Tuberculosis in Nonhuman Primates, - an Overview of Diagnostic Tools

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1. Introduction

In order to assess the status of a nonhuman primate (NHP) toward tuberculosis infection, several test methods with different informative value are available. The intradermal skin test is still the most commonly used and the only ILAR/CDC-approved method for tuberculosis testing of animals in import quarantine (Roberts and Andrews, 2008). However, there are severe limitations that make this test unsuitable as a single stand-alone test for tuberculosis surveillance in nonhuman primate (NHP) facilities. Advance in technology led to the development of new immune based screening assays, which should be included in the diagnostic screening program, while performances of direct and molecular tools are also improving. The use of several tests in combination can increase the sensitivity or specificity of screening and surveillance programs. Here, we summarize common diagnostic techniques, their application and limitations.

2.Non-specific approach

2.1. Clinical examination

Clinical status of infected animals may be altered, although this is more likely to happen in the active form than in the latent form of the disease. Weight loss may occur after the first month after infection, as well as initial hyperthermia (between 3 and 8 weeks after infection).

Other clinical signs are scarcer, but may include cough or abdominal/pleural effusion. On blood works, transient neutrophilia may occur within the first weeks. ESR, fibrinogen, C-protein and other inflammation biomarkers may increase as well, while the albumin/alpha-2 ratio could decrease on plasmatic protein electrophoresis.

Associated to the clinical examination, imagery tools other than X-rays/CT-scan can help, such as endoscopy or ultrasound, especially as they can help to visualize granulomas or suspicious lymph nodes and to perform biopsies.

2.2. Chest radiographs

A posterior-anterior chest radiograph can be used to detect chest abnormalities. Lesions may appear anywhere in the lung and may differ in size, shape, density, and the presence of cavitations. Thoracic radiography may provide confirmation of pulmonary disease but cannot distinguish between tuberculosis and other granulomatous or cavitary diseases of the lungs such as nocardiosis, aspergillosis, or echinococcosis. Therefore, chest radiographs may be used as an additional test procedure only for screening of TB. Although this additional test has a limited sensitivity, it is valuable for the identification of animals that have a negative tuberculin skin test (TST) reaction due to immunosuppression in an end-stage and fulminant disease. Compared to other species, nonhuman primates rarely develop calcified lesions. Therefore, chest radiographs can be difficult to interpret and ideally are presented to an experienced radiologist or lung physician for interpretation. There are no pathognomonic lesions, but enlargement of the bronchial lymph nodes may be an early albeit unspecific sign of pulmonary mycobacteriosis. Larger tubercles or cavitations may be appreciated radiographically. Abdominal radiography may help to identify or confirm splenomegaly or mesenteric lymphadenopathy. Computed tomography scanning or magnetic resonance imaging can give detailed real-time imaging of disease progression and reveal tiny

granulomas that may be overlooked despite regular X- ray (Lewinsohn et al. 2006).

3. Immunological based tools

3.1. Intradermal tuberculin skin test

In many facilities the *in vivo* diagnosis of tuberculosis is based upon the intradermal tuberculin skin test (TST) using mammalian old tuberculin (MOT) or purified protein derivates (PPD). The tuberculin is injected intradermally into an eyelid near its edge or into the abdominal skin, or both. The use of 25-27 gauge and 1/2 to 5/8 inches needles is recommended in order to reduce bruise risk. The test relies on the development of a delayed hypersensitivity response against mycobacterial antigens. In nonhuman primates this delayed-type hypersensitivity develops as part of the adaptive immune cascade within 3-4 weeks after infection. The protein fraction of the tuberculin is recognized by sensitized T-lymphocytes causing release of cytokines, localoedema and local cellular infiltration by inflammatory cell recruitment.

3.1.2. Method

For the palpebral TST, 0.1 ml of MOT (2.500 IU) has to be injected intradermally as close as possible into the edge of the upper eyelid using a sterile 25-27 gauge needle for each nonhuman primate (Fig.1). For the abdominal TST, the hair should be shaved off without traumatizing the skin, and the injection site should be marked with ink or permanent marker to ease the reading. A volume of 0.1 ml MOT (2.500 IU) has to be injected intradermally into the skin of the demarcated site on the abdomen. The abdominal skin test is most commonly used when nonhuman primates suspected of TB infection after a first TST are retested. The advantage of using the abdomen is that any indurations can be measured and a saline or buffer control injection can be used. According to the experience of some working groups, the abdominal TST in nonhuman primates seems to be less sensitive than the palpebral test.

