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Diagnostic Potential of GeneXpert MTB/RIF Assay For *Mycobacterium bovis* Infection in Cattle

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Mycobacterium bovis (*M. bovis*) is a bacterium that causes bovine tuberculosis (bTB) in cattle, and can infect humans. Accurate diagnosis of *M. bovis* infection is crucial for disease control in animals and in turn, protects people from getting the infection. This study aimed to evaluate the sensitivity and specificity of the so-called GeneXpert MTB/RIF assay in detecting *M. bovis* in the samples collected from cattle suspects. A retrospective study was conducted, including 50 dairy cows with a history of bTB. The GeneXpert technique, direct microscopy, and sample cultivation were carried out in parallel on all the samples to evaluate the diagnostic accuracy of each. GeneXpert showed higher positivity rates compared to the culture. Out of the examined 50 cattle lymph nodes from different slaughtered animals, 30 were GeneXpert-positive, and culture-positive, while 20 were GeneXpert-negative, and culture-negative. The performance of GeneXpert on animal lymph node samples demonstrated reliable detection capabilities. The study highlights the diagnostic potential of GeneXpert in detecting *M. bovis* infection, and its usefulness in diagnosing and monitoring infections in animal populations. GeneXpert offers improved sensitivity compared to conventional methods, and can aid in the accurate detection of *M. bovis* infection in clinical samples.

Keywords: Cattle, GeneXpert, Tuberculin-skin-test, *M. bovis*.

Introduction

Mycobacterium bovis, the causative organism of bovine tuberculosis (bTB), is a bacterium that primarily affects cattle, but can also infect other animals and humans. It has both economical and zoonotic importance due to its impact on livestock and public health. *Mycobacterium bovis* is closely related to *M. tuberculosis,* and poses a significant threat to the public health and agricultural economies worldwide. Human infection can occur through direct contact with infected animals or consumption of contaminated dairy and meat products [1, 2].

Mycobacterium bovis exhibits intracellular survival and replication within the host macrophages, primarily transmitted through the inhalation of aerosols containing the bacteria [3]. Upon inhalation, the bacterium is phagocytized by alveolar macrophages in the lungs, where it can evade the immune response and establish infection [4]. The interaction between *M. bovis* and the host immune system determines the outcome of the infection, which can range from clearance of the pathogen to the development of active disease, or establishment of latent infection [5].

Accurate and timely diagnosis of *M. bovis* infection in animal carriers is crucial for an effective disease control and prevention [6, 7]. Furthermore, previous studies have demonstrated the efficacy of GeneXpert in detecting extrapulmonary tuberculosis [8]. However, the sensitivity and specificity of GeneXpert in detecting *M. bovis* specifically in respiratory samples, both smear-positive and smear-negative, of bovine origin, need to be investigated.

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Accordingly, the objective of the present study was to evaluate the efficacy of the GeneXpert assay in detecting *M. bovis* in bovine lymph nodes with suspected lesions. The obtained results were then compared with direct smear microscopy using the Ziehl-Neelsen (ZN) stain, and sample cultivation, providing valuable insights into the diagnostic accuracy of different methods for detecting *M. bovis* in clinical samples.

Material and Methods

Study Design

This study focused on the examination of 50 lymph nodes from slaughtered animals that belonged to dairy herds with a documented history of bTB. Screening of the dairy herds was done using the comparative intradermal tuberculin test (CIDTT). All the samples were subjected to GeneXpert testing employing the real time PCR as directed by the kit supplier (Cepheid, CA, USA).

The comparative intradermal tuberculin test

After clipping and cleaning of the injection site, a fold of skin was measured with calipers and 0.1 ml of each of bovine and avian PPD was inoculated intradermally with an approximate distance of 12–15 cm between the two sites. The skin-fold thickness of each injection site was measured after 72 hours. The test was interpreted as positive, negative, or doubtful according to the difference in the skinfold thickness at both injection sites. By subtracting the thickness of avian PPD site from that of the bovine PPD sites, the test result was considered positive, negative or doubtful when the difference was >4 mm, <3 mm, or between 3 and 4 mm, respectively. Positive cases were subjected to culling and post mortem examination [9, 16].

