Vet.Med.J.,Giza. Vol.50, No.4. (2002): 721-732.

EVALUATION OF SENSITIVITY AND SPECIFICITY OF BOVINE GAMMA-INTERFERON *IN-VITRO* ASSAY FOR DIAGNOSIS OF TUBERCULOSIS IN BUFFALOES

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Received: 22.8.2002.
Accepted:24.9.2002.

SUMMARY

The sensitivity and specificity of the Bovine Gamma Interferon (IFN-γ) in-vitro assay (BOVIGAM) in the diagnosis of bovine tuberculosis in buffaloes as compared to the single intradermal cervical tuberculin test (SICT) and cultural isolation of *Mycobacterium bovis* were investigated. Thirty buffaloes from a dairy farm, in which tuberculin positive animals had been previously diagnosed, were tested. In the SICT test examined buffaloes 36.7% were positive and 23.3% were doubtful as compared with 53.3% positive and 16.7% doubtful results using the IFN-γ assay. Two animals were positive to avian tuberculin in the IFN-γ assay. The prevalence rate reached 60% and 70% using the SICT test and the IFN-γ assay respectively. The sensitivity of the SICT and the IFN-γ assay as compared with the cultural isolation of *M. bovis* were 75% and 95%, respectively, while the specificity were 70% and 80%, respectively. *M. bovis* was isolated from five buffaloes that were negative to SICT and one buffalo that was negative to the IFN-γ assay. In conclusion, the present study demonstrated that the in IFN-γ assay is a practical, more sensitive and specific in-vitro diagnostic test than the conventional tuberculin test for detection of bovine tuberculosis in buffaloes.

INTRODUCTION

Bovine tuberculosis is a devastating disease caused by *Mycobacterium bovis*, an organism that also affects other animal species and man (Morris et al., 1994 and Grange, 1996).

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The control and eradication programs of bovine tuberculosis are mostly based on tuberculin test and slaughter strategy. The standard test used for detection of bovine tuberculosis is the intradermal tuberculin test, first described by Koch (1891). This test relies on intradermal (I/D) injection of purified protein derivative (PPD) tuberculin into the animals and subsequent detection of swelling and erythema at the site of injection three days later (Caffrey, 1994). This test, however, lacks both sensitivity and specificity (Lepper et al., 1977, Francis et al., 1978 and Wood et al., 1991) and it alters the subsequent immune reactivity of the tested animals (Radunz and Lepper, 1985). Consequently, large efforts have gone into attempts to develop alternative diagnostic procedures, especially serological tests (Krambovitis, 1987, and Plackett et al., 1989).The serological tests, however, proved to be even less sensitive and specific than the conventional tuberculin test due to the wide range of antigenic cross-reactivity of mycobacterial antigens (Daniel and Janicki, 1978 and Thorns and Morris, 1983). Recent studies, however, have indicated the possibility of using certain recombinant mycobacterial antigens for serodiagnosis of tuberculosis (Pollock et al., 2000).

Among the other newly developed diagnostic tests for bovine tuberculosis, the bovine interferon gamma (IFN-γ) in-vitro assay appears to be a promising candidate with higher sensitivity and specificity (Wood et al., 1992, Neill et al., 1994a and Gonzalez et al., 1999). It depends upon the use of ELISA in detection and measurement of the IFN-γ released from sensitized lymphocytes of the infected animals following its exposure to mycobacterial PPD.

The aim of the present study was to compare between the conventional single intradermal cervical tuberculin (SICT) test and the in vitro IFN-γ assay for the diagnosis of bovine tuberculosis in buffaloes. Also to compare these techniques with the result of cultural isolation of *M. bovis* from tissue samples taken at the slaughter house.

MATERIAL AND METHODS

Tested animals: A total of thirty, 2-4 years old, buffaloes were used in this study. These animals were from a herd in which tuberculin positive animals had been previously diagnosed in the tuberculin eradication programs conducted at Suez Canal Governorate, Egypt during the period from May 2001 up to Oct. 2001.