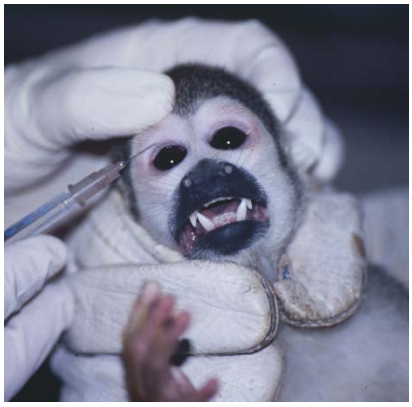
Fig. 1a  Fig.1b 

Fig. 1a: Intradermal application of 0,1ml MOT as close as possible to the edge of the upper eyelid.

Fig.1b: Directly after application a slight swelling of the upper eye lid is visible.

3.1.3. Reading TST

The animals must be observed for reactions at 24, 48, and 72 hours after injection under optimal lighting conditions. Animals, which are suspected to be anergic, should be observed after 2 to 8 hours and daily as "flash" reactions can quickly recede. A trained technician may do the initial readings, but any reaction or suspected reaction needs to be diagnosed and interpreted by an attending veterinarian. Close visual observation and even manual palpation of injection site in sedated animals may optimize the detection of a positive reaction (Panarella 2010). The following grading system is recommended:

Eyelid injection:

Score	Observations	Outcome
0	No reaction	Negative
1	Bruise, extravasation of blood into the eyelid	Negative

	associated with the injection of tuberculin	
2	Varying degrees of erythema of the palpebrum with minimal swelling	Negative
3	Moderate swelling with or without erythema	Positive
4	Obvious swelling of the eyelid with drooping and varying degrees of erythema	Positive
5	Marked swelling with necrosis and closed eyelid	Positive

. Abdominal injection

Score	Observations	Outcome
0	No reaction	Negative
1	Moderate swelling Height of induration 3-5 mm	Negative
2	Moderate swelling Height of induration 5-10 mm	Doubtful
3	Obvious swelling Height of induration >10 mm	Positive

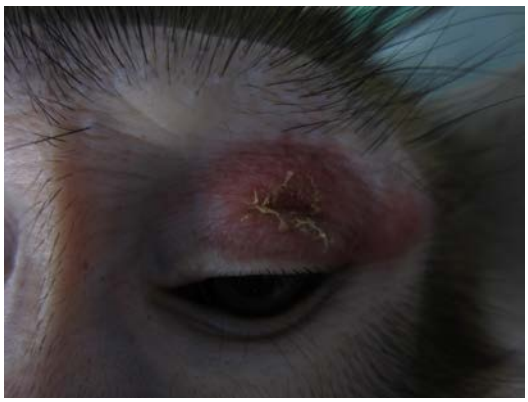


Fig. 2: Positive intracutaneous test, grade 4 reaction

Finally, again, visual scoring of the skin test is largely subjective promoting confusion between negative and positive results. Therefore, alternative diagnostic approaches for *ante mortem* TB testing are needed.

3.1.4. Frequency of TST

3.1.4.1. Before importation from non-European facility (= no agreement regarding directive 92/65 EEC):

Three negative tests with two-week interval are recommended. The last test should be done 10 days before shipment. Prior to importation the supplier should provide data on tests made (tuberculin used, etc.).

3.1.4.2. In quarantine:

The minimal duration of quarantine is 42 days. During this time period, three tests are required. The first test should be done one week after arrival to assure that the animals have a short acclimation period. In case there were any reports of TB infections in the origin/source of the monkey within the past year, a prolonged quarantine period of 60 days including 2 additional tests with bovine PPD is advisable. Some dedicated primate centers (e. g. BPRC in the Netherlands) install a three months (13 weeks) quarantine procedure regardless of the origin/history of the animals.

3.1.4.3 Post quarantine:

The following interval for TST of species or groups of nonhuman primates is recommended after quarantine for institutes with frequent animal transfer and close contact to humans: macaques and vervets quarterly, baboons, prosimians and New World monkeys semi-

annually, great apes annually. A so-called closed institute holding monkeys with limited exposure to humans may have a routine of TB testing every 12 months depending on the policy of the institute.

Because of a number of variables, the facility veterinarian may choose TST at less frequent time intervals. If nonhuman primates are shipped from a facility with less frequent TB testing than advised, the receiving veterinarian must be notified about the deviating procedure before the animals are transferred.

