International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.9, No.08 pp 123-128, 2016

Detection of *Mycobacterium avium subsp. paratuberculosis* in raw buffaloe's milk

[1]Nagah M. Hafiz, *[1]Saad, M.F., [2]Hanafy, M.H. & [1]Eman F. Abdel-Latif

[1]Cairo University, Faculty of Veterinary Medicine, Department of Food Hygiene & Control, Giza, Egypt
[2]Cairo University, Faculty of Veterinary Medicine, Department of Microbiology, Giza, Egypt

Abstract: *Mycobacterium avium subsp. paratuberculosis* (*MAP*) is the causative organism of paratuberculosis, a disease with extensive economic effect, superlatively on dairy cattle. One hundred ninety- two Egyptian buffaloes' milk samples were examined for *MAP*. Sixteen (8.33%) samples could be detected by milk- Enzyme- Linked Immunosorbent Assay (ELISA). No samples were found positive in milk-Polymerase Chain Reaction (PCR).
Keywords: Buffaloe's milk, *Mycobacterium avium subsp. paratuberculosis*, Enzyme- Linked Immunosorbent Assay, Polymerase Chain Reaction.

Introduction

Mycobacterium avium subsp. paratuberculosis is related to Crohn's disease in man, which characterized by Ulcerative Colitis, Inflammatory Bowel Disease and the modes of its transmission from animals to humans should be advised. Although fecal contamination of the udder may report for subsequent contamination of milk, animals infected with *M. paratuberculosis* shed it directly into their milk[1]. Johne's Disease (JD), caused by *MAP*, is a chronic infection of the intestinal tract of ruminants and the symptoms of this disease include diarrhea, reduced milk production, emaciation, death in infected animals, and infections result in heavy economic losses for dairy farms[2,3,4,5,6] .

In the advanced stages of infection, *Mycobacterium avium subsp. paratuberculosis* may be passed through the colostrum and cattle milk[1,7]. The practice of feeding pooled colostrum or waste milk from cattle may help the spread of infection to many calves during their most susceptible stage of life. *MAP* can survive pasteurization temperatures, so colostrum from infected dams should not be fed[8].

Some strains of *M. avium subsp. paratuberculosis* infect specific hosts. The two main types are the Type II (C strains), found in cattle, and type I (S strains), found in sheep. The C strains have a wide host range including cattle, goats, camels and wild non-ruminant. The S strains seem to mainly infect sheep and other small ruminants. Cross-species transmission of the S and C strains can occur between cattle and sheep[9].

Studies in many countries have been carried out to determine the prevalence of *MAP* infections[10]. Although some studies confirm the presence of JD in Egypt, the disease is currently neglected by the legal Egyptian agencies. There are no authorized data, no national control program, and no vaccination programs. Although JD is a notifiable disease in many countries, the disease is not a notifiable disease in Egypt. Owing to that, the target of scientific research should be directed to *MAP* as a pathogen with a probable for human

transmission. Extra investigations are needed to determine the probability of the existing epidemiological condition of *MAP* infections in Egypt to make a risk assessment. Specific and sensitive diagnostic tools as well as a superior understanding of the pathogenesis of JD are required to develop a practical program of control [11,12,13].

This study aimed to interrogate the prevalence of *Mycobacterium avium subsp. paratuberculosis* in Egyptian raw buffalo's milk; as a proof of the organism excretion in milk and assessment the possibility of using milk as an official tool for the detection of paratuberculosis in buffaloes.

Materials and Methods

1. Collection of milk samples

One hundred ninety- two individual buffalo's milk samples were taken from 15 small sized farms in Cairo, Giza and El Kaliobeya Governorates, Egypt after washing the udder and teat dipping. The first milk stream was discarded and the samples were collected (250 ml) into sterile containers, and then were transferred to the laboratory in an insulated ice box with a minimum of hindrance to be immediately analyzed for:

2. Detection of *MAP* antibodies by milk-ELISA (ID-vet kit, France)[14,15]

In a ninety-six well pre-dilution micro-plate, samples were diluted 1/2 and controls to 1/12 in dilution Buffer 6. Ten microliters of the negative control and 110 µl of dilution buffer number 6 were added to wells $A_1$and B_1. Ten µl of positive control and 110 µl of dilution buffer 6 were added to wells C_1 and D_1. 80 µl of dilution buffer 6 and 80 µl of each sample to be analyzed were added to the remaining wells and incubated for 45 min at 21°C. One-hundred microliters of the previously neutralized samples and controls were transferred to the coated ELISA micro-plates and incubated for 45 min at 21°C. The wells were empty and each well was washed three times with approximately 300 µl of the wash solution.

