats) separated by a transposase

gene. IS6110 typing is the most widely used method for molecular epidemiological studies because of high degree of discrimination obtained with this element. The procedure has been standardized (Van Embden *et al.,* 1993) so that results generated in different laboratories can be compared permitting national and international studies of disease transmission to be carried out (Kremer *et al*., 1999).

The major disadvantages is that this method requires a live culture, high quality DNA, and the procedure takes up to 5 days to complete (Kanduma *et al*., 2003). In some communities low copy number strains(<5 copies) make up to 25% of the strains. The band positions of low copy number strains show less polymorphism than high copy number strains and this coupled with the fact that there are fewer bands for similarity circulation means that IS6110 typing is less discriminatory when applied to these strains (Maguire *et al*., 2002). In addition some strains lack any copies of IS6110 (Van Soolingen *et al*., 1993), and some mycobacteria other than tuberculosis possess multiple copies of sequences that hybridize with the IS6110 probe and this will produce a pattern (McHugh *et al*., 1997). Thus care must be taken when performing studies to ensure accurate speciation (Kanduma *et al*., 2003). The consequence of this is that some matches in a large data base may arise through chance more frequently than would be expected (McHugh and Gillespie, 1998).

IS1081, direct repeat and major polymorphic tandem repeat : to overcome the problem of absence or low copy number, alternative molecular markers have been identified (Van Soolingen *et al.,* 1993), IS1081, identified by Collins and Stephens (1991) is a 1324-bp insertion sequence found ln *M. tuberculosis* complex.

The disadvantage of this method is that it has a lower degree of polymorphism than IS6110 because of its low transpositional activity (Van Soolingen *et al*., 1992, 1993). The copy number is lower than that of IS6110, limiting its use in epidemiological studies. Also, it cannot be used to differentiate *M. bovis*-BCG from the other members of *M. tuberculosis* complex (Van Soolingen *et al*., 1992).

Polymorphic GC-rich repetitive sequence: The most abundant repetitive element in the TB complex is a polymorphic GC-rich repetitive sequence (PGRS). It has numerous copies (De Wit *et al*., 1990; Ross *et al*., 1992; Poulet and Cole, 1994) consists of many tandem repeats of a 96 bp GC-rich consensus sequence. PGRS elements are present in 26 sites of *M. tuberculosis* chromosomes (poulet and Cole, 1995a) and have been detected in mycobacteria not belonging to the *M. tuberculosis* complex. Polymorphism in PGRS (Ross *et al*., 1992; Cousins *et al*., 1993; Doran *et al*., 1993) has been harnessed for typing and a recombinant plasmid pTBN12 containing the GC-rich consensus sequence as a probe has been used for secondary finger printing of *M. tuberculosis* with absent or slow copies of IS6110 (Yang *et al*., 1996; McHugh *et al*., 2000).

Amplification-Based Methods

More rapid typing techniques have been developed and most of them depend on PCR-based amplification of *M. tuberculosis* sequences including IS6110. PCR-based methods have the advantage of typing *M. tuberculosis* directly in clinical samples increasing the speed of identification of the organism. They can be used for non-viable isolates or when isolates cannot be resuscitated from archives. Some of these methods, lack reproducibility or have less discriminatory power than IS6110-RFLP (Kremer *et al*., 1999).

IS6110-based methods: One of the methods is ampli-typing which is based on the use of oligonucleotide primers hybridizing with ends of IS6110 and generating a PCR reaction directed away from the insertion sequence. The method is not suitable for comparison of a large number of strains as it lacks reproducibility because of non-specific amplification but can be used to investigate a suspected outbreak (Yuen *et al*., 1995).

Another method is based on the direction of differences in the distance between IS6110 and MPTR through unilateral nested PCR and hybridization analysis. Its draw back is the limited number and size of generated PCR products decreasing information on strain relatedness (Plikaytis *et al., 1993*).

The PCR using a primer complimentary to IS6110 has been used (Haas *et al*., 1993) and a second primer complimentary to a linker ligated to the genomic DNA digested with a restriction enzyme. This mixed linker-PCR typing sometimes generates more bands and can be applied directly on smear-positive clinical specimens. In an inter-laboratory comparison discriminatory power and reproducibility, mixed-linker PCR performed well (Kremer *et al*., 1999).

Double repetitive PCR based on amplification of IS6110 and PGSR generating a banding polymorphism because of distances between these elements has also been used

(Friedman *et al*., 1995). It is a predictive value of 96% and DNA patterns seem to be sufficiently stable to use the method for epidemiology (Kanduma *et al.,* 2003).

Hemi-nested inverse PCR analysis of IS6110 integration sites based on amplification of a part of the IS6110 sequence together with its flanking sequence has been developed (Patel *et al*., 1996). The method is technically simple and has excellent discriminatory power comparable with that of standard RFLP methods (Kanduma *et al*., 2003).

A method employing simple DNA extraction procedure followed by a PCR step involving a single primer aimed at inverted repeat sequence of IS6110 has been proposed (Yates *et al*., 2002). This method was not able to distinguish products of about the same size hence a further step of restriction was introduced giving results comparable with those obtained using standard RFLP (Kanduma *et al*., 2003).

