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Original Research Article

A qualitative immunoassay as complementary test with tuberculin skin test for detection of tuberculosis in dairy cattle

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ABSTRACT

Bovine tuberculosis, caused by *Mycobacterium bovis*, is a zoonotic disease causing approximately 6% of total human deaths. Its economic losses are not only a reduction of 10-20% in milk production and weight, but also infertility and condemnation of meat. Many serological tests are applied for detection of tuberculosis. ELISA test has the highest sensitivity and specificity than the other serological tests for the diagnosis of tuberculosis. Several forms of new technology were brought into the diagnostic approach to mycobacterial infection. The aim of this work was to detect bovine tuberculosis by application of different serological tests. Tuberculin skin test was applied on 2650 cattle, only 63(2.4%) were positive. Forty eight (76.2%) of the slaughtered positive animals showed visible lesions (VL) while the other 15 (23.8%) had non-visible lesions (NVL). The bacteriological examination of the 63 samples revealed isolation of *M. bovis* from 47 processed samples (74.6%). The results of the immunoassay test have detected 27 out of the tuberculin positive cattle, while the ELISA has detected 34 out of the positive reactor cattle. It was concluded that immunoassay and ELISA tests act as complementary tests for tuberculin skin test especially in anergic cattle.

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1. Introduction

Bovine tuberculosis (bTB) caused by *M. bovis*; a member of the *M. tuberculosis* complex, is a zoonotic disease having considerable economic and public health implications (Neill et al., 1994; O'Reilly et al., 1995). It is a worldwide disease that causes a great harm on dairy farms and poses health risks to the population that consumes products of animal origin. It infected 50 million cattle worldwide resulting in economic losses of approximately $3 billion (Hewinson, 2000).

The disease has been difficult to control in livestock because of the lack of an effective vaccine and the lack of a diagnostic assay with sufficient sensitivity and specificity to detect animals at all stages of infection. Currently the primary methods used for the detection of TB in cattle include the measurement of a delayed-type hypersensitivity (skin test) to purified protein derivative (PPD) (Monaghan et al., 1994).

Use of ELISA with the tuberculin skin test (Plackett et al., 1989) to overcome the problems of tuberculin development of an accurate serodiagnostic test requires a detailed understanding of the humeral immune response during bovine tuberculosis and, in particular, identification of the key antigens of *M. bovis* involved in antibody production (Lyashchenko et al., 1998).

Serological survey was carried out to determine the presence of antibodies against components of the culture filtrate protein extract by ELISA (Diaz-Otero et al., 2003), short term culture filtrate (ST-CF) was separated into molecular mass fractions and screened for recognition of ELISA (Pollock and Andersen, 1997).

Several forms of new technology were brought into the diagnostic approach to mycobacterial infection. Advances in humoral responses tests led to development of lateral flow tests which qualitatively detect *M. bovis* antibody in serum or plasma (Greenwood et al., 2003).

The aim of the study was to compare sensitivity of lateral flow test and ELISA for diagnosis of bovine tuberculosis.

2. Materials and methods

2.1. Animals

A total of 2650 cross-breed dairy cows from different governorates in Egypt were used in this study. All animals were tested with comparative tuberculin test (CTT) using bovine PPD (PPD-B).

2.2. Comparative Tuberculin Skin Test. (OIE, 2009)

Two sites on the right side of the mid-neck, 12 cm apart were shaved and the skin thicknesses were measured with calipers. One site was injected with 0.1ml Bovine PPD tuberculin; similarly 0.1ml avian PPD tuberculin was injected into the second site. After 72 hrs, the skin thickness at the injection sites was measured.

2.3. Serum Samples

From the positive reactors, about 10 ml of blood were obtained aseptically from the jugular vein. The blood samples were left at room temperature for 2hrs in a slope position, then kept at 4°C overnight, centrifuged at 3000 rpm for 15 min, serum was aspirated, labeled then kept at -20°C till use in serological test.