The conjugate was processed by diluting the concentrated conjugate 10X to 1/10 in dilution buffer number 3. One hundred microliters of the 1X conjugate was added to each well and incubated for 30 min at 21°C. The wells were empty. Each well was washed 3 times with 300 µl of wash solution.100 µl of the substrate solution was added to each well and incubated for 15 min at 21°C in the dark. 100 µl of the stop solution was added to each well to stop the reaction. The OD (Optical Density) was read and recorded at 450 nm. Sample/positive ratio percentage was calculated using control values: Sample/ positive ratio = OD (sample) – OD (negative control)/ OD (positive control) - OD (negative control) × 100. Higher than 15 % are considered positive.

3. Detection of paratuberculosis in the examined samples by milk-PCR[16,17,18]

3.1 Extraction of *MAP* genomic DNA from samples:

Fifty milliliter of milk sample was centrifuged at 4000 rpm for one hour. The pooled fat and pellet were washed four times with Phosphate Buffer Saline at 5000 revolutions per minute for 5 min, the mixture was resuspended in 450 µl TE buffer followed by the addition of 50 microliters lysozyme (10 mg/ml) and incubated overnight at 37ºC. After that, 100µl of 10% SDS and 50µl of proteinase K (10 mg/ml) were added & incubated at 55ºC for two hours and for 3-hour (with shaking and vortexing once per hour) in case of *Promega* method. Two hundred µl of 5M NaCl and 160 microliters Cetyl Trimethyl Ammonium Bromide were added, the suspension was incubated at 65ºC for 30 min. and this was followed by chloroform-isoamyl alcohol extraction and precipitation of isopropanol. The pellet was washed with 70 % ethanol, resuspended in 30 µl TE buffer, and stored at -20ºC for PCR. All these chemicals were produced by Sigma Aldrich (USA).

3.2 IS *900* specific primers for *MAP*:

Forward primer:

5`-CCGCTAATTGAGAGATGCGATTGG- 3`.

Reverse primer:

5`-AATCAACTCCAGCAGCGCGGCCTCG- 3`.

3.3 DNA amplification by conventional PCR:

PCR was performed in 25 microliters reaction volume in 200 microliter PCR tubes using 5 µl of extracted DNA from milk samples. Amplification cycles were carried out in programmable thermo-cycler (Brimus MWG, Germany). A number of experiments were performed to optimize the PCR method. The optimized PCR assay was finally established using a total volume of 25 microliters reaction mixtures contained 5 microliters of DNA as template, 20 pmol of each primer and 1X of PCR master mix (Dream Taq™ Green PCR).

3.4 Reaction conditions for IS*900* primer:

Initial denaturation was optimized at 95°C for 5 min as followed by 50 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 59 second. Finally, extension step at 72°C for 10 min was followed.

3.5 Visualization of the products of PCR:

After amplification, 10 microliters of the PCR product were electrophoresed on 1.5% agarose gel electrophoresis containing Tris- Borate- EDTA TBE buffer 0.5X and 0.5 µg/ml Ethidium Bromide for 60 min at 70 volts in an electrophoresis unit (Bio-Rad) containing 0.5X TBE as electrophoresis buffer. Amplification products were visualized using ultraviolet trans-illuminator and photographed using a digital camera (Fin-Pix S9600S). The size of the amplification products were compared with the Gene-Ruler™ 100 bp Plus DNA Ladder, Fermentas Life Science. Samples showing positive amplification of 229 bp for IS*900* primer.

Results and Discussion

Table (1): Prevalence of *Mycobacterium avium subsp. paratuberculosis* in the examined buffalo's milk samples based on serological method (ELISA) and PCR method (n=192).

