Exposure and Shedding in Milk of *Mycobacterium bovis* in Dairy Herds Using One-Step Anigen® Rapid Bovine Tuberculosis Antibodies Test and Ziehl-Neelsen Stain

[1]S. Danbirni, [1]A.K.B. Sackey, [2]J.O. Ayo, [3]E.K. Bawa,
[4]A.C. Kudi, [5]S.O. Okaiyeto and [6]S.B. Pewan
[1]Veterinary Surgery and Medicine, Ahmadu Bello University, Zaria, Nigeria
[2]Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria, Nigeria
[3]National Animal Production Research Institute, Ahmadu Bello University, Zaria, Nigeria
[4]School of Biomedical and Biological Sciences, Faculty of Sciences,
University of Plymouth, Drake Circus, Plymouth, PL4 8AA, United Kingdom
[5]Veterinary Teaching Hospital, Ahmadu Bello University, Zaria, Nageria
[6]National Animal Research Institute, Vom, Plateau State

Abstract: This study was carried out to detect exposure to *Mycobacterium bovis* (*M. bovis*) in four dairy herds consisting of a total of 200 cows, aged between $2^{1/2}$-10 years. The cows were of various breeds, namely: white Fulani (Bunaji), crosses of white Fulani and sokoto gudali, white Fulani and Friesian, semi-intensively managed and producing milk for yoghourt production in Kaduna and environs. Anigen® Rapid Bovine Tuberculosis Antibody Test (IQRT) specific for *M. bovis* antibodies in sera of cows were used in this study. The result showed 17.5% (35/200) positive for antibodies to *M. bovis*. Fresh milk from cows positive to the IQRT and also packed yoghourts made from milk obtained from those positive and negative cows in the sampled dairy herds were collected and subjected to Ziehl-Neelsen Stain (ZNS) in order to detect bacilli in fresh milk and packed yoghourts. The result obtained 17.1% (6/35) of these cows were shedding the bacilli in fresh milk while no bacillus detected in the packed yoghourts. The result showed that IQRT was sensitive in detecting *M. bovis* before they start shedding in milk while ZNS technique was found to be potentially useful in detecting *M. bovis* infected lactating cows that are shedding the bacilli in milk. This study has shown that apparently healthy lactating cows may shed viable *M. bovis* in milk there by posing a serious public health problem where unpasteurized milk is consumed. This calls for the need to ensure that only non-positive milking cows are milked for human consumption and the IQRT is the best of choice to determine that.

Key words: *M. bovis*, anigen® rapid bovine TB antibody test, Ziehl-Neelsen stain, lactating dairy dows, packed yoghourt, antibody

INTRODUCTION

Milk production has increased in most developing countries as a consequence of greater demand for milk for human consumption (Food and Agriculture Organization, 1993). Consumption of milk contaminated by *M. bovis* has long been regarded as the principal mode of transmission from animal to humans (Acha and Szyfres, 1987). The prevalence of Bovine Tuberculosis (bTB) within a country varies from area to area; the highest incidence of bTB is generally observed where intensive dairy production is most common, notably in the milk shades of larger cities (Acha and Szyfres, 1987). In some industrialized countries such as the United States where bTB is close to elimination, large dairy herds (i.e., 5,000 or more cows) that are crowded together represent the main source of infection (National Research Council, 1994). In Nigeria, despite increased importation of exotic dairy breeds of cattle (*Bos taurus*) to meet up with the demand for increase in milk production, the nomadic pastoralist indigenous zebu cattle (*Bos indicus*) constitute the larger proportion of dairy cattle (Ariyo, 2002). In those areas where extensive management is more common, animal crowding (near watering ponds, dip tanks, markets and corals) still plays major role in the spread of the disease (Ayele *et al.*, 2004). Milk obtained through hand milking from such infected cows is either taken as fresh raw or fermented (nono) without due processes of grading, classification and pasteurization. These unhygienic practices have led to the isolation of *M. bovis* in fresh raw and fermented milk nono in various parts of the country (Idrisu and Schnurrenberger, 1977; Abubakar, 2007;

Okaiyeto *et al.*, 2008). The basic strategies required for the control and elimination of bTB are well known and well defined (WHO, 1967) for instance in industrialized countries, bTB control and elimination programmes together with milk pasteurization have drastically reduced the incidence of the disease caused by *M. bovis* in both cattle and humans. In developing countries however, bTB is widely distributed and control measures are either not applied or applied sporadically and pasteurization of milk is rarely practiced. This is because of financial constraints, lack of political will as well as limitations of some diagnostic test in detecting early exposures before the tubercle bacilli begin to shed in milk and secretions to susceptible animals and humans (Cosivi *et al.*, 1998).