2.4. Post mortem examination

Careful inspection and examination was made simultaneously for the carcass, head and viscera of each slaughtered tuberculin positive reactor animals. The lung, liver, lymph nodes, spleen and heart received particular attention. Depending on the distribution of the lesions, the examined animals were categorized as: Animals with pulmonary TB lesions had lesions in the lung and related lymph nodes, animal with extra pulmonary lesions (had lesions in any parts other than thoracic cavity), animal with mixed TB lesions (had lesions in the lung and in any other organ of the body) and animals with generalized TB lesions.

2.5. Bacteriological isolation and identification of the mycobacterial isolates

The organs, lymph nodes showing growth lesions prepared and stained with Ziehl Neelsen stain. Samples were cultured on Lowenstein Jensen medium after being decontaminated with H_2SO_4. The isolates were identified by conventional methods according to Kubica (1973).

2.6. Enzyme linked Immunosorbent assay (ELISA)

According to Collee *et al*. (1996) using bovine PPD (B-PPD). The optical density was measured at 405 nm using spectra III ELISA reader. Sample was considered positive if yielded a mean OD equal to or greater than the cut off value that is calculated according to El-Seedy et al. (2013) which is equal to the mean OD of negative serum plus two standard deviations.

2.7. Immunoassay kits

- Remove the foil pouch of test kit and place it on a dry, flat surface.
- Label the test units with sample names.
- Add 4 drops of serum slowly to sample well with the specimen dropper and if migration is not appeared after one minute, add 1 more drop of the specimen to the sample well.
- The result is seen as a band in the result window of the kit.
- The results were interpreted within 20 minutes.

Interpretation.

- Negative result: presence of only one coloured band within the result window.
- Positive result: presence of two coloured bands (T and C bands) within the result window (even if the band colour intensity is faint).
- Invalid: if the colour band is not visible after performing the test and the specimen is re-tested.

3. Results

3.1. Tuberculin test

Tuberculin skin test was applied on 2650 cattle. Only 63 animals (2.4%) were positive reactors.

3.2. Post mortem slaughtered tuberculin reactor cattle

Out of 63 tuberculin-reactors; 48 (76.2%) showed visible lesions (VL); of which 40 (83.3%) were localized (respiratory, digestive or mixed} while 8 (16.7%) were generalized. On the other hand, 15 reactors (23.8%) showed NVL (Table 1).

3.3. Bacteriological examination of the tuberculin reactors

Bacteriological examination of the tuberculin reactors revealed that the total acid fast bacilli recovered from 63 slaughtered tuberculin reactors cattle were 50 (79.4%) isolates which were identified according to the morphological characters, growth rate, pigmentation, growth at different temperatures and biochemical tests into 47 *M. bovis* (74.6%) as well as 3 (4.8%) Mycobacteria other than TB (*MOTT*) (Table 2).

3.4. ELISA

The results of ELISA revealed that 34 out of 63 positive reactors (54%) were positive for ELISA using *PPD-B*; 8 with generalized lesions (100%), 22 with localized lesions (54%) and 4 (26.7%) with NVL (Table 3).

3.5. Lateral flow immunoassay

The results of lateral flow immunoassay revealed that 27 out of 63 positive reactors (42.9%) were positive including 7 with generalized lesions (87.5%), 18 with localized lesions (45%) and 2 (13.3%) with NVL (Table 4).

Table 1. The post mortem (PM) findings of tuberculin reactor cattle.

Total slaughtered animals (Positive reactors)	Visible lesions (VL)						Non Visible Lesions (NVL)	
	Total		local		general			
	No.	%	No.	%*	No.	%*	No.	%
63	48	76.2	40	83.3	8	16.7	15	23.8

%: Percentage according to the total No. of Positive reactors. %*: Percentage according to the total No. of VL.

Table 2. Bacterial identification of Mycobacteria isolates from tuberculin reactor cattle.

No. of Positive reactors	*M. bovis*		*MOTT*		Total isolates	
	NO.	%	NO.	%	NO	%
63	47	74.6	3	4.8	50	79.4

MOTT: Mycobacteria Other Than Tuberculosis. %: Percentage according to the total No. of Positive reactors.

Table 3. The results of ELISA test on sera of tuberculin reactor cattle.

