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Potential Application of Patho-TB test for Rapid Laboratory Diagnostic of Bovine Tuberculosis in Suspected Lesion

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Bovine tuberculosis is a zoonotic disease that is caused by *Mycobacterium bovis*. Rapid detection of disease can play an important role for controlling; Therefore researches for developing of rapid diagnostic tests with high sensitivity and specificity are considered. Patho TB test is one of considering tests for rapid detection in human that has been developed recently. In this study using of patho TB test for suspected lesion of bovine tuberculosis in cattle were examined. One hundred suspected samples that were transferred to bovine tuberculosis reference laboratory in Iran were evaluated by classic culture as gold standard, MGIT culture, PCR and patho Tb test; sensitivity and specificity of each one were determined. By performed investigations, sensitivity and specificity of MGIT culture were 100%. Also sensitivity and specificity of PCR and patho Tb test were evaluated 100% and 88% respectively. The results have been shown patho-TB test can be used for detection of bovine tuberculosis in suspected lesions with high viscosity either.

Keywords: Patho-TB Test, MGIT, PCR, *Mycobacterium bovis*.

Bovine tuberculosis (bTB) which caused by *Mycobacterium bovis* is one of the major animal diseases that are known in different countries of the world and is still highly regarded. The single intradermal comparative cervical tuberculin test (SICCT), more commonly known as the "Tuberculin skin test", is used in many countries to identify bTB "Reactor" animals, which are then compulsorily slaughtered. After scarification of animals and to confirm *M. bovis* infection, inspectors cut some observable tissues on lymph nodes of head, chest and lungs of animals which are suspected to *M. bovis* infection. Moreover, to compare the observable lesions taken from animals suspected to *M. bovis* infection, some samples are also provided from bTB-reactor animals with no visible lesions (NVL) and confirmation of diagnosis of bTB is reliant on successful isolation of *M. bovis* by culture; indeed Culture is the gold standard for the detection of mycobacteria with a sensitivity between 70% and 90%. Polymerase chain reaction (PCR) is another technique for the direct detection of MTB in clinical specimens. PCR sensitivity varies in this case so that it violates 95% for smear positive specimens but ranging 40%-77% for smear negative cases[1].

Besides, there are other diagnostic methods like utilization of various mycobacterial specific antigens by serodiagnosis which is used to detect tuberculosis. Since rapid diagnosis of tuberculosis, especially in humans can be very important for disease control, efforts to develop rapid diagnostic kits are considered; as most

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recently Patho- TB Test has been designed in humans.

Patho-TB test (Anda-RT mycobacteria Patho-TB;ANDA biologicals, Strasbourg, France) is a direct observational method which can detect bacilli retained on a filter with antibodies directed against them. The antibodies should be purified to have an appropriate efficacy against TB bacilli especially 35, 65 and 85 kDa mycobacterial antigens. In the following, these antibodies are made to react with a gold conjugate. The presence of bacilli is evidenced by the appearance of a central red-pink color on the filter. In this study, the application of the Patho-TB test for the diagnosis of bovine tuberculosis in the suspected lesions was assessed and compared with classic culture, Mycobacteria Growth Indicator Tube culture (MGIT) and PCR as conventional bacteriologic techniques[2,3,4].

MATERIALS AND METHODS

Collecting samples

One hundred samples of animal lesion suspected to Bovine tuberculosis which were sending from the General Administration of Veterinary Organization of Iran to Razi Vaccine and Serum Research Institute bovine tuberculosis reference laboratory were considered as target samples in the years 2004 and 2006 and to better evaluation they were classified in four groups of positive and negative tuberculin with visible and invisible lesions and were stored at -20°C.

II: Culture & Bacteria Isolation

There are important points that should be considered for collecting and maintaining samples; one of them is using sterile equipments. Pooling, grinding and homogenizing of each sample were done with applying sand in a mortar. The specimens were decontaminated using Petrof method; briefly by adding 20 ml NaOH 1M in a mortar and waiting for 15 minutes then about 5ml supernatant was neutralized in a universal tube containing hydrochloric acid and the provided mixture was centrifuged at 3500 rpm for 15min[5]. Supernatant was discarded and a suspension was provided from obtained plette and this suspension was used for cultures of classic and MGIT and also for PCR and patho Tb test.

