Agric Res
DOI 10.1007/s40003-016-0233-7

FULL-LENGTH RESEARCH ARTICLE

Microbiological and Molecular Investigation of Clinically Suspected Caseous Lymphadenitis Cases in Goats

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Received: 12 April 2015 / Accepted: 27 June 2016

Abstract Chronic suppurative lymphadenitis characterized by abscess formation is of economic significance in sheep and goats. It is principally caused by *Corynebacterium pseudotuberculosis* (caseous lymphadenitis, CLA) and *Staphylococcus aureus* subsp. *anaerobius*. Pus samples from superficial lymph nodes of 33 (4.38 %) out of 752 adult Sirohi goats showing clinical lesions similar to CLA were collected for laboratory investigations. Sixteen (48.48 %) bacterial isolates from pus were identified as *C. pseudotuberculosis*, 12 (36.36 %) as catalase-negative *S. aureus* subsp. *anaerobius* while samples from 5 cases were negative. The polymerase chain reaction (PCR) assays targeting putative oligopeptide/dipeptide ABC transporter, nicotinamide adenine dinucleotide phosphate oxidoreductase coenzyme F420-dependent and proline iminopeptidase genes of *C. pseudotuberculosis* when carried out directly on the DNA extracted from pus were positive in the same 16 goats. All the *C. pseudotuberculosis* isolates were also found to be positive for these genes in the PCR. Isolates of *S. aureus* subsp. *anaerobius* were found to be positive for 16S rRNA and nuclease (nuc) gene PCR. The present study shows the incidence of CLA as 4.38, 2.12 and 2.12 % based on the clinical, bacterial culture and direct PCR assay on pus samples, respectively. The incidence of abscess disease due to *S. aureus* subsp. *anaerobius* was 1.59 %. The three gene PCR assay developed in the study was found to be specific and rapid than the bacterial culture in detecting bacteria directly in the pus samples and can be applied for the diagnosis and control strategy of CLA.

Keywords *Corynebacterium pseudotuberculosis* · Caseous lymphadenitis · Polymerase chain reaction · Lymph nodes · *Staphylococcus aureus* subsp. *anaerobius* · Abscess disease

Introduction

Chronic suppurative lymphadenitis of external and internal lymph nodes has been described in the literature as caseous lymphadenitis (CLA) and abscess disease in sheep and goats [4, 19]. CLA caused by *Corynebacterium pseudotuberculosis* is a highly contagious disease and is of significant economic concern for goat and sheep husbandry due to reduced wool, meat and milk yield, loss of fertility, culling of affected animals, condemnation and downgrading of affected carcass at the time of slaughter and meat inspection [13, 28]. There are only few reports of its occurrences among sheep and goats in India [16, 18]. Similar pattern of abscessation indistinguishable from CLA has also been found to be associated with other bacterial infections such as *S. aureus*, S. *aureus* subsp. *anaerobius* thus necessitating laboratory diagnosis [4]. The abscess disease caused by catalase-negative *Staphylococcus aureus* subsp. *anaerobius* is a comparatively newly identified specific disease of sheep and goat [10]. This disease is drawing more attention in sheep and goat raising countries due to its mistaken diagnosis on the clinical grounds and poor response to CLA vaccination [5].

In goats, the typical pattern of clinical manifestation in the form of recurring abscessation of superficial lymph nodes in animals suggests the presence of *C.*

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Published online: 16 September 2016

pseudotuberculosis infection. In our recent study, we reported that only 51.9 % pus samples from suspected cases of CLA yielded *C. pseudotuberculosis* on culture. Since the attention was only on CLA, the remaining cases were not investigated for involvement of other bacteria [16]. In this study, we investigated involvement of other possible etiology in addition to *C. pseudotuberculosis*. The laboratory diagnosis of CLA is generally achieved by the bacterial culture and its confirmation by biochemical, serological and nucleic acid-based detection methods [6, 13, 15]. These tests are cumbersome and often suffer from the problem of cross-reactivity with other bacteria such as *Corynebacterium* spp., *Listeria monocytogenes* and *Mycobacterium avium* subspecies *paratuberculosis* [11, 17]. Polymerase chain reaction (PCR) has been used frequently for direct detection of microbes in a variety of clinical and postmortem samples. However, the efficacy of the PCR for direct detection of *C. pseudotuberculosis* in clinical pus samples has been less commonly evaluated [16, 21]. In the present study, we carried out the etiological investigation of suspected cases of CLA/abscess disease and developed a PCR assay amplifying three genes for rapid and specific detection of *C. pseudotuberculosis* in clinical samples, and its comparison with the bacterial culture.