Lesions	No. of reactors	ELISA	
		No.	%
General	8	8	100
Local	40	22	55
NVL	15	4	26.7
Total	63	34	54

%: Percentage according to the No. of reactors.

Table 4. The results of lateral flow immunoassay test on sera of tuberculin reactor cattle.

Lesions	No. of reactors	Immunoassay	
		No.	%
General	8	7	87.5
Local	40	18	45
NVL	15	2	13.3
Total	63	27	42.9

%: Percentage according to the No. of reactors.

4. Discussion

Bovine tuberculosis caused by *M. bovis*, characterized by progressive developed granulomatous lesions (tubercles) in any body organ, and affected a large number of species. Tuberculosis is now generally perceived to represent the greatest threat to cattle health and incidence of bovine tuberculosis is rising, both in numbers of herd affected and in the number of cases per affected herd (Cobner, 2003). Bovine tuberculosis infected so million cattle world-wide resulting in economic losses of approximately 3 billion (Hewinson, 2000).

Out of 63 tuberculin-reactor animals; 48 (76.2%) showed VL including 40 (83.3%) localized lesions; either respiratory, digestive or mixed, 8 (16.7%) generalized lesions and 15 (23.8%) NVL (Table 1). Such results are more or less similar to those recorded by Adawy (1986) where generalized TB lesions were seen in 9.07% of tuberculin positive cow. Moreover, Nasr (1997) reported that out of 66 reactor cattle, 60 cattle were slaughtered, 44 (73.4%) had VL and 16(26.6) with NVL. Hassan (2008) revealed that out of 115 tuberculin reactor animals, 85(73.91%) showed VL and 30(26.09%) had NVL. El-Seedy et al. (2013) detected VL in about 68.1% of the tuberculin reactor cattle while the NVL were seen in 31.9%.

Concerning the results of bacteriological examination of the tuberculin reactors cattle, the total acid fast bacilli recovered from 63 slaughtered tuberculin reactors were 50 (79.4%), 74.6% were *M. bovis* and 4.8% were *MOTT* (Table 2). Such results coincided with those recorded by Calaxton et al. (1979) who found that out of 642 lesions suspected to be tuberculous, 62% yielded *M. bovis* and 3.6% other than mycobacteria. Meanwhile, the results are in agreement with those given by El-Sabban (1980) who isolated *M. bovis* (71%) from tuberculous samples in Egypt. On the other hand, the present results differed from those reported by Choi (1981) who showed that bacteriological examination of 76 tuberculin reactors cattle and isolated 70 (92.1%) strain of mycobacteria, 33 (47.1%) strain identified as *M. bovis* and 37 strains other than Mycobacteria. The recovery rate of *M. bovis* figured up to 74.6% was nearly as that reported by Gouello et al. (1988) (69%). A lower *M. bovis* recovery rate (41%), (35.4%), (29.1%) and (20.2%) were reported by Beck and Bibrack (1971), Osman (1974) Gallo et al. (1983) and Lesslie and Birn (1970), respectively, Abou-Eisha et al. (1995) reported 42.9% recovery rate. On the other hand, Choi (1981*)* in Korea reported a much higher isolation rate amounting (92.1%). These results based mainly on the actual disease status present in the tested herd to some extent on the experience of the investigators as well as the technique used for decontamination of tissue specimens. Previous literature reported much lower *M. bovis* recovery (Parlas and Rossi, 1964; 14.8% and Payeur and Marquardt, 1988; 5.6%). A low *M. bovis* recovery of 14.8% rates may be on the expense of other mycobacteria, which may be noticeable in countries where *M. bovis* extirpated from their cattle population, whereas *M. avium* constitutes a problem among cattle herds, which is the case in Germany (Killian, 1982). The recovery rate of atypical mycobacteria was 6.3% and 3.1%, which is higher than that reported by Oliviera et al. (1983) (0.1%). However, Choi (1981) reported that 48.7% of the reactors were infected with atypical mycobacteria.