Methods	Positive (+)		Doubtful (±)		Negative (-)	
	No. of samples	%	No. of samples	%	No. of samples	%
ELISA	16	8.33	0	0.00	176	91.67
PCR	0	0.00	0	0.00	192	100.00

n= number of the examined samples.

Paratuberculosis is a chronic contagious bacterial enteric disease that affects ruminants; it is considered as important disease primarily in cattle, sheep & goats and is caused by *Mycobacterium avium subsp. paratuberculosis*. It was first described over 100 years ago in Germany. Milk has been reported as the main source of transmission of *MAP* to humans and animals[19].

Infected animals may excrete *MAP* in their feces & milk and spread the infection, which can cause financial losses due to premature culling and death [20]. This organism infects animals in the first months of life and causes a slowly progressive inflammation in the gastrointestinal tract that is not clinically proof until months to years later. Signs of this infection are weight loss chronically and diarrhea. In progressing cases, the organism can disseminate further away the gastrointestinal tract to other body organs [21].

Recent evidence suggests that *MAP* can induce the mucosal ulcerations in Inflammatory Bowel Disease. The possibility of a health risk for consumers resulting from viable *MAP*, DNA, inactive cells and even from their structural components[3,4,22,23].

Out of 192 milk samples tested, 16 (8.33%) samples could be detected by ELISA technique. However, no samples were found positive in Polymerase Chain Reaction method **(Table 1)**. Paratuberculosis is characterized by two phases; the first is the tuberculoid and is characterized by a strong cell mediated response. The second is the leproid, in which humoral response is high[24].

The ELISA method was first used for serum antibodies detection against *MAP* in cattle in the late 1980s and the indirect detection of antibodies in the blood serum of calves were later confirmed by the direct detection of anti-*MAP* antibodies in colostrum and milk[25].

Milk- ELISA is considered an alternative to fecal culture as cost-effective and accurate as paratuberculosis screening test [26]. The results of ELISA technique may be attributed to lower concentration of antibodies in milk than serum and the intermittent shedding in milk [27,28].

Molecular techniques based on the detection of *MAP* DNA. Polymerase Chain Reaction (PCR) was evaluated for detection of *MAP*. The rapidity and specificity of the PCR assay would greatly facilitate Johne's disease detection among susceptible ruminants and could be used for detection of this disease at an early infection [29].

Polymerase Chain Reaction is a rapid and specific method for the detection of *MAP* in milk. Mostly PCR protocols target the IS*900* insertion sequence, which has been agreeable as a standard marker for *MAP*. It can be seen that considerable variations exist among PCR methods. The analyzed volume of milk, state of centrifugation, the number of examined samples, Different techniques for extraction of genomic DNA from samples, also the sensitivity and detection limit of the used PCR technique differ from method to another [30].

Although molecular methods offer the benefit of rapid results they suffer from low sensitivity of detection[31]. Factors were reported to improve the recovery of *MAP* from raw milk: large sample volume, pooling the pellet & cream fractions and washing of pellet before DNA extraction for removing milk inhibitors (calcium ions) in PCR[32,33].

Due to the scarcity of information about paratuberculosis among the owners of buffaloes, lack of regulations on animal trade and standard husbandry practices, a continuous spread of paratuberculosis among flocks is highly common. The safety of raw milk came from *MAP* infected animal becomes a great attention for health of human, because *MAP* could be connected with Crohn's disease [34].

Conclusion

Adequate *MAP* control programs should be established to control such diseases among buffaloes and consumers. In a control programs, an ELISA test would be an excellent first procedure for identifying the affected animals and could be applied as a supplement tool for diagnosis of *Mycobacterium avium subsp. paratuberculosis* in susceptible buffaloes. Educational programs should be imposed for producers, processors and handlers to improve the quality of the buffalo's milk and to insure a maximum safety to consumers. Implementation of HACCP plan built upon a solid foundation of prerequisites program is required for the safety production of such milk.

