Open Access

Check for updates

Research Article

Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605
https://doi.org/10.51966/jvas.2024.55.3.631-634

Detection of replication-competent circular single stranded DNA from Deoni cattle#

M. V. Chinnu[1*], R. Uma[1], M. Shynu[1], Varuna P. Panicker[1], Deepthi Vijay[2], K. Roshna[1] and P. S. Megha

[1]Department of Veterinary Biochemistry, [2]Veterinary Public Health, [3]Department of Veterinary Physiology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur- 680651, Kerala Veterinary and Animal Sciences University, Kerala, India

Citation: Chinnu, M.V., Uma, R., Shynu, M, Varuna, P.P., Deepthi, V., Roshna, K. and Megha, P.S. 2023. Detection of replication-competent circular single stranded DNA from Deoni cattle.
J. Vet. Anim. Sci. **55** (3):631-634

Received : 15.03.2024 Accepted : 13.06.2024 Published : 30.09.2024

Abstract

Bovine meat and milk factors (BMMF) having structural similarity to plasmids of Acinetobacter baumanni and some single stranded (ss) DNA viruses are free circulating replication-competent circular ssDNA molecules which were first isolated from serum of Bos taurus animals. Isolation of BMMF from tissues of multiple sclerosis as well as some cancers led researchers to conclude that the consumption of these agents through milk and meat have a positive correlation with these diseases and that the absence of such molecules in B. indicus animals could account to the low occurrence of cancer in those areas consuming products of indigenous animals. Hence, the present study was designed to investigate its presence in Deoni, one of the indigenous cattle breeds of India. The DNA isolated from milk samples were subjected to rolling circle amplification (RCA) using exo-resistant random primers. The products of RCA were then used as template for PCR for amplification of circular DNA. The amplicons obtained of size around 2000bp were cloned in E. coli and sequenced. The BLASTn, multiple sequence alignment and phylogenetic analysis of the obtained sequences were carried out to identify the similarities of these isolates. The sequence showed maximum homology with either BMMF2 or plasmids of Acinetobactor spp. Replication initiation protein (Rep) which showed similarity to bacterial plasmids was present in the sequence. This is the pioneer report on the presence of BMMF in milk of indigenous cattle of India.

Keywords: *BMMF, replication-competent, circular ssDNA, Deoni*

Red meat and dairy products have often been accused as risk factors for cancer (Abid *et al*., 2014), chronic neurodegenerative diseases, autoimmune and cardiovascular disorders. Recently the discovery of single stranded (ss) circular DNA molecules from *Bos taurus* animals, named as Bovine Meat and Milk Factors (BMMF) having structural similarity to plasmids of *Acinetobacter baumanni* and some ssDNA viruses triggered the hypothesis that these could be novel pathogens, whose properties lie between those of viruses and bacteria (Funk *et al.*, 2014; Falida *et al*.,2017). The BMMFs are grouped based on their nucleotide similarity to known organisms into four categories - BMMF1, BMMF2, BMMF3 and BMMF4. The first three groups have a remarkable degree of similarity to plasmid of *A. baumannii*. The BMMF1 and BMMF2 also displayed many characteristic features of many single stranded circular DNA viruses (zur Hausen *et al.*, 2017; zur Hausen *et al.*, 2019).

#Part of Ph.D thesis submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala
** Corresponding author: chinnu@kvasu.ac.in , Ph.9562968495*

Copyright: © 2024 Chinnu *et al.* This is an open access article distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

The isolation of these agents from brain tissues of multiple sclerosis (Lamberto *et al*., 2014; Whitley *et al*., 2014) as well as peritumour tissues of colorectal carcinoma (de Villiers *et al.,* 2019) led to the conclusion that the consumption of these agents through milk and meat have a positive correlation with these diseases. They also suggested that the absence of such molecules in *Bos indicus* animals could account to the low occurrence of cancer in countries like India where products of indigenous animals are more preferred. Hence, the present study was designed to investigate the presence of free circulating replication-competent circular ssDNA in Deoni, the indigenous cattle breed of Maharashtra.

Materials and methods

Sample collection and preparation

For the isolation of circular DNA, milk samples were collected from each of six apparently healthy Deoni cattle maintained at Southern Regional Station, ICAR-National Dairy Research Institute, Adugodi, Bengaluru. The collected milk samples were subjected to lyophilisation before DNA isolation 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 (RCA) 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 pair- F5´AAGGCAGATCAACACAGG3´, R5´AGCAGATTGCAAAGCCTG 3´ (de Villiers *et al*., 2019).