It should be considered that intervals and frequency of TST may interfere with the outcome due to mechanisms of immunological sensitization or desensitization. So far, neither of these actions has been demonstrated in NHP, but a booster effect by repeated TST is sometimes elicited in human practice to reveal latent infections in elderly people. Moreover, desensitization through repeated tuberculin injection was described in cattle. TST overuse should therefore be avoided, especially within a shorter period of time.

It is important that each NHP tuberculin test is accurately recorded for each animal. An animal's clinical record should also include information about the building and/or room, in which it was kept, its social housing partners, and any movement (when it applies) during quarantine. A digital image and exact measurement of a suspect skin reaction is a useful tool to compare test results in repeated tests.

3.1.5. Interpretation issues

3.1.5.1. Tuberculins

The amplitude of the hypersensitivity response and, therefore, the accuracy of the TST reading may correlate with the number of (replicating) tubercle bacilli but also depends on various factors including the amount of circulating, primed, antigen-specific T-cells and the

amount of specific antigen within the tuberculin preparation that is used for the screening. Mammalian old tuberculin (MOT) is a poorly defined preparation composed of various mycobacterial antigens that are known to be highly cross-reactive regarding sensitization of hosts by environmental mycobacteria. In comparison, the purified protein derivatives (PPD) are a precipitated fraction of culture filtrates, but also comprise a mixture of mycobacterial antigens. MOT is less purified, but it holds more tuberculin units than PPD. In nonhuman primates, MOT has greater reactivity than PPD and is therefore preferred to PPD as the reagent to use in a TST to identify infected animals.

When using PPDs, one should be aware that potency of different PPDs differs. After MOT, the recommended PPD is bovine PPD (purified from *M. bovis*) at the usual dose of 2000 UI / 0.1 ml. *M. tuberculosis* ("human") PPD, also called RT23, could be used but reduces the sensitivity of the TST compared to bovine PPD. Moreover, it appears that tuberculin units needed to trigger TST reaction are 100 to 1000 times greater in primates than in humans, thus the use of RT23 could be questionable in terms of dosage.

A comparative TST could be performed using two PPDs such as avian PPD and bovine PPD injected simultaneously but into two separated sites (e. g.: one eyelid each). Differential analysis of reactions on the two sites gives information on the predominant immunological recognition by host. This TST is meant to differentiate between reactions to TB complex mycobacteria and mycobacteria other than tuberculosis (MOTT) and then increases the specificity of the skin test. However, it should be known that avian and bovine PPDs share at least 21 common antigens. Anecdotally, some other "in-house" tuberculins have been developed, like *M. scrofulaceaum* related ones, which could be used only in very specific studies or suspicion.

At least, quality, conservation, or suboptimal concentration of the tuberculin can profoundly influence results of TST usually producing false negative reactions. Despite OIE or EEC regulations discrepancies between the same tuberculin categories in different countries may

still occur, thus in-house re-testing remains of primordial importance.

3.1.5.2. Interactions

The TST is limited in its efficacy as animals with early or advanced infection may give false negative reactions because of a latency period upon infection or immunosuppression in case of progressive TB disease, respectively. The method relies on local immune cell recruitment. Therefore, the test will turn out negative, if the amount of local or systemic immune cells is decreased (Mahanta et al 1994). Concomitant diseases like measles or fungal infections may also result in a false negative TST reaction due to immunosuppression (Ganaway 1974, Potkay 1966, Tauraso 1973). Vaccination with polio, measles or yellow fever vaccine may have the same effect (Ott 1979, Staley 1995). Therapy with isoniazid or undergoing treatment with immunosuppressive drugs including corticosteroids will also negate the value of the tuberculin test through diminution of inflammatory capacity (Dillehay Huerkamp 1990). Furthermore, false negative reactions may result from incorrect injection and subjectivity in the interpretation of the skin test.

False positive reactions may result from previous exposure to environmental MOTT,, experimental injection of Freund`s complete adjuvant (see below) because it contains cell wall components of tubercle bacilli, trauma due to improper injection of the tuberculin, or nonspecific reactions. Regarding the latter, contaminants like phenolic components may cause (transient) allergic reactions, which can appear already 30 minutes after injection and may result in a false positive TST.

Tuberculous NHP can become anergic to mycobacterial antigens and appear negative in a TST during certain stages of the disease (e.g end stage). Therefore, tuberculosis should be considered and further tests performed when a TST negative NHP displays unexplained weight loss, non-healing wounds or any signs of distress or unexplained deviation from

normal behaviour. Additional tests may include: culture swabs of non-healing wounds, chest radiographs, selective culturing of a gastric or broncho-alveolar lavage.