Laboratory Examination

Subsequent postmortem examinations were carried out to examine the lungs, liver, kidneys, udders, and regional lymph nodes for any gross lesions associated with bTB, such as granuloma formation or caseation and calcification of the lymph nodes. Samples were collected from lesions and transported to the laboratory for bacteriological and molecular examination. All bacteriological examinations were carried out as described by the OIE manual [9]. Smears were prepared from the lesions and stained with the Ziehl-Neelsen method for the detection of the acid-fast bacilli of *M. bovis.* To proceed for culturing, the specimen was homogenized by grinding followed by decontamination with 3% sodium hydroxide and then neutralized. After centrifugation, the sediment was used for inoculation onto Lowenstein–Jensen medium bottles and incubation for 10–12 weeks at 37°C. *M. bovis* isolates were identified by determining traditional cultural and biochemical properties [9, 17].

Susceptibility testing of *M. bovis* isolates was performed according to the recommendations of Clinical and Laboratory Standards Institute (CLSI). Middlebrook 7H11 agar plates with and without the test drug were utilized with 1 mg rifampicin/ml of the medium After three weeks of incubation at 37 8C in 5% CO2, the isolate was considered resistant if the number of colonies on the medium containing rifampicin, relative to that observed on a drug-free medium was 1% [18].

GeneXpert MTB/RIF Assay

In this study, the bacterial load in animal lymph node samples was performed using the Xpert MTB/RIF Ultra assay (Cepheid, Sunnyvale, CA, USA), an automated nucleic acid amplification (NAA) test. This technology allows the detection of *Mycobacterium tuberculosis* complex (MTBC) deoxyribonucleic acid (DNA) using a standardized method.

Briefly, the quantification of mycobacterial DNA was determined using real-time PCR and reported as the quantification cycle (Cq). For targeting the *rpo*B gene, a set of five probes was employed. The GeneXpert (GX) instrument, in accordance with the manufacturer's instructions, was employed along with a single-use cartridge. Briefly, each sample (one part) was mixed with GX reagent (two parts), incubated for 15 min at room temperature with intermittent mixing, transferred to the cassette (2 ml aliquot), and subsequently ran on the GX device [10].

The reported Cq values from the analysis of the five probes were averaged to estimate the bacterial count. To analyze the data generated, the GX software version 4.3 was employed. The mean Cq values were categorized into four groups based on their magnitude: very low (Cq > 28), low (Cq 22-28), medium (Cq 16-22), and high (Cq < 16) [11].

Meanwhile, the extracted DNA was subjected to PCR amplification using the primers, Tbc15'-CGTACGGTCGGCGAGCTGATCCAA-3' and TbcR5 5'-CCACCAGTCGGCGCTTGTGGGTCAA-3', which were designed to amplify specific DNA fragments of the *rpo*B gene encoding the RNA polymerase β-subunit for MTBC (*M. bovis*), measuring 235 base pairs. The PCR amplification was carried out under the following conditions: an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 68°C for 30 seconds, extension at 72°C for 60 seconds, and a final elongation step at 72°C for 10

minutes. Subsequently, the PCR products were separated using 1.5% agarose gel electrophoresis [12].

Statistical analysis

The Pearson chi-square test was utilized to establish a meaningful comparison of values between culture and various other tests [13]. Furthermore, this test was employed to carefully examine the distribution of categorical variables within the culture-negative/GeneXpert-positive, and culture-positive/GeneXpert-positive sets. The Statistical significance was attributed to variations with a value less than 0.05. All statistical analyses were conducted utilizing SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Tuberculin test results

As depicted in table 1, out of 100 cattle tested with the comparative intradermal tuberculin test, 20 were negative, 18 were doubtful and 63 reacted positively (40 showed over reaction).