Materials and Methods

History and Clinical Examination

Animals were maintained under semi-intensive system in semi-arid tropical region of Rajasthan. The highest mean monthly temperature during April to June is about 42 °C. Rainfall is erratic and ranged from 200 to 500 mm per annum. This farm was infected following introduction of *Sirohi* goats from its original tract a decade ago. About 20–30 % adult males had shown suspected CLA cases every year. Surprisingly, females were rarely affected. For this study, a total of 752 *Sirohi* goats at the farm were clinically examined for the presence of abscessation of superficial lymph nodes. The consistency of enlarged lymph nodes at various locations varied from very hard to soft and did not evince pain on palpation. The location of the affected lymph nodes was also recorded.

Sample Collection

A small area on the enlarged lymph nodes or abscess of the affected animal was shaved and cleaned with antiseptic to avoid environmental contamination during sampling. Creamy to caseated odorless pus samples were aspirated aseptically from abscessed lymph nodes of 33 suspected cases using sterile disposable syringe and needle (18 gauges). The samples kept on ice were brought to the laboratory for analyses.

Bacterial Isolation

Pus samples were directly inoculated on blood agar base (Himedia, Mumbai, India) supplemented with 5 % defibrinated sheep blood and incubated aerobically at 37 °C for 72 h followed by further inoculation on cystine tellurite blood agar (cystine tellurite agar base added with 5 % defibrinated sheep blood and 5 % v/v of 1 % potassium tellurite) and incubated at 37 °C for 72 h. The resultant black colonies were subjected to routine biochemical tests and synergistic hemolysis with *Rhodococcus equi* (strain VTCC BAA67, courtesy Veterinary Type Culture Centre, Hisar, India) and antagonistic hemolysis with *S. aureus* for phospholipase D (PLD) production [13]. Catalase, PLD and urease-positive and nitrate-negative cultures were considered positive for *C. Pseudotuberculosis* [24].

From some cases, Gram-positive cocci in singles, pairs, tetrads and bunches morphology were isolated, which on biochemical and molecular characterization were identified as catalase-negative *S. aureus* subsp. *anaerobius* [24].

DNA Extraction from Pus Samples and Bacterial Isolates

DNA was isolated from all pus samples that had already been subjected to the bacterial culture. For isolation of DNA, approximately 100 mg pus was transferred into 1.5 ml Eppendorf tube and the DNA was extracted using QIAGEN DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, USA) as per manufacturer's instructions. DNA was also extracted from pure cultures of *C. pseudotuberculosis* and *S. aureus* subsp. *anaerobius* using same kit as mentioned above. After checking the DNA purity on 0.8 % agarose gel electrophoresis, it was stored at −20 °C until used.

Oligonucleotide Primers and PCR

The oligonucleotide primers from three most conserved genes of *C. pseudotuberculosis,* namely putative oligopeptide/dipeptide ABC transporter, nicotinamide adenine dinucleotide phosphate (NADP) oxidoreductase coenzyme F420-dependent and proline iminopeptidase (PIP) genes synthesized commercially from Sigma (UK) and 16S rRNA and thermonuclease (*nuc*) genes of *S. aureus* subsp. *anaerobius* from IDT (UK), were used for PCR amplification [9]. The oligonucleotide primer sequences for amplifying these genes along with their product sizes are listed in Table 1. The PCR was carried