Single Intradermal Cervical Tuberculin Test (SICT): 0.1-ml mammalian PPD tuberculin (obtained from Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt) was injected I/D in the tested buffaloes. The Veterinarians of the Official Veterinary Service, Ministry of Agriculture conducted the test. The inoculation site was examined approximately 72 hours later and any evidence of swelling due to

oedema or induration reaction greater than 4.0mm was defined as a positive reaction for mammalian PPD. Increase in skin fold between 3.0 and less than 4.0mm was considered doubtful reaction.

Assay for Bovine Interferon-Gamma: The kits of bovine IFN-γ measurement (BOVIGAM test kit) were purchased from CSL-limited, 45 polar RD-Parkville, Victoria 3052 Australia. The test was conducted according to the manufacturer regulation and as reported by Rothel et al. (1990) and Wood et al. (1990) as follows:

- Whole blood culture system for measurement of released IFN-γ: Heparinized blood samples (10ml/animal) were collected under sterile condition from tested animals just before tuberculin skin testing. The collected blood samples were transported to the laboratory in isothermal container (12-20°C) and processed within 6 hours from the time of collection. Each blood sample was gently shacked by inverting the tube 10 times before being dispensed in 6 wells (1.5ml/well) of a 24 well microtitre tissue culture plate. Each sample was examined in duplicates against PBS (100ul/well), bovine PPD (300ug/ml) and avian PPD (300ug/well). The blood cultures were then incubated 18 hours at 37°C and 5% Co2. After that the plasma supernatants were collected and assayed for bovine IFN-γ production using the supplied ELISA kits. The assay was carried out in duplicate for each plasma sample. According to the manufacturer instructions the results were validated if the mean of the optical density (OD) of the three negative control samples was <0.1, and the variation between individual results did not exceed 0.04 and at the same time, the mean of the optical density of the three positive control samples was >0.75 and the variation of the individual results did not exceed 30% of the mean value. The tested plasma samples were calculated according the following scheme:

Positive reaction : OD (bovine PPD) - OD (negative control) > 0.1, and the OD of bovine PPD should be higher than that of the avian PPD.

Negative reaction: OD (bovine PPD) - OD (negative control) < 0.05, and the OD (avian PPD) - OD (negative control) < 0.1

Avian reactors: OD (avian PPD) - OD (negative control) < 0.1, and the OD (avian PPD > OD (bovine control)

Doubtful Reaction: 0.1 > OD (bovine PPD) - OD (negative control) > 0.05, and OD (bovine PPD) > OD (avian PPD)

Necropsy: A detailed necropsy procedure was employed for all reactors. The non-reactor buffaloes of the examined group were also slaughtered and post mortem examination was done using the meat inspection procedures according to Anon (1976). From each reactor and according to Corner et al. (1990) tissues with high expectation of containing infection were collected for

bacteriological examination. It included the anterior and posterior mediastinal, left and right bronchial and left and right medial retropharyngeal lymph nodes (Table 1-A). These lymph nodes were collected using aseptic techniques, placed in sterile containers and submitted to the laboratory for bacteriological examination. Other tissues with a low expectation of infection (Table 1-B) were sliced in-situ and examined visually. When lesions resembling tuberculosis were found they were submitted for bacteriological examination. Up to a maximum of 3 lesions were examined from each animal.

Bacteriological examination of collected specimens (Marks, 1972): All tissues submitted to the laboratory were examined bacteriologically for *M. bovis*. The isolation of *M. bovis* was used as the definitive test for the diagnosis of bovine tuberculosis, whether from animal with suspected tuberculous lesions or from tissues without visible lesions. The collected tissue samples were dissected from fat and connective tissue and a 2 g portion of tissue from each specimen was homogenized with 2 ml of sterile distilled water in a sterile mortar containing sterile sand. Two ml of 4% H_2SO_4 were added to the mixture and incubated at 37°C for 30 minutes. The mixture was diluted with 16 ml of sterile distilled water and centrifuged at 3000rpm for 20 minutes. The supernatant fluid was poured off onto disinfectant and the obtained sediment was inoculated into four Lowenstein-Jensen slants (Bio Merieux), then incubated at 37°C for 8 weeks. The inoculated slants were examined daily over a week. Suspected colonies were subcultured and identified according to Chadwick (1981).