16S-and 23S rRNA-based methods: Amplification of the spacer region between the genes coding for 16S and 23S rRNA and digestion of the amplicon with restriction enzymes has also been performed for differentiation of *M. tuberculosis* strains (Abed *et al*., 1995a). Improved discrimination has been obtained using random amplified polymorphic DNA (RAPD) analysis of the amplified product (Abed *et al*., 1995b). This generates patterns that can be easily analyzed and seem to have high discriminatory power but reproducibility and the final discriminative power of the RAPD-based method was found to be limited (Frothingham, 1995; Glennon and Smith, 1995).

DR region-based methods: A method based on detection of DNA polymorphism is the DR cluster (direct variable repeat PCR) has been used (Groenen *et al*., 1993). It is based on the outward amplification of IS6110 into the direct repeat region generating a strain of specific banding pattern upon hybridization with a DR probe. It has good differentiating power when limited number of strains are being tested, but stability of the DR region is higher than that of IS6110 thus showing identity in otherwise different strains differentiated by IS6110-RFLP (Kanduma *et al*., 2003).

Spoligotyping: Spoligotyping is based on amplification of the DR region and subsequent differential hybridization of the amplified products with membrane bound oligonucleotides complimentary to the variable spacer regions localized between the DRs. Strains that are similar or different can be distinguished by their Spoligotype patterns which is characterized by the number and identity of spacers (Van Soolingen *et al*., 1995). The presence of the spacer sequences varies in different strains and are visualized by a spot on a fixed site of the hybridization membrane. The differentiating power of Spoligotyping is less than IS6110 typing when high copy number strains are being analyzed, but this method is superior for the evaluation of low copy number strains. It distinguishes *M. tuberculosis* and *M. bovis* and can be used with culture negative specimens (Kamerbreek *et al*., 1997). A simultaneous detection and strain differentiation based on this method has been developed (Kamerbreek *et al*., 1997). The method is simple, rapid and robust but lacks discrimination (Kanduma *et al*., 2003).The most widely used method is spoligotyping (from 'spacer oligotyping'), which allows the differentiation of strains inside each species belonging to the *M. tuberculosis* complex, including *M. bovis*, and can also distinguish *M. bovis* from *M. tuberculosis* (Heifets *et al*., 1998; Kamerbeek *et al*., 1997).

Minisatellite-based methods: Methods based on Minisatellite that contain variable numbers of tandem repeats (VNTRs) have been demonstrated to be effective and portable methods for typing *M. tuberculosis* (Kanduma *et al*., 2003). Supply *et al*., (2000) have identified 25 such loci in the *M. tuberculosis* genome and termed them mycobacterium interspersed repetitive units (MIRUs). Twelve loci were demonstrated to vary in tandem repeat numbers and in most, sequence between repeat units. This loci have formed the basis of PCR-based typing method that has discrimination similar to that of high IS6110 copy number strains and better for low copy number strains (Lee *et al.,*2002). This method can be automated for large scale typing projects using high throughput sequencing apparatus (Supply *et al.,* 2001). It is reproducible, sensitive and specific for *M. tuberculosis* complex strains at different levels of evolutionary divergence (Kanduma *et al.,* 2003). When laboratories have access to an automated sequencer, this method is relatively easy to set up, it yields result within a day and as it is a PCR-based. The infrastructure requirement means that this approach will be limited to large reference or research centres (Kanduma *et al*., 2003).

The MIRU-VNTR typing when compared with IS6110 RFLP and Spoligotyping produced more distinct patterns (Barlow *et al*., 2001; Cowan *et al*., 2002). Often a combination of techniques

may be used to gain the maximum discrimination between strains (Cousins *et al.,* 1998).

3. Application Of Typing Methodology

Large scale national and international studies: IS6110 is established as the international method for studying tuberculosis epidemiology (Kanduma *et al*., 2003). PGRS typing, which also uses an RFLP methodology can be used for conforming identity of strains matched by IS6110 or to be low copy number strains. However, the large number of bands produced by this technique makes the interpretation of the gels difficult limiting its application as a primary typing technique (Kanduma *et al*., 2003).

Local outbreak investigation: The RFLP has been used intensively for epidemiological purposes to trace outbreaks of disease (Van Soolingen *et al*., 1991). It is very valuable in situations where traditional contact tracing would not be able to identify the source of infection (Kanduma *et al*., 2003). Genotyping has facilitated the identification and characterization of strains associated with nosocomial transmission in hospitals (Valway *et al*., 1994; Bifani *et al*., 1996; Moss *et al*., 1997). In these studies, molecular markers have been used to confirm the outbreak and to elucidate the history of sequential acquisition of multiple drug resistance (Bifani *et al*., 1999). Molecular typing have been used to identify previously unrecognized point source outbreaks and has been used to confirm transmission in a social setting (Yaganehdoost *et al*., 1999; Sterling *et al*., 2000).

Detecting laboratory cross contamination: Possible cross-contamination can be confirmed using a definitive technique such as IS6110. In this context, Spoligotyping or MIRU-VNTR is more likely to be applicable combing the speed of PCR with the discrimination of IS6110 (Kanduma *et al*., 2003).

4. Conclusion

In conclusion, it is apparent that huge advancement has been made in the techniques available for the diagnosis of bovine tuberculosis compared to what was obtained some decades ago. The development of molecular techniques, amplification-based methods and minisetallite-based methods have indeed increased the spectrum of diagnostic techniques for bovine tuberculosis. However, in spite of this advancement made in the diagnosis of tuberculosis there is still need to develop techniques that are less expensive, less cumbersome with high specificity and sensitivity and especially applicable in less developed countries.

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11/5/2011