Table 1 Details of oligonucleotide primers used

Target gene	Primers	Sequence (5′–3′)	Length of PCR products (bp)	References
Putative oligopeptide/dipeptide ABC transporter	Forward	cct tac cga gac aac gtc at	285	D' Afonseca et al. [9]
	Reverse	gcc tgg tgc tta tca ttg at		
NADP oxido-reductase coenzyme F420-dependent	Forward	ctg cga cat agc tag gca ct	382	D' Afonseca et al. [9]
	Reverse	ccg cca gac ttt tct cta ca		
Proline iminopeptidase	Forward	aac tgc ggc ttt ctt tat tc	551	D' Afonseca et al. [9]
	Reverse	gac aag tgg gaa cgg tat ct		
16S rRNA	Forward	aactctgttattagggaagaac	756	Moussa et al. [19]
	Reverse	ccaccttcctccggtttgtcacc		
Thermonuclease	Forward	gcgattgatggtgatacggtt	270	Brakstad et al. [7]
	Reverse	agccaagccttgacgaactaaagc		

out in a final volume of 25 µl of reaction mixture containing 1× PCR buffer, 2 mM $MgCl_2$, 200 µM dNTPs mix, 0.2 µM of each of forward and reverse primers, 1.25 units of Taq DNA polymerase (Sigma) and 5 µl of DNA template (DNA from pus or bacterial isolates) in a thermocycler (MJ Research PTC200). The PCR cycling conditions were optimized by putting gradient PCRs with annealing temperatures ranging from 45 to 65 °C. The annealing temperature between 53.4 and 59.6 °C produced PCR products of expected length, of which 54 °C was found to be optimum and, therefore, selected for further reaction. The PCR conditions used to amplify all three gene fragments included a cycle of 95 °C for 5 min, followed by 35 cycles each of 94 °C for 30 s, 54 °C for 45 s and 72 °C for 45 s and a final cycle at 72 °C for 5 min. The PCR cycling conditions for 16S rRNA and *nuc* gene were optimized by putting gradient PCRs in a thermocycler (peqSTAR 96 universal gradient) as described previously [19]. Negative control consisting of all components of the reaction mixture without DNA template was included in the PCR. The PCR products were analyzed by visualization of desired size of DNA bands in the ethidium bromide-stained agarose gel (2.5 % w/v, 0.5× Tris borate EDTA buffer) under gel documentation system [25].

Sequencing of PCR Products

For sequencing of the gene fragments, standard PCRs were run using *Pfu* DNA polymerase (Fermentas, Maryland, USA) and PCR products were resolved on 2.0 % agarose gel. The specific sized bands were excised from gel under UV light and were subsequently purified using minielute gel extraction kit (Qiagen). The purified products were got sequenced by the Imperial Life Science Laboratories (Life Discoveries Pvt. Ltd.) and compared with sequence available in the NCBI database.

Results and Discussion

On clinical examination, 4.38 % (33/752) adult goats were found to exhibit affected superficial lymph nodes at various stages of enlargement and abscessation. These cases were suspected for CLA based upon the characteristic overt presence of palpable firm or slightly soft subcutaneous enlargement in the locations of superficial lymph nodes and in the brisket and neck regions. The affected lymph nodes were mainly parotid, superficial cervical, mandibular, prescapular and prefemoral (Table 2; Fig. 1). The size of the affected lymph nodes varied from 2 to 7 cm in diameter. These were painless on palpation and had varying degree of hair loss over or around the glands. All the cases yielded creamish-yellow pus of variable consistency (Fig. 1). Of these 33 animals, 16 (48.48 %) and 12 (36.36 %) were found to be positive for *C. pseudotuberculosis* and catalase-negative *S. aureus*, respectively, and none of them had mixed bacterial infection. Thus, based on the cultural examination, the overall prevalence of CLA was 2.12 %. All the isolates of C. *pseudotuberculosis* were Gram-positive pleomorphic rod positive for catalase, urease and PLD and negative for nitrate [24]. As all the *C. pseudotuberculosis* isolates were negative for nitrate reduction test, they belonged to biovar *Ovis* [13]. PCR was used for the detection of *C. pseudotuberculosis* directly from the clinical pus samples. The pus samples from all CLA suspected goats tested for the presence of putative oligopeptide/dipeptide ABC transporter, NADP oxidoreductase coenzyme F420-dependent and PIP genes of *C. pseudotuberculosis* by direct PCR, showed positivity in 16 (48.48 %) cases (Fig. 2) giving an overall proportion of 2.12 % of animals examined clinically. All *C. pseudotuberculosis* isolates were also positive for three genes in the PCR (Fig. 3). All *S. aureus* subsp *anaerobius* were positive for 16S rRNA and thermonuclease genes (Fig. 4).