References

1. Streeter RN, Hoffsis GF, Bech-Nielsen S, Shulaw WP and Rings DM. Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. Am. J. Vet. Res., 1995, 56(10): 1322–1324.
2. Ellingson JE, Anderson JL, Koziczkowski JJ, Radcliff RP, Sloan SJ, Allen SE and Sullivan NM. Detection of viable *Mycobacterium avium subsp. paratuberculosis* in retail pasteurized whole milk by two culture methods and PCR. Journal of Food Protection, 2005, 68 (5): 966–972.
3. Rath T, Roderfeld M, Blöcher S, Rhode A, Basler T, Akineden O, Abdulmawjood A, Halwe J, Goethe R, Bülte M and Roeb E. Presence of intestinal *Mycobacterium avium* subspecies *paratuberculosis (MAP)* DNA is not associated with altered MMP expression in ulcerative colitis. BMC Gastroenterology, 2011, 11(34): 1-19.
4. Dalton JP, Desmond A, Shanahan F and Hill C. Detection of *Mycobacterium avium* subspecies *paratuberculosis* in patients with Crohn's disease is unrelated to the presence of single nucleotide polymorphisms rs 2241880 (ATG16L1) and rs10045431 (IL12B). Med. Microbiol. Immunol. 2014, 203(3): 195-205.

5. Solanki R, Madat D, Chauhan K and Parmar L. Recent approaches in pathogenesis of Inflammatory Bowel Disease. International Journal of PharmTech Research, 2010, 2(3):1796-1809.
6. Rajinikanth B and Venkatachalam VV. Study on the effect of Amlodipine on chemically induced inflammatory bowel disease using animal model. International Journal of PharmTech Research, 2014-2015, 7(1): 119-124.
7. Sweeney RW, Whitlock RH and Rosenberger AE. *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph node of infected asymptomatic cows. J. Clin. Microbiol., 1992, 30: 166–171.
8. Chiodini RJ and Hermon-Taylor J. The thermal resistance of *Mycobacterium paratuberculosis* in raw milk under conditions simulating pasteurization. Journal of Veterinary Diagnostic Investigation.1993, 5: 629–631.
9. CFSPH, Center for Food Security & Public Health. Paratuberculosis, Johne's Disease. http://www.cfsph.iastate.edu/Factsheets/pdfs/paratuberculosis.pdf 2007.
10. Okura H, Toft N and Nielsen SS. Occurrence of *Mycobacterium avium subsp. paratuberculosis* in milk at dairy cattle farms: A systematic review and meta-analysis. Vet. Microbiol., 2012, 157 (3-4): 253-263.
11. Salem M, Zeid AA, Hassan D, El Sayed A and Zschoeck M. Studies on Johne's disease in Egyptian cattle. J. Vet. Med., 2005, 52:134–137.
12. Abd El-Malek SA and Mohamed KF. Phylogenetic Analysis of *Mycobacterium avium subsp. paratuberculosis* of Some Egyptian Isolates Isolated from Clinically Infected Dairy Cattle. Int. J. Microbiol. Res., 2011, 2(1): 49-53.
13. Collins MT. Johne's disease, an Issue of Veterinary Clinics: Food Animal Practice. Elsevier Health Sciences. 2011.
14. Milner AR. Analysis by ELISA and Western blotting of antibody reactivities in cattle infected with *M. paratuberculosis* after absorption of serum with *M. phlei*. Res. Vet. Sci. 1987, 42:140–144.
15. Singh SV, Singh AV, Singh PK, Gupta VK, Kumar S and Vohra J. Sero-prevalence of paratuberculosis in young kids using "Bison type", *Mycobacterium avium subsp. paratuberculosis* antigen in plate ELISA. Small Rumin. Res. 2007, 70: 89–92.
16. Vary P, Andersen P, Green E, Hermon-Taylor J and McFadden J. Use of highly specific DNA probe and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. J. Clin. Microbiol., 1990, 28: 933–937.
17. Paolicchi F, Zumarraga M, Gioffre A, Zamorano P, Morsella C, Verna A, Cataldi A, Alito A and Romano M. Application of different methods for the diagnosis of *Mycobacterium avium subsp paratuberculosis* in a dairy cattle herd in Argentine. Journal of Veterinary Medicine Series ″B″, 2003, 50(1): 20–26.
18. Promega. Wizard genomic DNA purification kit. Technical manual. Promega Corporation. 2800 Woods Hollow Road· Madison, WI 53711-5399 USA. Revised 12/14, 2014.
19. OIE, World Organization for Animal Health. OIE Terrestrial Animal Health Code: www.oie.int/en/internationalstandard-setting/terrestrial-code/access-online/ 2015.
20. Ayele WY, Svastova P, Roubal P, Bartos M and Pavlik I. *Mycobacterium avium* subspecies *paratuberculosis* cultured from locally and commercially pasteurised cow's milk in the Czech Republic. Applied and Environmental Microbiology, 2005, 71(3): 1210 –1214.
21. Whitlock HR and Buergelt C. Preclinical and clinical manifestations of paratuberculosis (including pathology). Vet. Clin. North America Anim. Pract., 1996, 12(2): 345-356.
22. Juan J, Estiarte R, Colome E, Artes M, Jimenez FJ and Alonso J. Burden of illness of Crohn's disease in Spain. Digestive and liver disease J., 2003, 35(12): 853-861.
23. Ebinger M, Leidl R, Thomas S, Von Tirpitz C, Reinshagen M, Adler G, and Konig HH. Cost of outpatient care in patients with inflammatory bowel disease in German university hospital. J. Gastroenterol. and Hepatol., 2004, 19(2): 192-199.
24. Slana I, Paolicchi F, Janstova B, Navratilova P and Pavlik I. Detection methods for *Mycobacterium avium* subsp. *paratuberculosis* in milk and milk products: a review. Veterinarni Medicina., 2008, 53(6): 283–306.
25. Jorgensen JB and Jensen PT. Enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to *Mycobacterium paratuberculosis* in cattle. Acta Veterinaria Scandinavica, 1978, 19: 310–312.