Analysis of Data: In all analysis undertaken, an infected animal is defined as as one from which *M. bovis* was isolated (a true positive). The sensitivity was defined as the number of diseased animals detected by the test divided by the number of diseased animal in the population being examined (Cochrane and Holland, 1971). The specificity was defined as the number of non-diseased buffaloes identified as non-diseased by the used test divided by the number of non-diseased buffaloes on the population being examined. Apparent prevalence rate was defined as the number of diseased animals detected by the test divided by the total number of animals in the population being examined (Smith, 1995).

Table (1): Tissues examined for lesions of tuberculosis in animals

1-A	Tissues collected for bacteriological examination:
	medial retropharyngeal lymph nodes*
	mediastinal lymph nodes-anterior and posterior
	tracheobronchial (bronchial) lymph nodes
1-B	Tissues examined for lesions by incision at the time of slaughter
- Head	mandibular (submandibular) lymph node
	parotid lymph nodes
	lateral retropharyngeal (atlantal) lymph nodes
	tonsils
- Thorax	tracheobronchial lymph nodes-cranial and medial
- Lungs	
- Abdomen	liver
	hepatic lymph nodes
	spleen
	mesenteric lymph nodes
	kidneys
-Carcase	caudal cervical (prescapular) lymph nodes
	subiliac (precrural or prefemoral) lymph nodes
	medial iliac lymph nodes
	superficial inguinal (supramammary) lymph nodes
	popliteal lymph nodes
	internal iliac lymph nodes
	gluteal (ischiatic) lymph nodes
	lateral iliac (deep inguinal) lymph nodes

* Includes left and right nodes except where otherwise indicated.

RESULTS

Results shown in Table (2) demonstrate the reactivity of the tested 30 buffaloes to the SICT and bovine IFN-γ assay. 53.3% of the buffaloes were positive using the IFN-γ assay as compared with 36.7% positive reactors in the SICT test. The doubtful reactions measured by the IFN-γ assay (16.7%) were lowered than that recorded by the SICT test (23.3%). Two buffaloes (6.7%) reacted positively to the avian PPD the IFN-γ assay.

Table (2): Results obtained by the single intradermal tuberculin test and the bovine IFNγ - assay in buffaloes.

		Sict Test Results %*			
		Positive	Doubtful	Negative	Total
IFN- γ Assay** Results (%)	Positive	10 (33.3)	4 (13.3)	2 (6.7)	16(53.3)
	Doubtful	1 (3.3)	2 (6.7)	2 (6.7)	5 (16.7)
	Negative	0	0	7 (23.3)	7(23.3)
	Avian	0	1 (3.3)	1 (3.3)	2 (6.7)
	Total	11 (36.7)	7 (23.3)	12 (40)	30(100)

* The tuberculin used is mammalian PPD.

** Bovine tuberculin was used in the tes.

To facilitate the statistical analysis of the obtained results and according to the manufacturer interpretation guidelines of the IFN-γ kits, the doubtful reactors using IFN-γ assays were classified as positive reactors, also the same was done for the SICT assay. Accordingly the apparent prevalence rates were 60% and 70% using SICT test and the IFN-γ assay, respectively (Table 3).

Comparison between the results obtained by the SICT test, IFN-γ assay and the cultural isolation method (the gold standard test) are shown in Table 4 and 5, respectively. The sensitivity rates of SICT test and the bovine IFN-γ assays as compared with the cultural isolation of the *M. bovis* were 75% and 95%, respectively, while the specificity were 70 % and 80%, respectively.

