International Journal of Veterinary Sciences and Animal Husbandry

ISSN: 2456-2912
VET 2024; 9(2): 300-302

www.veterinarypaper.com
Received: 19-12-2023
Accepted: 23-01-2024

MV Chinnu
Department of Veterinary Biochemistry, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala Veternary and Animal Sciences University, Kerala, India

R Uma
Department of Veterinary Biochemistry, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala Veternary and Animal Sciences University,Kerala, India

K Roshna
Department of Veterinary Biochemistry, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala Veternary and Animal Sciences University, Kerala, India

Ammu Ramakrishnan
Kerala Livestock Development Board, Mattupetty, Kerala, India

Corresponding Author:
MV Chinnu
Department of Veterinary Biochemistry, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala Veternary and Animal Sciences University, Kerala, India

Detection of replication competent circular DNA from cattle serum

MV Chinnu, R Uma, K Roshna and Ammu Ramakrishnan

Abstract
Recently the discovery of novel infectious agents in dairy and meat products from cattle of European origin (Bos Taurus) has been reported. These pathogens called as "Bovine Meat and Milk Factors (BMMFs)" are small single stranded circular DNA molecules that have sequence similarity to plasmids of Acinetobacter baumanni and some viral agents. Four groups of BMMFs have been so far reported to be isolated from serum, milk and meat of large and small ruminants. The present study was carried out to standardise a PCR for the detection of circular DNA in the serum of cattle. Serum samples were collected from apparently healthy purebred Holstein Friesian cattle and DNA was isolated. The DNA was then subjected to rolling circle amplification (RCA) and PCR was carried out with specific primers for BMMF. The annealing temperature was standardised at 58ºC. The amplified products were sent for sequencing. Sequencing of the amplicons revealed a product of about 2kb with 99% sequence similarity to previously isolated BMMF2.

Keywords: BMMF, RCA, Circular DNA, *Acinetobacter baumanni*

1. Introduction
Red meat and dairy products have often been accused as risk factors for cancer, chronic neurodegenerative diseases, autoimmune and cardiovascular disorders. High incidence of colon and breast cancer has been reported in countries where large quantities of products of European cattle are consumed in contrast to their low incidence in Asian countries which mainly consume products of zebus. Investigations for the presence of new infectious agents in dairy and meat products sourced from European-origin cattle (*Bos Taurus*) leads to the discovery of "Bovine Meat and Milk Factors (BMMFs)", which are small single stranded circular DNA molecules that have sequence similarity to plasmids of *Acinetobacter baumanni* and viral agents like transmissible spongiform encephalopathy (TSE) - associated circular DNA isolates. Genetic analysis of the BMMF reveals that it carries a gene that encodes "Rep" protein (replication initiator protein). The *Rep* gene has a very high sequence identity to bacterial replication initiation genes (De Villiers *et al*., 2019) [6]. The sequence also contains a putative origin of replication which makes them similar to circular rep-encoding single-strand DNA (CRESS) viruses. So the present study aims to standardize a PCR reaction for the isolation of circular DNA from cattle serum and is reported to be the first work of this kind in India.

2. Materials and Methods
Sample collection
To standardise the procedure for the isolation of circular DNA, serum samples were collected from purebred Holstein Friesian cattle maintained at Kerala Livestock Development Board, Mattupetty. The collected serum and milk samples were subjected to lyophilisation before DNA isolation in a lyophiliser (Operon FDU7003, Korea) at Central Instruments Laboratory, College of Veterinary and Animal Sciences, Mannuthy in order to concentrate the samples for maximum recovery of circular DNA present in them.