Nonhuman primates display positive TST upon experimental injection of Complete Freunds Adjuvant (CFA), because it contains cell wall components of tubercle bacilli. Ideally, other non-mycobacterium-based adjuvants should be selected so that the diagnostic power of the TST is not compromised. If the use of CFA is unavoidable (e. g. use of porcine zona pellucida contraceptive vaccine), the individual animal has to be tuberculin tested the week before the CFA is injected, or it has to be isolated from the colony. Because CFA application will induce a positive TST, other indirect measures like weight monitoring, chest X-ray or bacterial screening of biofluids might indicate TB infection. If tuberculosis is diagnosed in any other animal in the holding room housing a CFA exposed NHP, the nonhuman primate(s) that previously received CFA should be regarded as potentially infectious and monitored carefully. Because of the economic risk and possible ethical implication when a case of TB remains unidentified, some dedicated primate facilities imply a stricter regime and do not allow animals to be in contact to their regular colony facilities at any time after the injection of CFA.

It should be noted that some NHP species such as the orangutan (*Pongo (P.) pygmaeus*) have been reported to react false positive in the TST (Calle et al. 1989, Wells et al., 1990). In Calle's study, 60 % (12/20) of the orang-utans responded positively to at least one tuberculin that was injected intradermally. Most animals reacted positive to MOT followed by *M. avium* PPD and *M. bovis* PPD. Since none of the 20 tested individuals had evidence of tuberculosis, as revealed by physical examination, hematologic and serum biochemical tests, thoracic radiography, mycobacteriological examination of gastric lavage samples and ELISA, it is postulated that immunological response was associated with the presence of non-tuberculous

mycobacteria isolates. Eleven of twelve individuals were positive for mycobacteria other than tuberculosis (MOTT) in gastric lavage samples, and nine of those reacted positively in at least one TST. It is therefore strongly recommended to carefully interpret single test results in species that have not been tested as yet, or those with high levels of false positive results. A positive reaction is not necessarily a positive TB case, but in any way a challenge for the responsible veterinarian or official. It is open to question, how, for example, orangutans infected with MOTT react in the interferon-gamma test.

Given all these issues, a positive TST has to be confirmed by more definitive diagnostic methods like thoracic radiographs, broncho-alveolar or gastric lavage, serology, and, finally, isolation and culture of the bacteria.

3.2. Interferon-gamma test

Whole blood interferon gamma release assays (IGRAs) should be considered as an alternative or as a complementary tool to the skin test. Both tests assess the cellular mediated immunity towards mycobacteria. IGRAs detect cellular reactivity to antigens through the measurement of interferon-gamma (IFNγ) production by live lymphocytes contained in a whole blood sample. IFNγ is an important cytokine involved in the cell-mediated immune response to mycobacteria. The assays may discriminate between *M. bovis* and *M. avium* infection by exposing blood samples to avian and bovine PPD. Instead of PPDs, single antigens (specific of mycobacteria groups or complex) may be used in the test to provide more specificity.

Recently, a whole blood stimulation assay has become commercially available as the so-called PRIMAGAM® test licensed for use in cynomolgus and rhesus macaques. The assays may discriminate between *M. tuberculosis* complex and *M. bovis* infection by including avian and bovine PPD separately in the test. The amount of interferon produced in response to

stimulation with different antigens can be used to differentiate a reaction due to *M. bovis/tuberculosis* or atypical mycobacteria (Lerche et al. 2008). It is a quantifiable diagnostic test with good sensitivity and specificity compared with disease status determined by pathologic examination (Garcia et al. 2004 a, b Vervenne et al. 2004). Recent results suggest that the IFNγ response to tuberculin antigen may not be reliable in cynomolgus macaques and that another cut-off should be used to read the test (Garcia et al 2004a).

Compared to the TST-test the PRIMAGAM test has a low sensitivity and high specificity (Garcia et al. 2004b, Vervenne et al. 2004, Lin et al. 2008). A combination of both tests may increase the overall sensitivity and specificity.

Whole blood with anticoagulant, preferably lithium-heparin, is needed for testing. As the test relies on (re)stimulation of primed T-lymphocytes, the first compulsory step is to keep blood cells viable for *in vitro* culture. Thus, samples must be available for testing not later than 8-10 hours after sampling and meanwhile should be kept at ambient temperature. Particular care must be paid to homogeneous and full mixing of blood and anticoagulant (heparin) at collection.

