



A Novel Evidence of Immunological and Molecular Detection of Brucella Species in Camels

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Authors' contributions

This work was carried out in collaboration between all authors. Author KM designed the study, managed the analyses, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors MR and HA designed and supervised the study, wrote the protocol, interpreted the data, and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Objectives: Brucellosis is a zoonosis with severe complications for both humans and animals. In this work, we intended to examine the Brucella infection in dromedary camels in Qatar by using different analysis.

Materials and Methods: A total of 203 samples of dromedary camels were randomly collected from the nearby farms in Qatar. Real-time PCR for the genus specific Brucella cell surface salt extractable bcs31 kDa protein gene were performed on DNA extracted from camel samples. Rose Bengal and rivenol tests were performed to detect the Brucella species. The milk samples were collected from the camels and utilized for the milk ring test.

Results: The outcomes of RT-PCR analysis illustrate the presence of Brucella spp. in 170 samples (83.74%) out of 203 samples. The findings of immunological assays also proved the presence of Brucella spp. such as Rose Bengal (67.14%), ELISA (71.42%), and precipitation assay (65.71) in both serum and blood samples of the dromedary camels, which were collected from the Qatar.

Conclusions: In conclusion, it was clear that the incidence of the brucellosis in camels is significantly rising in Qatar region and there is a need to control the spread of the disease from camels to camels as well as from camels to humans.

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1. INTRODUCTION

Brucellosis is a zoonotic disease, and an important public health threat in many parts of the world, especially in the Middle East countries and central Asian regions [1,2]. This bacterial ailment is caused by numerous *Brucella* species, which mostly infect the camels, goats, sheep, cattle, swine, and dogs [3]. Brucellosis of cattle is an extremely transmittable disease caused by *Brucella abortus* and is distinguished by abortion in late pregnancy and augmented incidences of infertility [4]. Humans commonly acquire the disease via straight contact with diseased livestock, consumption of infected dairy products, and/or by inhaling the airborne agents in the slaughter houses. The mainstream of human incidences is caused by consuming the unpasteurized milk and the uncooked meats of goats, sheep, camels or cattle. Brucellosis is a categorized as a serious public health issue with worldwide distribution. Brucellosis causes a huge economic loss to the livestock industries via an abortion, infertility, and birth of defected or dead offspring, augmented calving interval and diminution of milk yield [5].

Many countries around the world has established the control programs for domestic livestock for the effective suppression of brucellosis, however clinical presentation of the disease is nonspecific, and may be very unusual. Hence, the laboratory confirmation by isolation or detection of specific anti-brucella antibodies is necessary for the final confirmation of the disease. Due to the increased economic importance of livestock in developed and under developed nations, means for *B.abortus* diagnosis and prophylaxis was extensively investigated, and numerous serological and non-serological examinations are developed for cattle brucellosis have been found useful for the diagnosis of brucella infection in livestock [6,7].

The genus *Brucella* is a facultative intracellular pathogen, which includes 11 accepted nomo-species at present, based on the primary host species specificity. The classical species of *Brucella* are *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. ovis*, and *B. neotomae* which are primarily isolated from the ruminants, bovines, pigs, dogs, sheep and desert wood rats, respectively [8]. The serological examinations like ELISA, serum agglutination test, rose Bengal test, complement fixation test, can be nonspecific due to the cross-reaction with other common

antigenically related bacteria, or high immunity reactions, based on the endemic prevalence of the ailment [9,10]. A further limit of the serological assay is that they cannot distinguish between infected and exposed individuals [11,12]. Polymerase chain reaction test was shown to be a valued approach for the detection of DNA from the various fastidious and non-cultivable pathogens, having the further advancements of permitting the detection of small numbers of pathogens, being reproducible and easily standardized, minimizing the risk of infection to laboratory staffs, and having the total processing time of about 2 to 3h [13]. These features can be highly imperative when rapid and precise detection of *Brucella* spp. is needed.