Culturing the obtained suspension was done in slant of Lowenstein– Jensen (LJ) medium complemented with glycerol (LJG) and one more slant complemented with pyruvate (LJP) and incubated for eight weeks at 37 °C.

For preparing the MGIT culture tubes (Becton Dickinson, Maryland, USA), a vial of 15 mL OADC enrichment (oleic acid, albumin, dextrose and catalase) was well-mixed with a vial of PANT antibiotics (polymixin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin) before culturing and 0.8 ml was added to each MGIT culture medium by an insulin syringe. At the end, 200 Micro liter of previous suspension was added to MGIT culture and was evaluated during 2 weeks[6,7].

DNA extraction and PCR test

For DNA extraction, Vansoolingen et al method was applied and Forward16SrRNA and 16SrRNA-Reverse primers and INS1 and INS2 primers (according to standard procedures) were used respectively in order to identify the Mycobacterium family by observing a band with the weight of 543bp and *Mycobacterium tuberculosis* Complex by observing a band with 245bp[8].

The characteristics of the reaction are as follow

Denaturation at temperature of 94°C for three minutes along with 25 cycles at temperature of 94°C as long as 60 s, 65 °C for 1 minute, 72 °C for 1 minute and the ultimate extention at temperature of 72 °C for 4 minutes.

Patho- TB test

Patho-TB test was done concurrent with MGIT Culture and Classic culture. This test was carried out in accordance with the manufacturer's instructions. The suspension which used for previous was boiled for 5 minutes and 100 micro liter was transferred to filtering cartridge. Once the sample absorbed, the prefilter was washed. Then, the prefilter is discarded and the filter washed. In this experiment, it was found that the presence of bovine TB bacilli leads to immobilization of sample available on the filter.

Then rabbit immunoglobulin G (IgG) antibodies that react with all *mycobacterium tuberculosis* antigens was added, creates the formation of antigen-antibody complex which were revealed in the last step by addition of a gold conjugate. Once more, red–pink color at the central part of the filter acts as a good indicator to evidence the test positivity.

Statistical analysis

Investigation of Sensitivity and Specificity by formula "Sensitivity= true positives/ (true positive + false negative)" and "Specificity=true negatives/(true negative + false positives)" was determined. Classic culture was used as the "gold standard".

RESULTS

In categorization of samples, 15 samples of positive tuberculin cows with visible lesions (Group I), 50 samples of positive tuberculin cows with invisible lesions (Group II), 4 samples from negative tuberculin cows with visible lesions (Group III) and 31 samples of negative tuberculin cows with invisible lesions (Group IV) were determined. In the first group all 15 samples were evaluated positive in classical culture, MGIT, 16sRNA-PCR, IS6110-PCR and rapid diagnostic Patho TB test(Table 1).

In the second group, 42 same samples in classical culture, MGIT, PCR16srNA, IS6110-PCRs and patho Tb test were evaluated positive, and 8 same samples were evaluated positive in assessing PCR16srNA, IS6110-PCRs and in patho TB test that did not correspond with classical culture and MGIT cultures. In the third group, all 4 samples were evaluated positive after assessing tests and in the fourth group all samples were identified negative in all four tests. With regard to the classical culture as the gold standard, the sensitivity and specificity of MGIT culture of both of them was calculated as 100 and the sensitivity and specificity of 16sRNA, IS6110-PCRs and patho Tb test of both was estimated at 88 and 100 respectively(Table 2).