Isolation of circular DNA
DNA from all the samples were extracted using QIAamp DNA Mini Kit (Qiagen, Hilden,

Germany) according to the manufacturer's instructions and subjected to rolling circle amplification using exo-resistant random primers. Template DNA (50 ng) was incubated in a total volume of 10µL (1x phi29 DNA polymerase buffer) with 25µM exo-resistant random primer (Thermo Fisher Scientific) at 95°C for 3 min. followed by cooling on ice for DNA denaturation. This denatured sample was then diluted to 20µL by adding 10 mM dNTPs (Takara) each and 10U phi29 DNA polymerase (New England Biolabs) in 1X phi29 DNA polymerase buffer. The mixture was then incubated sequentially at 30°C for 18 h. and at 65°C for 10 min. The RCA products were amplified for the BMMF sequence using the primer
(Forward primer-5´AAGGCAGATCAACACAGG3´, Reverseprimer-5´AGCAGATTGCAAAGCCTG 3´).
The PCR conditions were standardised to minimise nonspecific amplification and to get maximum amplification of the desired product. The PCR was performed in a total volume of 20µL with 10 µM each of forward and reverse primers. The thermal cycling profile consisted of denaturation at 94°C for 30 sec, annealing at 58°C for 1 min and extension at 58°C for 2 min for 34 cycles followed by final extension at 72°C for 10 min. The amplicons were electrophoresed in 0.6 percent agarose gel for 40 min.

Sequence Analysis
The amplicons were purified and sequenced at the DNA sequencing facility of Gene Spec Labs Private Limited, Kochi, Kerala using the primer (Forward primer 5´AAGGCAGATCAACACAGG3´,Reverseprimer-5´AGCAGATTGCAAAGCCTG 3´). The sequence similarity search was performed using Basic Local Alignment Search Tool (BLASTn) provided by the National Centre for Biotechnological Information (NCBI). NCBI ORF finder tool was used to identify the Open reading Frame of the sequence. BLASTp analysis was carried out to find out the similarity of proteins encoded by the ORF region.

3. Results and Discussion
Serum samples collected were lyophilised in order to concentrate their contents for recovery of maximum amount of circular DNA present in them, if any. Isolated DNA was further subjected for RCA. Rolling circle amplification is an isothermal amplification process in which circular DNA template will be converted to linear DNA using the enzyme phi 29 DNA polymerase and exo-resistant random primers. During RCA the concentration of DNA will get increased by many folds. Figure 1 shows the electrophoretogram of RCA product. After RCA, the circular DNA obtained was amplified using back to back primers. Initially, gradient PCR was done using RCA product as template to standardise the annealing temperature which was standardised at 58°C for 1 min. Further amplification resulted in amplicons of size 2100-2500bp.

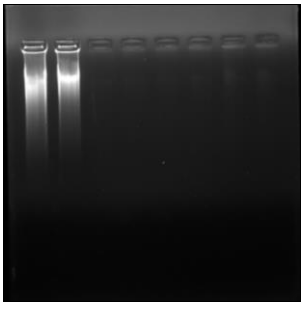
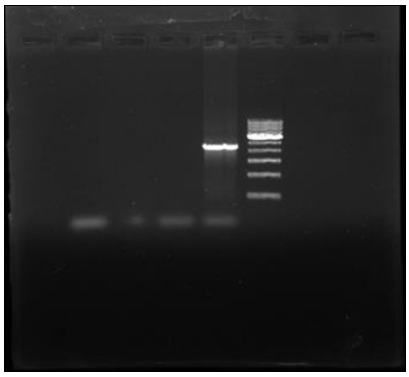


Fig 1: Electrophoretogram of RCA product



Lane M: 500 bp ladder,
Lane L: Amplicons of BMMF

Fig 2: Amplicons of BMMF

The amplicons were sequenced and the sequences were analysed using NCBI BLASTn (www.ncbi.hlm.nih.gov/BLASTn). The isolates obtained from pure bred HF serum displayed 99.18 percent identity and 100 percent query coverage with Sphinx/BMMF group2 DNA sequence as reported by Whitley *et al*., 2014 [13] and Zur Hausen *et al*., 2017 [15]. These isolates also shared sequence similarity with the plasmids of bacterium *Acinetobacter baumanni.*
The obtained sequences from the study were analysed for the presence of putative proteins by finding the ORF present in them using NCBI ORF finder. The parameters were set to find ATG as initiation codon by setting standard genetic code as reference and 75 nucleotides as minimal ORF length. The BLASTp analysis of protein encoding ORF sequences revealed that the identified single stranded circular DNA potentially encodes at least one replication initiation protein, which shared similarity with the rep protein of a bacterium *Acinetobacter* and early detected SPHINX/BMMF group.