Successful conduct of a test and slaughter policy requires sustained Purified Protein Derivatives (PPD) production and supply, cooperation of national and private veterinary services, meat inspectors and farmers as well as adequate compensation of the farmers by governments. Only a few developing countries can adhere to these requirements (Cosivi *et al.*, 1998). Moreover, most countries have stopped producing PPD at commercial quantities either because it is not economical or they have controlled or eliminated the disease or have developed a newer specific and sensitive diagnostic test than PPD. IQRT is one of such newer serological test that is specific and sensitive to *M. bovis* antibodies; rapid, portable and does not require electricity (Anigen Animal Genetics Incorporation, 2005).

The aim of this study is to detect the exposure and shedding in milk of *Mycobacterium bovis* and its public health significance in dairy cows using Anigen® Rapid Bovine Tuberculosis Antibody Test as a screening procedure in the surveillance of bTB in four yoghourt producing dairy herds in Kaduna and environs with a view to enlightening the public and recommending its use for further application in Nigeria.

MATERIALS AND METHODS

The study areas were selected using convenient sampling method and were Igabi, Giwa and Sabon-Gari Local Government Areas (LGAs). A total of four dairy herds were selected for the study based on the cooperation of the herdsmen and 200 lactating cows were sampled (Table 1). The farms were semi-intensively managed with high half-roof pens except farm C that had low-roofed pen. In semi-intensive system of management, animals were allowed to go outside the pen to graze from time to time in paddocks and sometimes in communal grazing land while water is provided in the pens.

Blood sample collection: Lactating cows were identified using their ear tags while those without ear tags were marked with a permanent marker. Each animal was physically restrained and about 5 mL of blood was aseptically collected from the jugular vein with a sterile hypodermic 18 G needle attached to 10 mL syringe. The blood was placed into sterile 10 mL plastic tube and allowed to clot in a slanting position before transporting on iced packs to the laboratory for centrifugation at 2000 g for 5 min to obtain serum. The serum obtained was stored at 2-8°C until used within 3 days (Anigen Animal Genetics Incorporation, 2005).

Anigen® Rapid Bovine Tuberculosis antibodies test (IQRT) procedure: Anigen® Rapid Bovine tuberculosis antibodies test kits specific for *M. bovis* antibodies containing the test devices and specimen droppers procured from Anigen® Animal Genetics Inc. in South Korea were used in detecting *M. bovis* antibodies in the sera collected. The sera samples were taken out of the freezer and allowed to attain room temperature (15-30°C) before use (Anigen® Animal Genetics Incorporation, 2005). The procedure was as follows:

- The test kit was removed from the foil pouch and placed on a flat, dry surface to attain room temperature
- Four (4) drops of the test serum were added slowly to the sample hole using the specimen dropper. Where the migration did not appear after 1 min, one more drop of the test serum was added to the sample hole
- The test result was interpreted within 20 min. Result interpreted beyond 20 min was invalidated

Table 1: Dairy herd composition and farm management system in sampled farms

| Dairy farm No	Location (LGA)	Herd composition (breed of cattle)	Farm management system	No. of lactating cows on farm	No. of lactating cows sampled
A	Igabi	White fulani × Sokoto gudali	Semi-intensive, High half-roofed pen	53	53
B	Igabi	White fulani	Semi-intensive High half-roofed pen	78	78
C	Giwa	Friesian × White fulani	Semi-intensive Low half-roofed pen	06	06
D	Sabon-Gari	White fulani	Semi-intensive High half-roofed pen	63	63
Total	4			200	200

LGA = Local Government Area, X = Crossed
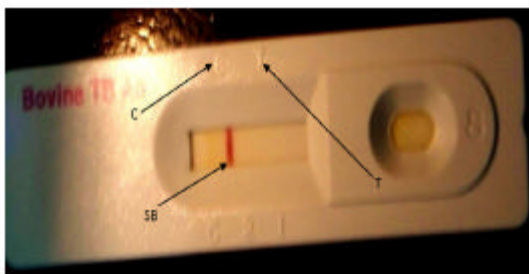
Fig. 1: A Negative IQRT result showing a single band (control) in the result window; C = Control band position, T = Test band position, (SB) Single Band
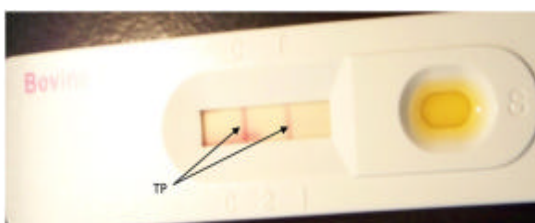