IGRA is an *in vitro* test, and thus discards some effects of *in vivo* tuberculin injections (e. g. sensitisation/desensitization). Over the past few years, comparable assays for humans (Quantiferon®, Quantiferon Gold®, T-SPOT-TB®) and cattle (Bovigam®) gave reliable and informative results on the status of TB infection. It is a quantitative diagnostic test with good sensitivity and specificity values. There is only sparse information about the use of Quantiferon Gold® test in nonhuman primates. A comparison of this test with the TST-test showed that the Quantiferon Gold® test might be a highly sensitive tool for the diagnosis of mycobacterial infection in rhesus macaques but it was not possible to detect specific infection with *M. tubucolusis* with this test (Parsons et al. 2010).

However, three important caveats should be known about IGRAs used in NHP. First, there are several different interpretation guidelines, based on different calculation methods

with various cut-off thresholds. In humans, IGRAs are known to have a high variability (>80 %) between individuals. Cut-off values, therefore, might be influenced by the species as well as the individual knowledge.l Experienced technicians are required for the completion of IGRAs, and raw data (optic density) shall be communicated and documented properly. It is essential to have an equalized interpretation scheme. The test implies the use of a mitogen as a positive control. An aliquot of the sample is exposed to this mitogen, which in turn elicits an unspecific application and IFNγ production in lymphocytes. However, this mitogen is usually not provided within the test and should be selected by the lab. Different mitogens can be used (ConA, PHA, SEB, Pokeweed etc.). The level of unspecific stimulation is species dependant and needs to be assessed when an IGRA gets established. For instance, a human IGRA (Quantiferon®) could be considered to be used for some species of NHP (apes) but contains PHA as a mitogen, which is unfortunately known to be a weak stimulant in NHPs. Lab technicians who perform IGRA must be aware of species/mitogen differences. Unfortunately, yet, the majority of NHP species lacks such information. It is therefore recommend to use two or more different mitogens to test NHP species with paucity of information about the most suitable mitogen.

Third caveat is the detection of IFNγ itself. IGRAs rely on ELISA or ELISPOT that are design to reveal IFNγ molecules for certain species. Through all the NHP taxonomy, IFNγ varies in composition and structure, and there is no broad ELISA that is able to detect interferon of all species. For instance, ELISA embedded in the PRIMAGAM® test is a "monkey INFγ" ELISA, but unable to detect IFNγ in most New World monkeys.

Therefore the parallel use of TST and PRIMAGAM® test is advisable for an optimal coverage in a diagnostic regimen. TST might influence IGRA results when performed a few days before blood is sampled. As observed in other species (cattle, human), a "booster effect" could occur with the previous injection of tuberculin strengthening the immune response obtained on subsequent IGRA. Said to increase sensitivity of the test, questions may arise on

the influence on its specificity, as the boost effect may be created only by tuberculin sensitization. Therefore, the use of a booster procedure cannot be recommended at that stage. Further studies are needed to answer those questions.

3.3. Serological tests: antibody detection

Recent work suggests that the serological detection of antibodies against *Mycobacterium*-specific antigens might be a useful tool for the development of an immunodiagnostic method for tuberculosis (Kanaujia et al. 2003). There is a strong association between tuberculosis in NHPs and an immune response against early secretory antigenic target-6 (ESAT-6), a protein especially secreted by the metabolically active virulent bacilli belonging to the tuberculosis complex. Some indirect ELISA tests are designed and marketed to detect ESAT-6. Other laboratories are proposing ELISA targeting on single antigens like the 10-kDa culture filtrate protein (CFP-10) or broader antigenic compounds from culture filtrates. Purified single antigens are known to increase specificity. However, ESAT-6 has been reported to be present in mycobacteria out of the tuberculosis complex, which might cause cross-reactions that potentially produce false positive results.

In a comprehensive approach, ESAT-6 is one of several antigens used in the PrimaTB STAT-PAK® assay, a new lateral flow test that has been developed as a TB specific serodiagnostic. The test was evaluated in comparison with the intradermal palpebral tuberculin test on NHPs of two different species of macaques and on green monkeys. Some of the animals were experimentally infected with *M. tuberculosis*, and serologic evaluation demonstrated high diagnostic sensitivity (90 %) and specificity (99 %) (Lyashchenko et al. 2007).