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 per cent agarose gel for 40 min. The amplicons obtained after PCR were then subjected to cloning using pCR®2.1 cloning vector and TOP 10 *E.coli* cloning vector. The ligation mixture was prepared at an insert vector ratio of 1:1 and was used to transform competent TOP 10 *E.coli* cells. The transformed cells were selected by blue-white screening. The bacterial cells were confirmed for insert by colony PCR and were subjected to plasmid isolation. The isolated plasmids were again confirmed for the presence of insert by plasmid PCR and were further chosen for commercial sequencing at the DNA sequencing facility of Gene Spec Labs Private Limited, Kochi, Kerala using the abovementioned primer pair.

Sequence analysis

The sequence similarity search was performed using Basic Local Alignment Search Tool (BLASTn) provided by the National Centre for Biotechnological Information (NCBI). Tandom repeats in the sequences was determined using TR finder. The 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 the putative proteins encoded by the ORF region.

Results and discussion

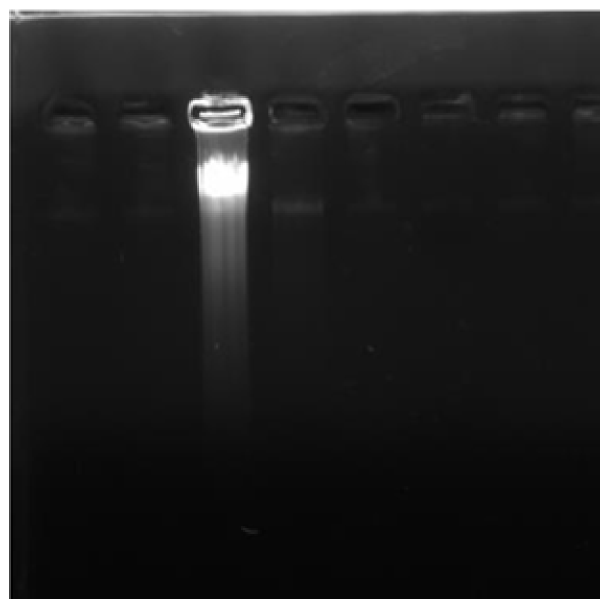

Fig. 1. Electrophoretogram of RCA products from Deoni milk sample

Table 1. Tandem repeat analysis data of circular DNA sequences obtained from Deoni milk

Sample	Sequences	Positions	Length of repeat unit	No. of repeats
Deoni Milk	TGCAAA	413, 613, 635	6	3
	CTGCAA	5, 78, 634	6	3
	TTTTA	360, 379, 431	5	3
	AAACT	2, 537, 594	5	3
	CAATG	189, 219, 470	5	3
	CAAAT	85, 140, 637	5	3
	TATA	230, 434, 550	4	3
	TAAT	121, 252, 403	4	3
	CACG	161, 545, 605	4	3

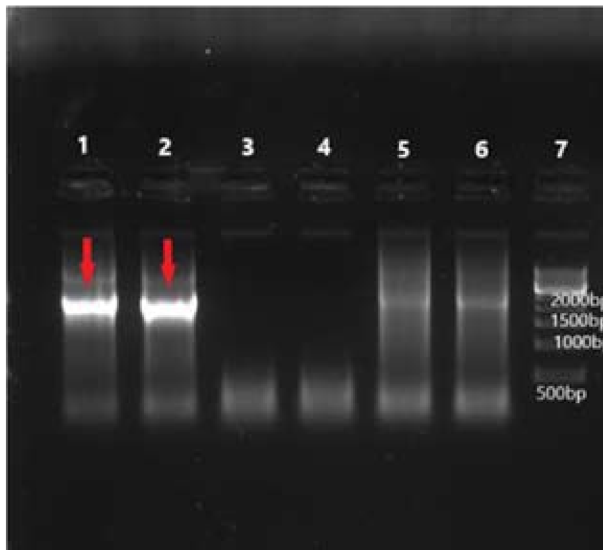

Fig. 2. Electrophoretogram of PCR products
Lane 1&2 : Deoni milk isolate
Lane 7 : 500bp Ladder