Fig. 2 : A Positive IQRT result showing two purple bands in the result window; TP = Two Purple bands

- A test result was seen as a purple band in the result window of the kit (Fig. 1)
- The right section of the result window indicated the test results. If another colour band appeared in the right section of the result window, this band was the Test line (T) (Fig. 1)

Interpretation of the IQRT result: The presence of only one purple colour band within the result window indicated a negative result (Fig. 1). While the presence of two colour bands (T band and C band) within the result window, no matter which band appeared first indicated a positive result even if the intensity of the purple band colour was faint, it was interpreted as positive, if it appeared within 20 min based on the recommendation of the manufacturer (Fig. 2).

Where the purple colour band was not visible within the result window after performing the test, the result was considered invalid (Fig. 1). This is because directions might not have been followed correctly or the test kit might have deteriorated. Based on the manufacturer's recommendation the specimen was re-tested.

Collection of fresh raw cattle milk and yoghourt samples: Lactating cows that previously tested positive for *M. bovis* antibodies using IQRT were identified using ear tags and permanent marker numbers from the rest of the herd. The teats of each of these cows were washed with clean warm water to reduce contamination and to stimulate milk let-down prior to milk collection. A total volume of about 16 mL of milk were expressed from the 4 quarters of each cow's udder manually into sterile screw-capped universal bottle, then properly identified and labeled. The fresh raw milk and 4 packs each of yoghourt (collected randomly) from 3 yoghourt producing plants located within Kaduna and environs that obtain fresh raw milk from these selected farms were collected and conveyed to the laboratory within 24 h of collection on ice-cooled (4°C) sterile screw capped universal bottle and stored in the freezer until processed.

Ziehl-Neelsen staining technique: Ziehl-Neelsen staining was carried out using standard protocol as described by Kazwala *et al.* (1998) to detect acid-fast bacilli in fresh raw milk and yoghourt.

RESULTS AND DISCUSSION

The percentage of positive reactivity's of sampled dairy cows to IQRT across farms were, 7.5% (4/53), 15% (12/78), 50% (3/6) and 25% (16/63) for farms A-D, respectively (Table 2). Farm C has the highest prevalence level and Farm B had the least.

The percentage of positive reactivity's of sampled dairy cows to ZNS technique that previously tested positive to IQRT across farms were, 0% (0/4), 8.3% (1/12), 33% (1/3) and 25% (4/16) for farms A-D, respectively (Table 3). Farm C has the highest prevalence level while Farm A had the least.

Tubercle bacilli were not detected in packed yoghourt from 3 yoghourt producing plants that do obtained fresh milk from IQRT positive and negative lactating cows.

A comparison of the percentage exposure of the sampled dairy cows to *M. bovis* using IQRT and shedding of the tubercle bacilli in fresh raw milk using ZNS technique across Farms were 7.5 and 0.0, 15 and 8.3, 50 and 33, 25 and 25% for farms A-D, respectively (Table 4).

The percentage of lactating cows in the farms investigated that reacted positively to IQRT was higher than the 14.3% reported in a similar study by Abubakar (2007) in Kaduna state and the Federal Capital Territory (FCT) Abuja using PPD.

The difference in the percentages obtained may be due to the difference in the type of test used. PPD is a less specific test for detection of any *Mycobacterium species*

Table 2: Detection of antibodies to *M. bovis* in four dairy farms using IQRT

Dairy farm	No. of cows tested	No. of cows positive	Percentage of cows positive
A	53	4	7.6
B	78	12	15.0
C	06	03	50.0
D	63	16	25.0
Total	200	35	

Table 3: Detection of Mycobacterium in fresh raw milk using Ziehl-Neelsen stain in four dairy farms previously tested positive to IQRT

Dairy Farm	No. of cows tested	No. of cows positive	Percentage of cows positive
A	4	0	0.0
B	12	1	8.3
C	3	1	33.0
D	16	4	25.0
Total	35	6	-

Table 4: A comparison of exposure and shedding of bacilli across Farms using Immunochromatographic qualitative rapid test and Ziehl-Neelsen staining technique