Table 2 Distribution of clinical lesion and laboratory findings

Sr. no	Abscessed lymph node/region	Bacterial isolation		*C. pseudotuberculosis* specific PCR	
		C. pseudotuberculosis	*S. aureus* subsp. *anaerobius*	Pus	Culture
1	Parotid	+	−	+	+
2	Parotid and mandibular	+	−	+	+
3	Parotid and mandibular	−	+	−	n.a.
4	Parotid	−	+	−	n.a.
5	Parotid	+	−	+	+
6	Superficial cervical and parotid	+	−	+	+
7	Superficial cervical	+	−	+	+
8	Superficial cervical	+	−	+	+
9	Superficial cervical	−	+	−	n.a.
10	Superficial cervical	−	+	−	n.a.
11	Superficial cervical	−	+	−	n.a.
12	Mandibular	+	−	+	+
13	Mandibular and prefemoral	+	−	+	+
14	Mandibular	+	−	+	+
15	Mandibular	+	−	+	+
16	Mandibular	+	−	+	+
17	Prescapular	+		+	+
18	Prescapular	−	+	−	n.a.
19	Prescapular	−	+	−	n.a.
20	Prescapular	−	+	−	n.a.
21	Prefemoral	+	−	+	+
22	Prefemoral	+	−	+	+
23	Prefemoral	+	−	+	+
24	Prefemoral	−	+	−	n.a.
25	Prefemoral	−	+	−	n.a.
26	Prefemoral	−	−	−	n.a.
27	Prefemoral	−	−	−	n.a.
28	Supramammary	−	−	−	n.a.
29	Supramammary	−	−	−	n.a.
30	Brisket region	−	+	−	n.a.
31	Brisket region	+	−	+	+
32	Brisket region	−	−	−	n.a.
33	Brisket region	−	+	−	n.a.

n.a. not applicable

All the three nucleotide sequences were deposited in the NCBI GenBank (accession Nos. JF901918, JF901920 and JF901922). Sequences of the amplified products revealed 98–99 % homology with published sequence of PIP (CP003077.1, CP002251.1, etc.,), NADP oxidoreductase coenzyme F420-dependent (CP003082.1, CP003152.1, etc.,) and ABC transporter (CP001809.2, CP002924.1, etc.,) genes of *C. pseudotuberculosis* available in the NCBI database, respectively.

The present study shows the incidence of CLA in goats as 4.38, 2.12 and 2.12 % based on the clinical examination, bacterial culture and pus PCR assay, respectively. The incidence of CLA varying from 1.6 to 30 % has been reported from Turkey, Egypt and Australia [1, 2, 8, 20, 22]. The characteristics of the organism and the insidious nature of the infection in the animal make the diagnosis and control of CLA a difficult proposition. In a number of previous studies, the clinical diagnosis of CLA was not always confirmed by the

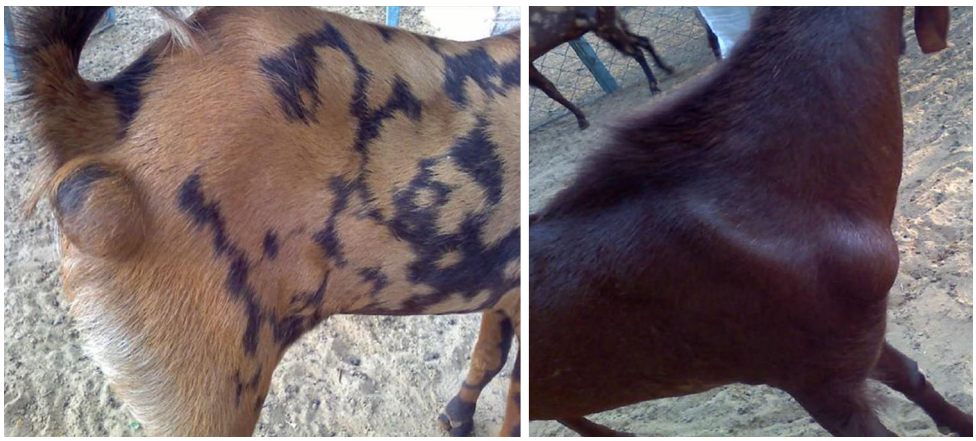

Fig. 1 Photographs of CLA affected goats. Note abscess formation in thigh and pre-scapular regions