M. bovis isolation and rifampicin susceptibility

Out of 50 lymph node samples, 30 resulted in the recovery of *M. bovis* of which 2 and 5 were obtained from negative and doubtfully reacted animals, respectively. Meanwhile, 14 and 9 isolates were recovered from over reactor and positive animals, respectively. Concerning the susceptibility to rifampicin, only one isolate was resistant and 29 were susceptible (table 1).

Performance of the GeneXpert assay

The performance of the GeneXpert assay on 50 animal lymph node samples collected from suspected post mortem lesions in slaughtered cattle (table 1). Out of 42-suspected samples based on acid fastness, 38 were found positive with the GeneXpert assay (90.47%).

Discussion

The accurate diagnosis of *M. bovis* is the key for devising programs for eradication of tuberculosis from cattle and other domestic animals as well as to protect humans from the zoonotic transmission of the disease. Isolating the causative organism from post-mortem specimens is the gold standard test for diagnosis of bTB as it identifies all infected cases with 100% sensitivity and specificity [19, 20].

Accordingly, the aim of this study was to evaluate the diagnostic potential of GeneXpert in the detection of *M. bovis* in bovine samples. Samples were examined using direct microscopic smearing to identify acid-fast bacilli (AFB). Direct microscopy of Ziehl-Neelsen stained smears is the simplest way for the detection of acid-fast *M. bovis* in tissue impressions. However, this method was found of low significance in bTB control programs [21]. In addition, bacterial culture and GeneXpert amplification were performed to enhance the diagnostic process.

In this study, out of 50 lymph node samples, 30 resulted in the recovery of *M. bovis* of which 2 and 5 were obtained from tuberculin-negative and doubtfully reacted animals, respectively. Meanwhile, 14 and 9 isolates were recovered from over reactor and positive animals. This confirms, once more, the importance of tuberculin test in leading bovine tuberculosis eradication programs in Egypt [16].

Concerning bacteriological isolation, accurate diagnosis is based on the ideal performance of all steps of the process including samples number, collection manner and cultivation methods. In most instances, there may be little doubt as to the accuracy of a positive isolation, except for the risk of cross-contamination between samples. If suboptimal steps are performed, severe bias in the cases detected is suggested with possible undetected early or latent infected animals. On the other hand, an insensitive immunological assay may lead to arguments about infection prevalence as well as sensitivity and specificity **[22]**.

To establish the veracity of the mycobacterial culture, a comprehensive analysis utilizing differential PCR was conducted on the entirety of the isolated DNA derived from all the samples encompassed within the present investigation. The results of PCR analysis substantiated that every single sample procured from animal origin unequivocally demonstrated the presence of *M. bovis*.

What is called GeneXpert amplification kit was produced to target human samples. To ensure a comprehensive evaluation, we extended our investigation to include the performance of GeneXpert on 50 animal lymph node samples collected from suspected post mortem lesions in slaughtered cattle (table 1). Out of 50 lymph nodes, 42 were positive on acid fastness of which 38 were found positive with the GeneXpert assay (90.47%). As compared with the results of bacteriological isolation (71.42%), the GeneXpert was found more sensitive. Of the 38 positive GeneXpert samples, 30 showed positive culture results, confirming the presence of the target organism while, 8 samples resulted in negative cultures, indicating a higher sensitivity of GeneXpert in tuberculosis detection when compared with the conventional culture techniques.

It has been mentioned that that PCR improved the detection of *M. bovis* in tissues of infected cattle. In

addition, the diagnosis time was reduced from 8 weeks to 48 hours. The GeneXpert assay is a real time PCR method which is even faster and more sensitive than the ordinary PCR [17].

In terms of drug resistance, 97.36% (37/38) of the positive samples exhibited susceptibility to rifampicin, while only one sample demonstrated drug resistance. This finding can be due to absence of a control regimen of bTB by treatment of the infected animals. Consequently, drug resistance phenotype is rarely encountered in *M. bovis* recovered from animals. Treatment of bTB is prohibited by EU legislation as it is not effective. Alternatively, TB-positive reactors must be slaughtered, and movement restrictions are placed on the herd **[23]**.