The distribution of both the false positive and false negative results obtained with both immunological tests as compared to the cultural isolation are listed in Table (4). Out of the 18 buffaloes that were positive to SICT test from which samples were collected at the slaughterhouse. A total of 3 buffaloes were considered as false positive reactors, because *M. bovis* could not be isolated from them. Similarly in 2 out of the 21 buffaloes that were positive with the IFN-γ assay, *M. bovis* were not recovered. Regarding the false negative reactors, *M. bovis* was isolated from 5 buffaloes that showed negative response to human tuberculin in the SICT test and from one buffaloes, which were negative responders to the bovine IFN-γ assay.

Table (3): Comparison between the prevalence rate determined by the single intradermal cervical tuberculin (SICT) test and the bovine IFNγ - assay in buffaloes.

		Sict Test Results %*		
		Positive	Negative	Total
IFN- γ Assay** Results (%)	Positive	17 (56.7)	4 (13.3)	21 (70.0)**
	Negative	1 (3.3)	8 (26.7)	9 (30.0)
	Total	18 (60.0)**	12 (40.0)	30 (100.0)

* For statistical purposes the responder to the avian PPD in the IFN-γ were considered as negative reactors. While the doubtful reactors to either the SICT test or the bovine IFN- γ assay have been considered as positive reactors.

** Prevalence rate = Test positive animals/total number of examined animals.

Table (4): Sensitivity and specificity of SICT and IFNγ - assay in the diagnosis of tuberculosis in buffaloes with reference to cultural isolation of *M. bovis* as a definitive diagnosis

		Thirty Buffaloes Tested with		
		Cultural isolation of *M. bois*	SICT	IFN-γ assay
Positive (+ ve)	True +ve	20	15	19
	False +ve		3	2
	Total Positive		18	21
Negative (+ ve)	True -ve	20	7	8
	False -ve		5	1
	Total Negative		12	9
Test sensitivity			75%	95%
Test specificity			70%	80%

Test sensitivity = Test positive/Test positive + False negative

Test specificity = Test negative/Test negative + Galse positive

DISCUSSION

Diagnosis of tuberculosis in animals is still an important health problem especially in developing countries. The SICT is the current conventional in vivo test used for diagnosis of bovine tuberculosis in cattle and buffaloes. This test, however, lacks both sensitivity and specificity and 3 days are required to obtain results (Wood et al., 1991). Also, the I/D injection of tuberculin induces desensitization of tested animals to repeated tuberculin testing for at least 2 months (Radunz and Lepper, 1985).

In buffaloes tuberculin testing is rather problematic. where it is usually associated with low specificity and the skin reactivity to tuberculin is higher than in cattle. Also in buffaloes, the skin is relatively more thick than in cattle, which render the process of testing difficult. For all these reasons we have studied, in the present work, the applicability of the in vitro IFN-γ assay for diagnosis of tuberculosis in buffaloes as compared with the SICT.

A total of 30 buffaloes selected from a herd suffering from tuberculosis were used in this work. Using the bovine IFN-γ assay, the recorded apparent prevalence rate of tuberculosis among the tested buffaloes (Tables, 2 and 3) was always higher than those obtained using the SICT. Similar results have been reported in other countries (Wood et al., 1991, Neill et al., 1994b, Domingo et al., 1995 and Gonzalez et al., 1999), where the IFN-γ assay proved to be significantly sensitive than SICT test. In other studies, however, the apparent prevalence rates recorded for the SICT test were higher than that of the bovine IFN-γ assay (Wood et al., 1991 and 1992). On the other side, Ryan (1994) reported a similar sensitivity of SICT and IFN-γ assays.