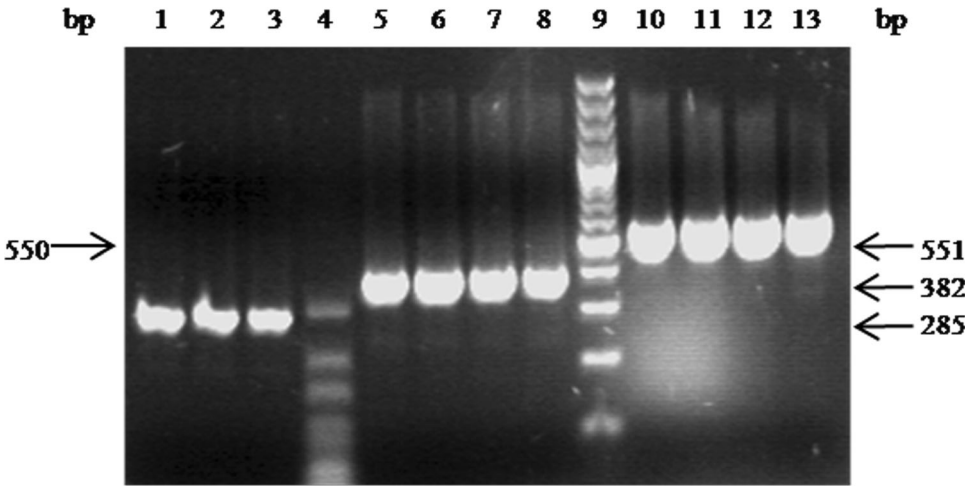

Fig. 2 PCR amplification profile *lane 4*, *9* molecular size marker, *lane 1*, *2*, *3* 285 bp product of oligopeptide/dipeptide ABC transporter, *lane 5*, *6*, *7*, *8* 382 bp product of NADP oxidoreductase, *lane 10–13* 551 bp product of PIP gene for direct detection of *C. pseudotuberculosis* in pus samples

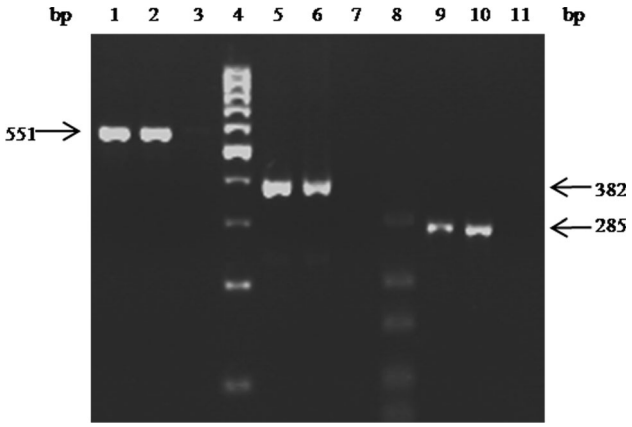

Fig. 3 PCR amplification profile *lane 4*, *8* molecular size marker, *lane 3*, *7*, *11* negative control, *lane 1*, *2* 551 bp product of PIP gene for identification of *C. pseudotuberculosis. Lane 5*, *6* 382 bp product of NADP oxidoreductase, *lane 9*, *10* 285 bp product of oligopeptide/dipeptide ABC transporter

laboratory diagnostic methods such as ELISA and the bacterial culture [3, 27]. The bacterial culture requiring isolation and characterization by biochemical tests, synergistic hemolysis test with *R. equi* and antagonistic hemolysis test with *S. aureus* is a cumbersome and time-consuming and less sensitive method. Therefore, in the present study, we used PCR assay based on three specific genes to confirm *C. pseudotuberculosis* isolates and to detect the bacterial genome directly in the pus samples. The PCR carried out on the pus samples detected all 16 cases positive that has been confirmed by the bacterial culture. Thus, sensitivities of the bacterial culture and the PCR were same in the present study suggesting the adoption of PCR as an alternative test to cumbersome bacterial culture procedure.