26. Hendrick S, Duffield T, Leslie K, Lissemore K, Ar- chambault M and Kelton D. The prevalence of milk and serum antibodies to *Mycobacterium avium* subspecies *paratuberculosis* in dairy herds in Ontario. Canadian Veterinary Journal- Revue Veterinaire Canadienne, 2005, 46(12): 1126–1129.
27. Van der Giessen JW, Haring RM, Vauclare E, Eger A, Haagsma J and Van der Zeijst BA. Evaluation of the abilities of three diagnostic tests based on the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in cattle: application in a control program. J. Clin. Microbiol., 1992, 30(5): 1216 – 1219.
28. Whipple DL, Kapke PA and Andersen PR. Comparison of a commercial DNA probe test and three cultivation procedures for detection of *Mycobacterium paratuberculosis* in bovine feces. J. Vet Diagn Invest., 1992, 4(1): 23–27.
29. Ibrahim A, El-Sanousi S and Aradaib I. Detection of *Mycobacterium avium* subspecies *paratuberculosis* using nested polymerase chain reaction. Veterinarski Arhiv., 2004, 74(1): 27-35.
30. Englund S, Bolske G and Johansson KE. An IS*900* - like sequence found in a *Mycobacterium* sp. other than *Mycobacterium avium subsp. paratuberculosis*. Federation of European Microbiological Societies (FEMS) Microbiology Letters, 2002, 209(2): 267–271.
31. Giese S and Ahrens P. Detection of *Mycobacterium avium subsp. paratuberculosis* in milk from clinically affected cows by PCR and culture. Vet. Microbiol., 2000, 77: 291–297.
32. Pillai S and Jayarao B. Application of IS*900* PCR for detection of *Mycobacterium avium subsp. paratuberculosis* directly from raw milk. J. Dairy Sci. 2002, 85:1052–1057.
33. Herthnek D, Englund S, Willemsen P, Bölske D. Sensitive detection of *Mycobacterium avium subsp. paratuberculosis* in bovine semen by real time PCR. J. Appl. Microbiol., 2006, 200:1095–1102.
34. Paolicchi F, Cirone K, Morsella C and Gioffre A. First isolation of *Mycobacterium avium subsp. paratuberculosis* from commercial pasteurized milk in Argentina. Brazilian journal of microbiology, 2012: 1034-1037.

*****