The percentage of doubtful reactors detected by the SICT was considerably higher as compared with the IFN-γ assay. This result is not in agreement with what reported by Gonzalez et al. (1999), where the percentage of doubtful reactors were almost four time higher for the bovine IFN-γ assay. The difference in the rate of doubtful reactor reported in the present investigation using both tests might be attributed to the nature of the used tuberculin. Mammalian PPD was used in the SICT and bovine PPD (supplied with the IFN-kits) was used in the IFN-γ assay, this might renders the later more specific with low rate of doubtful reactors. The response of two buffaloes to the avian tuberculin in the IFN-γ assay, might be attributed, as stated by Thorns and Morris (1983), to the presence of cross-reactive epitopes on the used tuberculin.

In the present study, the criterion used for definitive diagnosis of bovine tuberculosis in buffaloes was the cultural isolation of *M. bovis* from tissue specimens collected from the 30 examined buffaloes after slaughtering. Relating the results ob-

tained by the used immunological tests (SICT and IFN-γ assay) with that of cultural isolation enabled us to determine the percentage of both false positive and false negative reactors in both tests. As demonstrated in Table (4), The IFN-γ assay was found to have significantly higher sensitivity (95%) as compared with the SICT (75%). Similar differences in the sensitivity of both tests were reported (Woods and Rothel, 1994 and Domingo et al., 1995). In Australia (Wood et al., 1991) detected a sensitivity of 63.4% for the intradermal test, 81.6% for the IFN-γ assay and 89.6% for the combination of two tests when 6264 cattle were tested. On the other hand, slightly higher (Monaghan et al., 1995) or similar (Neill et al., 1994a) sensitivity percentages compared to those reported in our study have been obtained in Ireland using the intradermal test. In other investigations, however, the figures obtained for the bovine IFN-γ assay and for the combination of both tests were similarly high 87.6% and 93.5%, respectively (Domingo et al., 1995). Because the present experiment was designed to evaluate the sensitivity and specificity of both immunological tests, it was essential to compare the number of false positive animals detected in the SICT test and the bovine IFN-γ assay using the bacteriological examination as the gold standard test. The higher number of IFN-γ +/culture- false positive animals than SICT+/culture- false positive reactors obtained in this study suggests that the IFN-γ assay is more sensitive than the skin test. In this respect, some authors have reported that animals react sooner to the IFN-γ assay than to the SICT test (Neill et al., 1994a, Domingo et al., 1995 and Gonzalez et al., 1999).Therefore, recently infected animals could have been detected by means of the IFN-γ but not by SICT test.

The high number of tuberculin+/ culture- and IFN-γ + /culture- false positive animals in our study (Table 4) could be explained firstly by cross-reactivity due to bacteria other than *M. bovis* but also to possible failure in the culture isolation method. As could be expected, the number of tuberculin-/culture+ false negative reactor was higher in SICT test than that of IFN-γ-/culture+ false negative animals (Table 4). Suppression of immunological reactivity is behind the false negative cases. Such immunosuppression is found to be associated with military tuberculosis, which was not reported in the present examined cases. It may be attributed also to other environmental immunosuppressive drugs as dexamethasone. Exposure to this glucocorticoides immediately before or after the tuberculin inoculation has been proved to reduce cell-mediated immune responses in animals sensitized to *M. bovis*, resulting in false negative reactivity to SICT test and in a lesser extent, to the IFN-γ assay (Doherty et al., 1995, Dondo et al., 1995 and Goff, 1996).

Although the unit cost of testing animals with the IFN-γ assay will be higher than tuberculin skin

test, its greater sensitivity and specificity renders the total costs of tuberculosis eradication from a herd much reduced due to reduction of testing rounds and decrease of false positive rate. Also the fact that testing with the IFN- γ assay does not compromise the immune system of the animals as does the current in vivo tuberculin test (Radunz and Lepper, 1985) and immediate retesting of animals can be done with rapid results. All these advantages nominate the IFN-γ in vitro assay a useful alternative test for diagnosis and earlier detection of bovine tuberculosis.

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