Camels are either transporter, vulnerable or suffering from a huge number of infectious and parasitic ailments. The brucellosis is such ailment and having considerable public health importance. Brucellosis was reported in camels as early as 1931 [14,15]. Camels are not only reported as a primary hosts of *Brucella*, but they are highly vulnerable to both *B. abortus* and *B. melitensis* [16]. The clinical signs of brucellosis in camels are not clearly defined. Many infected camels are silent carriers of brucellosis [17]. Camel's meat is one of the most important commercial food products in Middle East countries. Commonly the males and infertile female camels are sold as slaughter animals by pastoralists. Nonetheless, selling these animals for the purpose of meat can present an imperative source of income [18,19]. Camels are not only reported as a primary hosts of *Brucella*, but they are highly vulnerable to both *B. abortus* and *B. melitensis*. Since then, it has been testified by all the camel rearing countries like Ethiopia, Sudan, Somalia, Kenya, Jordan, Nigeria, and Egypt but not yet in Qatar [20].

The clinical pictures of brucellosis in camels can differ from the asymptomatic to abortion, retention of fetal membranes, weak or defected offspring, impaired fertility and delayed sexual maturity in females accompanied by lameness in males [21,22]. Outbreaks of human brucellosis by consumption of infected raw camel milk have been reported in Qatar, Israel and countries of the African continent. Brucellosis identified to be a severe occupational health hazard to livestock handlers particularly slaughterhouse workers [23-25]. In this present research work, we intended to examine the *Brucella* infection in camels in Qatar

by using the serological assays and real time polymerase chain reaction analysis. The serological and molecular techniques were applied to identify the *Brucella* species involved in the infection of camels in Qatar.

2. MATERIALS AND METHODS

2.1 Study Area

The study was conducted from September 2018 to 2020 and collected different location in Qatar, which showing hereunder.

2.2 Collection of Samples

A total of 203 (serum – 45, placental tissue – 40, blood – 25, placental fluid – 13, milk – 50, uterine swab – 30) samples of dromedary camels were randomly collected from nearby farms, Al-shahaniya province, Qatar. None of the animals tested had administered any vaccinations, collected samples were utilized for the detection of *Brucella* species in both serological and RT-PCR analysis techniques.

2.3 Preparation of DNA from the Samples

DNA was extracted from the blood samples with the aid of Magnapure compact Nucleic acid extraction kit (Roche) and Qiagen extraction kit and DNA was extracted from tissues, placental fluid and swabs with the help of Qiagen extraction kit (Roche Life Science, Penzberg, Germany) according to the manufacturer's instructions for bacteria and then re-suspended in 200 µl of phosphate buffered saline (PBS) after heat inactivation for two hours at 80°C. DNA quantity and purity was determined by using Quawell 5000 Nano drop (Nano-Drop Technologies, Wilmington, DE, USA) to get the DNA number. Serial dilutions (1:10) ranging from 100 ng to 1 fg/µl of DNA was prepared for qPCR efficiency and specificity testing.

2.4 Multiplex Real Time Polymerase Chain Reaction (RT-PCR) Analysis

Real-time PCR for the genus specific *Brucella* cell surface salt extractable bcsp31 kDa protein gene was performed on DNA extracted from camel samples using the following primers for *Brucella* species IS711 forward: 5'-GCTTGAAGCTTGCGGACAGT-3' and reverse: 5'-GGCCTACCGCTGCGAAT-3' (amplicon size: 63bp); probe:-FAM-GCCAACACCCGGCCATATGGT-Tamra
B.abortus forward: 5'-