4. Conclusion
The present study was conducted to standardise a PCR protocol for detection of circular DNA from cattle serum and annealing temperature was standardised at 58ºC for one minute. Analysis of the sequences revealed their similarity to the previously isolated BMMF. The study reports for the first time the presence of BMMF in India.

5. Acknowledgements
The facilities provided by College of Veterinary and Animal Sciences, Mannuthy, Kerala Veterinary and Animal Sciences University is duly acknowledged.

6. Conflict of interest
The authors declare that they have no conflict of interest

7. References
1. Abid Z, Cross AJ, Sinha R. Meat, dairy, and cancer. Am. J Clin. Nutr. 2014;100:386S-393S.
2. Ali I, Wani WA, Saleem K. Cancer scenario in India with future perspectives. Cancer Ther; c2011, 8.
3. Ali MM, Li F, Zhiqing Zhang Z, Zhang K, Kang DD, *et al*. Rolling circle amplification: A versatile tool for chemical biology, materials science and medicine. Chem. Soc. Rev. 2014;43(10):3324-3341
4. Bund T, Nikitina E, Chakraborty D, Ernst C, Gunst K, Boneva B, *et al*. Analysis of chronic inflammatory lesions of the colon for BMMF Rep antigen expression

and CD68 macrophage interactions. PNAS. 2021;118(12):1-9.
5. Dean FB, Nelson JR, Giesler TL, Lasken RS. Rapid amplification of plasmid and phage DNA using PHI29 DNA polymerase and multiply-primed rolling circle amplification. Genome Res. 2001;**11**:1095-1099.
6. De Villiers EM, Gunst K, Chakraborty D, Ernst C, Bund T, Hausen ZH. A specific class of infectious agents isolated from bovine serum and dairy products and peritumoral colon cancer tissue. Emerg. Microbes Infec. 2019;8(1):1206-1218.
7. Eilebrecht S, Wagenblatt HA, Sarachaga V, Burk A, Falida K, Chakraborty D, *et al*. Expression and replication of virus-like circular DNA in human cells. Sci. Rep. 2018;8(1):1-15
8. Funk M, Gunst K, Lucansky V, Müller H, Hausen Z H, De Villiers E. Isolation of protein-associated circular DNA from healthy cattle serum. Genome Announc. 2014;2(4):e00846-14.
9. Salazar GHR, Cruz AA, Pérez MD, Hernández SAD, Esquivel GJ, Galindo MDA, *et al*. Association of milk and meat consumption with the development of breast cancer in a Western Mexican Population. Breast Care. 2015;10:393-396
10. Linos E, Willett WC, Cho E, Colditz G, Frazier LA. Red meat consumption during adolescence among premeno-pausal women and risk of breast cancer. Cancer Epidemiology Biomarkers Prev. 2008;17:2146-2151.
11. Konig M, Fux RR, Link E, Sutter G, Märtlbauer EE, Didier AA. Circular rep-encoding single-stranded DNA Sequencesin milk from water buffaloes (*Bubalusarnee* F. Bubalis). Viruses. 2006;13:1-13.
12. Vaughan H, Chalker VJ, Mee Z, Rossouw A, James V. Stability of lyophilised specimens for the molecular detection of viral DNA/RNA. Journal of clinical virology. 2006;35(2):135-140.
13. Whitley C, Gunst K, Müller H, Funk M, Hausen ZH, De Villiers EM. Novel replication-competent circular DNA molecules from healthy cattle serum and milk and multiple sclerosis-affected human brain tissue. Genome Announc. 2015;2(4):e00849-14
14. Hausen Z H, De Villiers EM. Dairy cattle serum and milk factors contributing to the risk of colon and breast cancers. Int. J Cancer. 2015;137:959-967.
15. Hausen ZH, Bund T, De Villiers EM. Infectious agents in bovine red meat and milk and their potential role in cancer and other chronic diseases. Curr. Top. Microbiol. Immunol. 2017;407:83-116.
16. Hausen ZH, Bund T, De Villiers EM. Specific nutritional infections early in life as risk factors for human colon and breast cancers several decades later. Int. J Cancer. 2019;144:1574-1583