Dairy farm	Percentage of cows positive to IQRT	Percentage of cows positive to ZNS
A	7.6	0.0
B	15.0	8.3
C	50.0	33.0
D	25.0	25.0

(e.g., *M. bovis*) because of cross reaction with environmental *Mycobacteria* (Buddle *et al.*, 2002). Thus, the 17.5% prevalence obtained in this study was most likely a true representation of the herds' prevalence status to *M. bovis*. Perhaps, if IQRT was used instead of PPD, the percentage positive reported by Abubakar (2007) could have been lower for *M. bovis* in the study areas because other acid-fast bacilli could have been screened out leaving *M. bovis*. Also, the percentage prevalence obtained in this study is >5% reported by Okaiyeto *et al.* (2008) in a study carried out on a dairy farm in Kaduna state using PPD, lending further credence to the low sensitivity of PPD compared to IQRT as earlier pointed out.

The prevalence of *M. bovis* obtained using IQRT varied from farm to farm. Farm C had the highest percentage reactivity of 50% (3/6) while farm A had the least value of 7.5% (4/53) (Table 2). The reason for the variation in the percentage reactivity across farms could be because farm C had cross breeds of Friesian and White Fulani kept under semi-intensive system with a small and low half-roofed pen. The small and low half-roofed pen reduces air-flow (ventilation) and increases the closeness of the animals to each other and thus could be facilitating the transmission of *Mycobacterium* infection by inhalation within the pen. This finding based on the type of management practice agrees with the report of MacIntyre and Plant (1998) that the risk of acquiring tuberculosis in both animals and humans increases with closeness. In addition, the only watering trough for the

animals in this farm takes several days before being washed; thereby providing a potential source of acquiring the infection as all the animals drink from one and only drinking trough. This may agree with the findings of Radostits *et al.* (2003) that stagnant drinking water may cause infection up to 18 days after its last use by an infected animal. Farm A had the least percentage reactivity 7.6% (4/53). This may be due to the relatively adequate ventilation as a result of adequate space within the pen with several watering points. These two management factors might have reduced the risk of the animals exposure to the *Mycobacterium* through inhalation or drinking water as the animals were spread reducing close contact with each other.

The situations in farms A, B and D (Table 2) disagrees with the report of Salisu (2007) in a comparative study of exotic and local breeds that the local breeds were more affected with bTB than the exotic breeds. Although, farm C comprised of crosses of Friesian and White fulani breeds, yet had 50% of the lactating dairy cows in the farm positive to IQRT than the rest of the farms that had local breeds.

Farm A was not truly negative for *M. bovis* using ZNS though the cows in this farm previously tested positive to *M. bovis* with IQRT (Table 3) but it indicated that they were not shedding the bacilli in milk (*M. bovis* may be in a dormant form). These animals if allowed to stay long in the farm particularly under stressful conditions could eventually start shedding the bacilli because bTB is a chronic and progressive disease (Cadmus *et al.*, 2004). This study showed that ZNS was less potentially sensitive for early detection of *M. bovis* exposed animals before the animal starts shedding the *Mycobacterium* in milk.

Farm C had the highest percentage reactivity to ZNS (Table 2). Although, 50% of the cows were exposed to *M. bovis* (IQRT), only 33% (1/3) were shedding the bacilli in milk. Other animals may not be shedding the bacilli due to the progressive and chronic nature of the disease (Cadmus *et al.*, 2004). Farm D had equal number of percentage reactivity with both IQRT and ZNS 25% (16/63) and 25% (4/16).

This may indicate that all the cows exposed to *M. bovis* were infected and were shedding the bacilli in milk a potential danger to any individual consuming raw or inadequately pasteurized milk. Farms B, C and D showed that the cows were not only infected but were shedding *M. bovis* in milk (Table 3) even though the cows appeared apparently healthy. Thus, could be a potential danger to calves and the public consuming unpasteurized milk. This result agrees with those of Corner *et al.* (1990), National Research Council (1994) and Kazwala *et al.* (1998) that apparently healthy cows may shed *Mycobacterium* in milk without showing clinical signs of the disease.

Yoghourt obtained from the three yoghourt producing plants tested negative to ZNS. This may be attributed to adequate pasteurization, sample size (limited number of yoghourt sampled) or small volume of bacilli in a large milk pool examined.

CONCLUSION

In this study, apparently healthy lactating cows may shed viable *M. bovis* in milk posing a health problem for man and calves and hence not safe for public consumption and this call for the need to pasteurized milk meant for public consumption.