This commercial test is handy and easy to use and works with serum, plasma or possibly any other antibody containing body fluid. It may, therefore, be an attractive option for institutes with limited capacity. This serodiagnostic test employs a selective array of recombinant *M.*

tuberculosis proteins covering several immunodominant mycobacterial antigens for immune detection. A combination of PrimaTB STAT-PAK® assay and the TST seems to be a sensitive and reliable diagnostic approach for the detection of TB in NHPs, as it includes both ways of immune-reaction towards tuberculosis infection. Yet, scientific validation of the test was assessed for three species of Old World monkeys only. Several studies, unpublished or in press, report its use in free ranging and captive primate species with lower sensitivity and specificity than in *Macaca* species. However, as antibody detection in culture positive NHP species, other than the three published species, was already noticed, serological tests remain an interesting ancillary tool for the TB screening in monkey species.

As for IGRAs, an anamnestic rise in antibody titres has been observed in some species (cattle, elephant) on serum samples collected between 1 and 3 weeks after tuberculin injection. Even if not yet reported in NHP, it is likely to occur in primate species as well and, in absence of any validation of this effect, serologic evaluation should be separated from TST by at least 2 months.

4 Direct examination

4.1. Culture: the Gold standard

The presence of acid-fast-bacilli (AFB) on a sputum smear or other specimen may indicate TB disease. Acid-fast microscopy is easy and quick, but has its limitations. Some acid-fast-items are not *M. tuberculosis* (*Nocardia*, some coccidian, sperm heads). Ziehl-Neelsen is the stain of choice. A positive result has to be confirmed by culture,

Bacterial culture is required on all initial samples to confirm the diagnosis of a positive suspicion. A positive culture for *M. tuberculosis* confirms the diagnosis of TB infection. Culture examinations should be completed on all suspected samples regardless of AFB smear results. Culture should last at least 8 weeks before a final conclusion can be made. Samples to be cultivated for mycobacteria from clinical materials using Löwenstein-Jensen agar or other

suitable cultivation media (liquid, semi liquid) include

- gastric lavage with acid fast cytology and culture of gastric mucus
- tracheal or broncho-alveolar lavage with acid fast cytology and culture of tracheal mucus
- fecal examination with acid fast staining and culture
- biopsy of altered or unaltered organs (lymph nodes) with acid fast stain and culture
- bio-fluids such as urine, pleural or abdominal effusion, cerebrospinal fluid, milk etc.

Although they belong to the group of “slow-growing mycobacteria”, culture methods are highly standardized for *M. tuberculosis* and *M. bovis* with significant reduction of culture delay. However, for other mycobacteria from the TB complex, such as *M. pinnipedii* or Dassie Bacillus (*M. tuberculosis* complex sp.), a waiting period of at least 8 weeks is strongly recommended before declaring the culture negative.

Finding NTMs out of gastric lavage or stools is common. In the case of NTM discovery on a suspected or TST positive animal, the conclusion of false positivity due to cross reaction should not be taken too quick, as NTM and TB complex mycobacteria infection can occur simultaneously.

4.2. Molecular biological diagnostic

Polymerase chain reaction (PCR) can be used to detect mycobacterial DNA in any biological samples and intrinsically has the advantage of being much quicker than the conventional culture methods of diagnosis (one day *versus* several weeks). Detection of infection by screening faeces or sputum for mycobacterial DNA may therefore be considered as a rapid and alternative diagnostic tool. Commercial laboratories often consider PCR on pure culture, only, and therefore may refuse to run PCR on non-purified sample material. Moreover, sensitivity of PCR will be likely lower compared to pure culturewhen applied to biopsies or

stools (presence of inhibitors etc.).

Beyond PCR, other DNA fingerprinting methods are available such as spoligotyping or other strain identification process. Their main interest is to identify strains of mycobacteria, and then track epidemiological circulation and origin of the infection. These methods are mainly applied on pure culture, but some laboratories accept to perform it on samples, provided that the number of bacilli inside is high enough.

5. CONCLUSION

Intradermal testing alone is not sufficient to adequately diagnose tuberculosis. Each of the methods listed include the risk of false positive or false negative reaction. Therefore, a combination of the TST test with an IFNγ assay or a lateral flow assay is advisible to increase the sensitivity and specificity. In doubtful cases, a range of test methods relying on different aspects of the pathogen (immunology, serology) should be applied and the results should be evaluated critically. During evaluation, the veterinarian has to set established laboratory and species specific cut-off values for most of the available tests, and this is directly influencing the predictive positive and negative values.

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