GCACACTCACCTTCCACAACAA-3' and reverse: 5'-CCCCGTTCTGCACCAGACT-3'; probe:FAM-

TGGAACGACCTTTGCAGGCGAGATC-BHQ1:
B. melitensis forward: 5'-TCGCATCGGCAGTTTCAA-3'; reverse: 5'-CCAGCTTTTGGCCTTTTCC-3'. Probe: cyS-CCTCGGCAGGCCCGCAA-BHQ2. The primers and probes were obtained from TIB MOLBIOL (Berlin, Germany). The real-time PCR assay was prepared using the TaqMan™ Universal Master Mix (Applied Biosystems, New Jersey USA) containing the following components per reaction: 12.5 µl Taq-Man™ Universal Master Mix(2X) (Applied Biosystems), 0.75 µl of each primer (0.3 µM) and 0.25 µl probe (0.1 µM). 2 µl of bacterial DNA was used as target and nuclease-free water sum up to a total reaction volume of 25 µl. No Template Controls (NTC) that contained 2 µl of water instead of DNA and positive controls that contained DNA of *Brucella* were included in each run to detect any amplicon contamination or amplification failure. The real-time PCR reaction was performed in duplicate in optical 96-well microtitre plates (q PCR 96-well plates, Micro Amp™, Applied Biosystem) using a Quant studio 6 Flex thermocycler system (Stratagene, La Jolla, Canada) with the following run condition 1 cycle of 95°C for 15 min, 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The extracted DNA from the bcsp31 positive samples were examined with the *Brucella* IS711 species specific real-time PCRs for *B. abortus* and *B. melitensis* using the primers and probe as described previously for typing [26]. Amplification reaction mixtures were prepared in volumes of 25 µl containing 12.5 µl TaqMan™ Universal Master Mix (Applied Biosystems) 0.75 µl of each primer (0.3 µM) and 0.5 µl TaqMan probe (0.2 µM), 5 µl of template, and nuclease-free water sum up to a total reaction volume of 25 µl. Optimization resulted in reaction condition of 2 min at 50°C, 10 min at 95°C, followed by 50 cycles of 95°C for 15 s and 57°C for 1 min. Cycle threshold values below 40 cycles were interpreted as positive. The threshold was set automatically by the instrument. The samples scored positive by the instrument were additionally confirmed by visual inspection of the graphical plots showing cycle numbers versus fluorescence values.

2.5 Rose Bengal Test for Brucellosis

Rose Bengal test for the detection of *Brucella* antibody in the collected samples was performed

using commercially available antigen from IDEXX (IDEXX Lab, Inc., Westbrook, Maine, United States) as per the instructions of the kit. Samples were diluted 10 times in sterile phosphate buffer. An aliquot of 30 μ L of sample to be tested was mixed with the equal volume of Brucella RBT reagent on a glass slide and spread to produce a zone of about 2 cm in diameter. The mixture was then agitated gently on a rocker (Cole-Parmer, Illinois, Chicago, USA) for 4 min. A visible precipitation was recorded as positive. Proper negative and positive control purchased from IDEXX was also included in RBT tests.

2.6 Milk Ring Test (MRT)

The test was executed via adding 30 μ l of hematoxylin-stained *B. abortus* antigen and the height of the milk column in the tube was kept up to 25 mm. The milk-antigen mixtures were incubated at 37°C for 1 h, along with positive and negative control samples. Agglutinated *Brucella* cells were picked up by fat globules as they rose, forming a dark cream layer on the top of the sample. A strongly positive reaction was indicated by formation of a dark blue ring above a white milk column. The test was considered negative if the color of the underlying milk exceeded that of the cream layer and when the cream layer was normal.

2.7 Detection of Brucella Using ELISA

Commercially available kit from Sigma-Aldrich, (Sigma-Aldrich, St. Louis, Missouri United States) for the detection of *Brucella* spp. was used. Samples were diluted 10 times in sterile water and the assay was performed following the instructions of the kit. Reactions were recorded using microtiter plate reader (Multiskan Ascent, Labsystems, Finland).

2.8 Rivenol Precipitation

For the detection of *Brucella*, the rivenol (2-ethoxy-6,9-diaminoacridine lactate) was mixed to the collected samples, which cause the precipitation of high molecular weight glycoproteins. Then the precipitate was eliminated by centrifugation and a rapid agglutination test b using the diluted samples i.e. 1:25, 1:50, 1:100, and 1:200. This experiment was extensively used as a confirmatory test [27].

2.9 Statistical Analysis

All the experiments data were performed in triplicates and each treatment had six replicates. The data was analyzed using analysis of variance (ANOVA), and the means were compared using SPSS (version 16.0) at 5% level of significance ($p < 0.05$).