Immunochromatographic qualitative rapid test in this study has been found to be sensitive for the detection of early exposure of cows to *M. bovis* in the tropic and comparison with other test would be required to determine its sensitivity and specificity.

Ziehl-Neelsen Staining technique though less sensitive in detecting early exposure of animals to *M. bovis* was found to be useful in determining the status of cows that were not apparently infected but were shedding the bacilli in milk.

ACKNOWLEDGEMENTS

REFERENCES

Abubakar, I.A., 2007. Molecular epidemiology of human and bovine tuberculosis in the federal capital territory and kaduna state, Nigeria. Ph.D. Thesis, Plymouth University, UK.

Acha, P.N. and B. Szyfres, 1987. Zoonoses and Communicable Diseases Common to Man and Animals. 2nd Edn., Pan American Health Organization/World Health Organization; Scientific Publication, Washington, DC., pp: 181-190.

Anigen Animal Genetics Incorporation, 2005. Anigen rapid bovine TB Ab test kit. Product Name: RB 23-02. http://anigen.cafe24.com/bbs/bbs/board.php?bo_table=eje1&wr_id=3#.

Ariyo, A.J., 2002. Livestock, Africa atlases: Atlas of Nigeria. 1st Edn., Bietlot, Belgium, pp: 98-99.

Ayele, W.Y., S.D. Neill, J. Zinsstag, M.G. Weiss and I. Pavlik, 2004. Bovine tuberculosis: An old disease but new threat to Africa: Review article. Int. J. Tuberculosis Lung Dis., 8: 924-937.

Buddle, B.M., B.J. Wards, F.E. Aldwell, D.M. Collins and G.W. DeLisle, 2002. Influence of sensitization to environmental Mycobacteria on subsequent vaccination against bovine tuberculosis. Vaccine, 20: 1126-1133.

Cadmus, S.I.B., N.N. Atsanda, S.O. Oni and E.E.U. Akang, 2004. Bovine tuberculosis in one cattle herd in Ibadan, Nigeria. Vet. Med. Czech, 49: 406-412.

Corner, L.A., L. Melville, K. McCubbin, K.J. Small, B.S. McCormick, P.R. Wood and J.S. Rothel, 1990. Efficiency of inspection procedures for the detection of tuberculosis lesions in cattle. Aus. Vet. J., 67: 389-392.

Cosivi, O., J.M. Grange, C.J. Daborn, M.C. Raviglione and T. Fujikura *et al.*, 1998. Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries. Emerg. Infect. Dis., 4: 59-70.

Food and Agriculture Organization, 1993. FAO Animal Production and Health Series No. 33. In: FAO/OIE/WHO Animal Health Year Book, Welte, W.R. (Ed.). The Organizations, Washington.

Idrisu, A. and P. Schnurrenberger, 1977. Public health significance of bovine tuberculosis in four northern states of Nigeria: A Mycobacteriological study. Nig. Med. J., 7: 384-387.

Kazwala, R.R., C.J. Darborn, L.J.M. Kusiluka, S.F.H. Jiwa, J.M. Sharp and D.M. Cambarage, 1998. Isolation of *Mycobacterium species* from raw milk of pastoral cattle of Southern highlands of Tanzania. Trop. Anim. Health Prod., 30: 233-239.

MacIntyre, C.R. and A.J. Plant, 1998. Tuberculosis in South-East Asia refugees: Can prevention be improved by better policy and practice? Prev. Med., 27: 815-820.

National Research Council, 1994. Livestock disease eradication: Evaluation of the co-operative State Federal bovine tuberculosis eradication programme. Aetiol. Diagn. Detection Tuberculosis, 2: 13-34.

Okaiyeto, S.O., L. Allam, E. Akam and G. Sabo, 2008. Investigation of the prevalence of bovine tuberculosis in a dairy farm in kaduna state. Res. J. Dairy Sci., 2: 27-29.

Radostits, O.M., D.C. Blood, C.C. Gay and W.H. Kenneth, 2003. Veterinary Medicine: A Text Book of Diseases of Cattle, Sheep, Pigs, Goats and Horses. 9th Edn., Harcourt Publisher Ltd., London, pp: 909-919.

Salisu, I., 2007. The epidemiology of human and bovine tuberculosis in jigawa state. M.Sc. Thesis, Ahmadu Bello University, Zaria.

WHO, 1967. Third Report of the Joint FAO/WHO Expert Committee on Zoonoses. World Health Organization, Geneva.