Serological assays are generally simple, rapid and inexpensive, but the development of improved serodiagnostic assays also require understanding of the bTB humeral immune mechanism as it is characterized by highly heterogeneous antigen recognition (Lyashchenko et al., 1998).

Findings of ELISA on sera of tuberculin reactor cattle showed that 34 (54%) out of 63 positive reactors were positive for ELISA using *PPD-B* arranged as follow; 8 (all) generalized lesions (100%), 22 (54%) out of 40 localized lesions and 4 out of 15 NVL (26%) (Table 3).

Advances in humeral based responses tests have led to the development of lateral flow test kit among others, to capture and detect *M. bovis* antibodies (Garnier et al., 2003). These chromatographic immunoassays employ unique cocktails of selected *M. bovis* antigens as both qualitative captures and detectors of specific antibodies against *M. bovis* in plasma, serum, and whole blood (Lyashchenko et al., 2004; Wernery et al., 2007). MPB83, ESAT-6, 14-kDa protein, CFP-10, MPB70, MPT63, MPT51, MPT32, MPB59, MPB64, Acr1, PstS-1, *M. bovis* purified protein derivatives, ESAT-6/CFP10 fusion protein, 16-kDa alpha-crystallin/MPB83 fusion protein, and *M. bovis* culture filtrate have been identified as the common sero-reactive antigens in bTB (Lyashchenko et al., 2004; Waters et al., 2006; El-Seedy et al., 2013). The bound antibodies are visualized with the naked eye as colour band at the test device within some minutes of application (Lyashchenko et al., 2004; Wernery et al., 2007).

Regarding the lateral flow test, 7 (87.5%) out of 8 with generalized positive to lateral flow, 18 (45%) out of 40 with localized lesions positive to lateral flow, 2 out of 15 (13.3) with NVL positive to lateral flow (Table 4).

Comparing ELISA and lateral flow on sera of tuberculin reactors cattle in ELISA, 8 (100%) out of 8 with generalized TB positive, but 7 (87.5%) out of 8 with localized lesions positive to lateral flow, 22 (54%) out of 40 with localized lesions positive to ELISA, but 18 (26%) out of 40 with localized lesions positive to lateral flow, 4 (26%) out of 15 with NVL positive to ELISA, but 2 (13.3%) out of 15 with NVL positive to lateral flow. The current results not coincide with the conclusion of Ritacco et al. (1990) who concluded that the lower sensitivity of ELISA compared with that of tuberculin test make it of low value as an alternative to tuberculin test. On other hand these results coincide with the results reported by (Reggiardo et al., 1981), as they recorded that the sensitivity of ELISA was 86%. The previous results coincided with several authors, Thoen et al. (1983) recorded positive results of 80%. Comparing tuberculin test, Hall and Thoen (1985) recorded 100% positive ELISA in calves, Auer and Schleehauf (1988) recorded 88.7% ELISA sensitivity, Ayanwale (1987) recorded 98% and 65% for sensitivity and specificity of ELISA, respectively for *M. bovis*, Dimitri (1987) recorded that sensitivity and specificity of ELISA used in tuberculosis reached 100% in cattle. Lilenbaum et al. (2001) reported the sensitivity and specificity 91.3% and 94.8% in bovine tuberculosis. Nasr et al. (2005) reported 76% sensitivity and 100% specificity for ESAT-6 *in vivo* diagnosis of bovine tuberculosis.

False negative ELISA results explained by the fact that low titer of antibodies to mycobacterial antigens which may be associated with heavy infection and that antigens may be released into the blood circulation and cause temporary suppression of antibody formation (Krambovitis, 1986) and that agree with Thorns and Morris (1983) who cleared the level of specific antibodies in many *M. bovis* infected cattle may be low or undetectable. Again this is supported with Amadori et al. (1998) who pointed that antibodies to mycobacterial antigens were investigated with various rates of success since the humeral immune response to *M. bovis* is late and irregular during the course of the disease. In the current study there were not false negative results.

5. Conclusion

It is concluded that the lateral flow assay is rapid, simple and safe and gives results within short period but not enough alone to detect the disease in concern but can act as complementary for tuberculin skin test especially in anergic cattle.

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