Fig. A . Map showing the study Location

3. RESULTS AND DISCUSSION

Brucellosis is a zoonosis with severe consequences for both humans and animals, affecting predominantly sexually mature individuals. The primary clinical signs of infection are abortion in females, and orchitis and epididymitis with frequent sterility in males, because of the localization of brucella within the male and female reproductive organs. Large numbers of brucellae can be excreted in fetal fluids and mammary secretions [28,29]. This research work was carried out on A total of 203 (serum – 45, placental tissue – 40, blood – 25, placental fluid – 13, milk – 50, uterine swab – 30) samples of dromedary camels suspected to be or had a history of brucellosis from Qatar. Also, these dromedary camels had no history of vaccination against the brucellosis. RT-PCR and serological tests including Rose Bengal, ELISA, and rivenol precipitation assays were utilized in this work.

When the blood and serum samples were examined for brucellosis using Rose Bengal test, 47 samples (67.14%) out of total 70 tested positive for the presence of *Brucella* spp. When ELISA was performed to detect the IgM antibodies against the *Brucella* spp. in the samples, 50 (71.42%) samples constituting positive results Table 1. These outcomes reveal a higher occurrence of brucellosis among the dromedary camels in Qatar region. Even though detailed reports on camel brucellosis are not reported yet from Qatar, higher incidence of the

disease in dromedary camels is reported from the neighbouring region. Previous report suggested that the 15.8% of the camels were found to be infected with *Brucella* in Jordan [30]. Whereas, in another investigation executed in Sudan revealed that the 40% of the tested samples were positive for *Brucella* when 2,000 camel serum and milk samples were examined [31].

Table 1 depicts the findings of Rose Bengal, ELISA, rivenol, and RT-PCR assays of both blood and serum samples. The outcomes of RT-PCR analysis illustrate the presence of *Brucella* spp. in 20 blood and serum samples (28.57%) out of total 70 samples. The findings of immunological assays also proved the presence of *Brucella* spp. such as Rose Bengal (67.14%), ELISA (71.42%), and rivenol precipitation assay (65.71%) in the blood and serum samples of dromedary camels, which was collected from the Qatar Table 1. The results of serological analysis and RT-PCR analysis of serum samples of aborted dromedary camels were illustrated in Table 1. The findings of Rose Bengal test illustrated the presence of *Brucella* spp. in 31 (68.88%), ELISA 32 (71.11%), and rivenol precipitation assay 29 (64.44%) in the serum samples of aborted dromedary camels.

Table 2 reveals the presence of *Brucella* spp. in the placental tissue samples of aborted dromedary camels. The presence of *Brucella* spp. in 14 (35%) placental tissue samples was identified by using RT-PCR analysis Fig. 1.

Table 1. Total number of positive results in both blood and serum samples per serological test and real-time PCR used for the detection of brucellosis in dromedary camels

Tests	Total number of samples	Number of positive samples	Number of negative samples	Correlation (%)	
				Positive	Negative
Real-time PCR	70	20	50	28.57%	71.42%
Rose Bengal test	70	47	23	67.14%	32.85%
ELISA	70	50	20	71.42%	28.57%
Rivenol precipitation assay	70	46	24	65.71%	34.28%

Table 2. Number of positive results of real-time PCR used for the detection of brucellosis in placental tissue samples of dromedary camels

Tests	Total number of samples	Number of positive samples	Number of negative samples	Correlation (%)	
				Positive	Negative
Real-time PCR	40	14	26	35	65

The 16 (64%) blood samples demonstrated the existence of *Brucella* spp. that was identified through the Rose Bengal test, 18 (72%) samples showed the presence of *Brucella* spp. by ELISA test, and 17 (68%) samples exhibited positive results for the presence of *Brucella* spp. by rivenol precipitation assay. The control of brucellosis in cattle as well as humans relies on the trustworthiness of the techniques utilized for detection and identification of the pathogens. But, diagnosis of brucellosis in dromedary camels is often problematic. The disease can mimic numerous infectious and non-infectious diseases. Typical clinical signs of brucellosis in camels are often lacking and diagnostic techniques are not evaluated yet. In the present study, the findings indicate that the different samples of camels showed the presence of *Brucella* spp. and many infected camels might be silent carriers for brucellosis and their products may pose a severe health issues for consumers. The findings of this study were supported by a previous researcher Alshaikh et al. (2007) [32]. He demonstrated that non pregnant camels experimentally infected with a field strain of *B. abortus* had no clinical signs and only negligible pathological changes were present.