In previous studies, genes such as *rpoB*, *pld* and 16S rRNA have been employed in the PCR for identification of *C. pseudotuberculosis* with a sensitivity of 94.6 % in detecting bacteria from pus samples [8, 21, 23]. In the

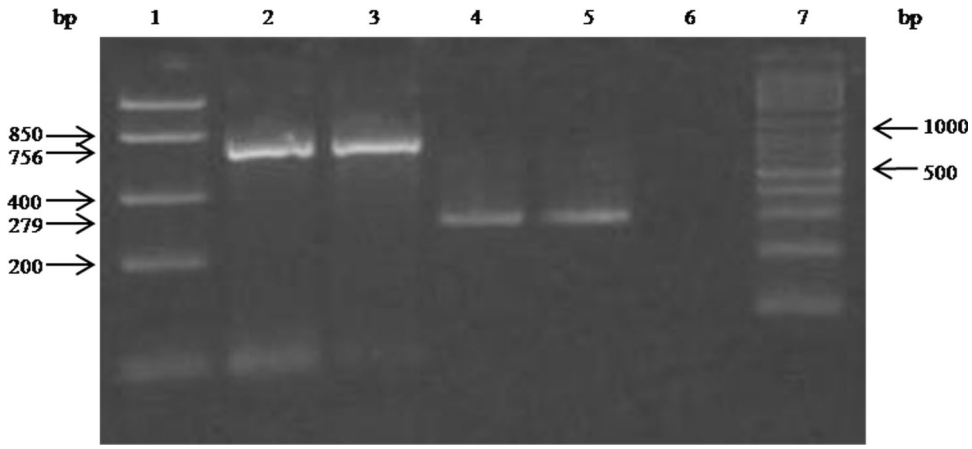

Fig. 4 PCR amplification profile *lane 1*, *7* molecular size marker, *lane 2*, *3* 756 bp product of 16 s rRNA, *lane 4*, *5* 270 bp product of nuclease (nuc) gene for identification of *Staphylococcus aureus* subsp. *anaerobius* and *lane 6* negative control

present study, one step PCR assay confirms *C. pseudotuberculosis* and the PCR employing three genes distinguishes *C. pseudotuberculosis* from other species of genus *Corynebacterium* of veterinary importance and also from genetically related pathogens such as *R. equi*. The assay also circumvents the problem of clinical diagnosis that is mostly dependent on less reliable serological tests and highly variable cultural and biochemical characteristics of *Corynebacterium* spp. used for differentiation [14].

It is evident from the results that all 33 clinically suspected cases did not turn out to be the CLA cases and showed involvement of catalase-negative *S. aureus* in 36.36 % cases. The recovery of live organisms in the culture depends on the stage and nature of infection, immune status of the animal, and distribution of the organism within the lesions as well as the presence of other bacterial agents, which could be the reasons for non-isolation of any bacterial agent from 5 cases [6, 12]. Suppuration of lymph nodes or subcutaneous tissues by other bacteria like *Staphylococcus* spp., *S. aureus* subsp *anaerobius*, *Erysipelothrix* spp., *Streptococci* has been reported to complicate the diagnosis of CLA cases [3, 26]. Thus, it is most likelihood that clinical diagnosis by morphological characteristics may lead to an overestimate of incidence of CLA. It is, therefore, suggested that the suspected CLA cases must be confirmed by the bacterial culture or the molecular test for correct diagnosis. This observation is supported by a few previous studies, wherein the estimates of CLA incidence on clinical basis have always been higher than on the bacteriological and molecular bases [5, 8].

The findings of the present study suggested that all cases clinically indistinguishable from CLA could not be confirmed by the bacterial culture and PCR. Therefore, the latter methods should be invariably applied to confirm the clinical diagnosis of CLA. The sensitivities of PCR and bacterial culture were quite comparable in confirming CLA diagnosis. The PCR, however, offers advantages over the bacterial culture in terms of rapidity, simplicity and its ability to also detect non-viable or lesser number bacteria in the pus samples. Inclusion of three specific genes as target confers specificity to the assay and precludes several step confirmation procedures of cultural isolation and identification. The assay has a potential for detection of *C. pseudotuberculosis* in other type of clinical sample from other host species.

Acknowledgments The authors thank the Director and In-charge, Livestock Farm Section of the institute for providing facilities and support to collect clinical samples. The work has been conducted in the Disease Investigation Project and the Network Project on Veterinary Type Culture Centre (ICAR-NRCE, Hisar).

Compliance with Ethical Standards

Conflict of interest Authors do not have any conflict of interest for publication of this article.

Ethical Approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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