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 (Ali *et al*., 2014). During RCA the concentration of DNA will get increased by many folds. After RCA (Fig.1), 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 around 2000bp (Fig.2). The products were cloned using pCR®2.1 cloning vector and TOP 10 *E. coli* cloning vector. The clones were sequenced and the sequences were analysed using NCBI BLASTn (www.ncbi.hlm.nih.gov/BLASTn). Deoni milk isolate (DM1) showed maximum query coverage and identity with TSE-associated circular DNA isolate Sphinx 2.36 followed by SPHINX/BMMF group 2 DNA sequence. Similar sequences were isolated from serum and milk samples of Eurasian cattle (Whitley *et al*., 2014; Zur Hausen *et al*., 2017). Multiple sequence alignment and phylogenetic analysis also revealed the homology of obtained sequence with that of previously isolated BMMF2.

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 *Acinetobacter* spp and early detected SPHINX/BMMF2 group. The sequences obtained contained multiple tandem repeats as shown in table 1.

The biggest ORF identified in the sequence had a length of 516nt/171aa, starting from 191nt to 706nt and it showed maximum similarity with replication initiation protein [SPHINX/BMMF group 2 DNA sequence] (100% query coverage and 90.1 identity) and replicase 1-like protein [TSE-associated circular DNA] (97% query coverage and 97.67% identity).

The findings from this study prove that BMMF which were claimed to be solely found in milk and meat products of taurine cattle (zur Hausen *et al*., 2017) are seen in milk of zebu cattle as well. The present study is the pioneer work on the presence of BMMF in zebu cattle of India. Further detailed *in vitro* and *in vivo* analyses will be needed to prove the role of circular ssDNA molecules in the development of cancer and neuro degenerative diseases.

Conclusion

The present study confirmed for the first time the presence of single stranded circular DNA in the milk of Deoni cattle. The detailed analysis of the sequences revealed maximum similarity to the previously isolated BMMF2 group sequences. Further studies are needed to analyse the pathogenicity of the isolates.

Acknowledgements

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

Conflicts of interest

The authors declare that they have no conflict of interest.

References

Abid, Z., Cross, A.J. and Sinha, R. 2014. Meat, dairy, and cancer. *Am. J. Clin. Nutr.* **100**: 386S-393S.

Ali, M. M., Li, F., Zhang, Z., Zhang, K., Kang, D., Ankrum, J.A., Le, X. C. and Zhao, W. 2014. Rolling circle amplification: a versatile tool for chemical biology, materials science and medicine. *Chem. Soc. Rev.* **43**: 3324-3341.

de Villiers, E. M., Gunst, K., Chakraborty, D., Ernst, C., Bund, T. and zur Hausen, H. 2019. A specific class of infectious agents isolated from bovine serum and dairy products and peritumoral colon cancer tissue. *Emerg. Microbes Infec*. **8**: 1206-1218.

Falida, K., Eilebrecht, S., Gunst, K., Zur Hausen, H. and de Villiers, E.M. 2017. Isolation of two virus-like circular DNAs from commercially available milk samples. *Genome announc*. **5**: 10-1128.

Funk, M., Gunst, K., Lucansky, V., Müller, H., Zur Hausen, H. and de Villiers, E.M. 2014. Isolation of protein-associated circular DNA from healthy cattle serum. *Genome Announc*. **2**: 10-1128.

Lamberto, I., Gunst, K., Müller, H., Zur Hausen, H. and de Villiers, E.M. 2014. Mycovirus-like DNA virus sequences from cattle serum and human brain and serum samples from multiple sclerosis patients. *Genome Announc.* **2**: 10-1128.

Whitley, C., Gunst, K., Müller, H., Funk, M., Zur Hausen, H. and de Villiers, E.M. 2014. Novel replication-competent circular DNA molecules from healthy cattle serum and milk and multiple sclerosis-affected human brain tissue. *Genome Announc.* **2**: 1110-1128.

Zur Hausen, H., Bund, T., and de Villiers, E.M. 2017. Infectious agents in bovine red meat and milk and their potential role in cancer and other chronic diseases. *Curr. Top. Microbiol. Immunol*. **407**: 83–116.

Zur Hausen, H., Bund, T. and de Villiers, E.M. 2019. Specific nutritional infections early in life as risk factors for human colon and breast cancers several decades later. *Int. J. Cancer*. **144**: 1574-1583. ■