Overall, the GeneXpert performance on animal lymph node samples demonstrated reliable detection capabilities, allowing for accurate identification of the target gene. These results emphasize the importance of GeneXpert as a valuable tool in diagnosing and monitoring infections in animal populations [7, 14, 15].

TABLE 1. Characteristics of animal samples, to evaluate the performance of GeneXpert, in comparison with mycobacterial culture for the diagnosis of bovine tuberculosis

Characteristics	Lymph nodes# (%)		Lymph nodes# (%)		OR (95% CI)	P-value
	Culture positive	Culture negative	GeneXpert positive	GeneXpert negative		
Skin Test						
Negative (0)	2 (2.0)	8 (8.0)	4(40.0)	6(60.0)	Ref.	-
1 – 4	5 (55.55)	4 (44.44)	6(83.33)	3(16.67)	0.25 (0.03–2.07)	0.17
>4	9 (81.8)	2 (18.2)	11(100.0)	0 (0.00)	0.31 (0.04–2.55)	0.25
Over reactors (>10)	14 (70.0)	6 (30.0)	17(85.0)	3(15.0)	5.34 (2.51–11.35)	0.75
Total	30	20	38	12		
Antibiotics sensitivity (Rifampicin susceptibility)						
Sensitive	29 (59.18)	20(40.81)	37(75.51)	12(24.89)	Ref.	-
Resistance	1 (100.00)	0(0.00)	1(100.00)	0(0.00)	0.25 (0.03–2.07)	0.17
Total	30	20	38	12		

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الكفاءة التشخيصية لطريقة جين إكسبرت MTB/RIF لعدوى ميكوباكتيريام بوفيس في الأبقار

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إن ميكوباكتيريام بوفيس هي بكتيريا تسبب السل البقري في الماشية و قد تعدي للإنسان. و يعتبر التشخيص الدقيق لعدوى السل البقري في الماشية أساسي للسيطرة على المرض في الحيوان و بالتبعية منع انتقاله للإنسان. وقد استهدفت هذه الدراسة تقييم حساسية و نوعية مايسمى باختبار جين إكسبرت MTB/RIF للكشف عن ميكوباكتيريام بوفيس في عينات مأخوذة من أبقار مشبتبه بإصابتها. وقد تمت الدراسة على عدد بقرة متوقع إصابتها بالسل البقري. وتم اختبار العينات باستخدام طقم الجين إكسبرت التشخيصي بالإضافة إلى الفحص المجهري للعينات والزرع البكتيري لعزل الميكروب المسبب و ذلك لتقييم الكفاءة التشخيصية لكل طريقة. أظهرت طريقة الجين إكسبرت كفاءة عالية لتشخيص السل البقري في العينات المختبرة حيث كانت نسبة الإيجابية عالية مقارنة بالعزل البكتيري. حيث أنه من بين 50 عقدة ليمفاوية لحيوانات مختلفة، كانت 38 إيجابية مع جين إكسبرت مقابل 30 فقط إيجابية لعزل بكتيريا السل البقري. و قد أظهرت طريقة الجين إكسبرت قدرة عالية مع العقد الليمفاوية مباشرة. و قد سلطت الدراسة الضوء على الكفاءة التشخيصية لطريقة الجين إكسبرت في الكشف عن عدوى ميكوباكتيريام بوفيس و أهميتها في الكشف و التحري عن العدوى بين التجمعات الحيوانية. وقد حسنت طريقة جين إكسبرت من حساسية الكشف عن الميكوباكتيريام بوفيس مقارنة بالطرق التقليدية و بالتالي ستساعد على الكشف الدقيق عن وجود بكتيريا السل البقري في العينات الإكلينيكية.

الكلمات الدالة: الماشية، جين إكسبرت، اختبار تيوبركلين، ميموباكتيريام بوفيس.