DISCUSSION

Trying to design cheap and quick methods of diagnostic tuberculosis has always been considered by the World Health Organization (WHO) and World Organization for Animal Health (OIE). In fact, the rapid detection of pathogenic Mycobacteria can play a very important role in reducing the spread of disease in humans and also for evaluating tuberculin test and judging on other livestock of herd(9), for example, the experience of using MGIT culture media during 2000-2009 has caused a 20% reduction of disease in humans[10]. The sensitivity and specificity of diagnostic methods is calculated with regard to the classical culture as the gold standard and despite grouping was based on the tuberculin test and observing or not observing visible lesions but tuberculin test was not examined in this study in terms of sensitivity and specificity. Regarding the MGIT culture, sensitivity and specificity was calculated as much as 100%. However, in other reports on clinical and non-clinical samples of Tuberculosis

Table 1. The results of MGIT culture, PCR, Patho-TB test with classic culture in four groups

	Group 1	Group 2	Group 3	Group 4	Total
Number of Samples	15	50	4	31	100
Positive Classic Culture	15	42	4	0	61
Negative Classic Culure	0	8	0	31	39
Positive MGIT Culture	15	42	4	0	61
Negative MGIT Culture	0	8	4	31	39
Positive PCR	15	45	4	0	69
Negative PCR	0	5	0	31	31
Positive Patho- TB test	15	45	4	0	69
Negative Patho- TB test	0	5	0	31	31

Table 2. Correlation between results of patho-TB test and classic culture

Patho TB-test	No of cattle (n=100) Positive classic Culture (n=61)	Negative Classic Culture(n=39)
Positive	61	8
Negative	0	31

illness, MGIT sensitivity than LJ environment for clinical and subclinical cases is 80 to 90 %for MGIT and 59 to 87 & for the subclinical[11,12]. In this study, considering the type of sample that were suspicious lesions, such results are not far-fetched. The sensitivity and specificity obtained in PCR despite using Vansooligen method for DNA extraction that provides us with the greatest amount of DNA were assessed 90 and 74 percent respectively. This study aims at investigating Patho-TB kit for diagnosing Bovine TB and comparing it with culture examination and PCR in our research area. There are some features of the test that make it very suitable for this goal; rapidity and easiness to perform can be pointed out. Besides, it does not require any special equipment or proficiency and gives us the chance of researching on many samples in a short time[13,14]. Patho-TB test is very important and efficient in diagnosing TB rapidly compared to microscopic examination of ZN-stained smears that takes much time in particular for low-loaded MT slides. Molecular methods such as polymerase chain reaction are complicated and expensive and need skillful experts.

In this study as Gold standard was classic culture, sensitivity and specificity could be problematic for a variety of reasons in classical culture:

1. Small numbers of mycobacteria in the selected tissue leading to false negative in classic culture.
2. Chemical decontamination particularly during the decontamination period can significantly kill viability of *M. bovis* and reduce living bacteria.

Some points are noteworthy in interpreting the results of patho TB test. Firstly, this test is designed to detect the disease in humans and the used samples like Sputum, CSF and gastric lavage have low viscosity.

But in the animal waste with high viscosity, boiling method mentioned for human subjects in Kit instruction did not have a good performance (results not shown) and the suspension obtained from grinding and decontamination in classical culture was used. In fact, this study is the first report to evaluate this kit for samples with high viscosity (such as suspicious tissue lesions) and introduces a new recipe for these samples.

Sensitivity and specificity of this test with considering the results of classical culture as the gold standard was estimated as 88 % and 100 % and these numbers were estimated as 95 % and 100 % in other studies. A similar study in this regard for human tuberculosis indicated numbers 90 % and 100 % respectively[2]. The difference of the specificity obtained in this study can be due to the viscous properties of tissue samples compared with human samples and changes in the method of providing suspension or related to the problems of culture (low number and killing *Mycobacterium bovis* in the process of decontamination) which was noted before.

The sensitivity and specificity of Pato-TB test is perfectly matched with PCR sensitivity and specificity that with considering the high cost of PCR and requiring special laboratory facilities in versus to easy use and not requiring special laboratory facilities are considered in this test. The results show that this kit can be used with the modified instruction changed in this study for samples with high viscosity.

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