Table 3 exhibits the findings of RT-PCR analysis of placental fluid samples of aborted dromedary camels in Qatar. The positive results for the presence of *Brucella* spp. were identified in 6 (46.15%) placental fluid samples out of total 13 samples, which were investigated through RT-PCR analysis Table 3.

Table 4 demonstrated the findings of RT-PCR assay for the detection of *Brucella* spp. in the milk samples of dromedary camels. The result of RT-PCR analysis exhibited the presence of *Brucella* spp. in 5 (10%) milk samples out of total 50 milk samples. The outcomes of MRT demonstrated the positive results for the presence of *Brucella* spp. in 6 (12%) milk samples out of 50 milk samples Table 4.

The 30 uterine swab samples of aborted dromedary camels from the Qatar were investigated by using RT-PCR assay for the detection of *Brucella* spp. The result of RT-PCR assay showed the positive results for the presence of *Brucella* spp. in 5 (16.66%) uterine swab samples out of total 30 uterine swab samples Table 5.

Table 3. Number of positive results as per real-time PCR used for the detection of brucellosis in placental fluid samples of dromedary camels

Tests	Total number of samples	Number of positive samples	Number of negative samples	Correlation (%)	
				Positive	Negative
Real-time PCR	13	6	7	46.15	53.84

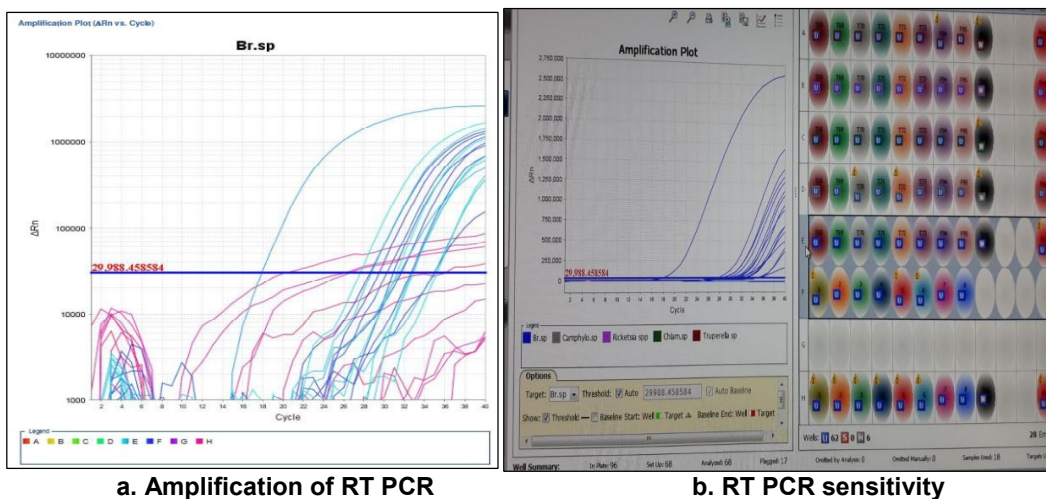


Fig. 1. Amplification curve of real-time PCR used for the detection of brucellosis in placental tissue samples of dromedary camels

The weight marker utilized to examine all the sample genes for the detection of PRC products in agarose gel electrophoresis. Whichever sample gene with a molecular weight equal to the expected PCR product size was judged positive. The fluorescent line that illustrating the molecular weight, which was over or under that measure was considered a negative result. The outcomes were investigated separately per sample throughout the study and RT-PCR yielded positive results. This outcomes were coincides with the previous research reported by Al-Majali et al. (2009)[33]. In this exploration, the outcomes of RT-PCR test demonstrated the existence of *Brucella* spp. in the aborted dromedary camels. Followed by the investigation of results of RT-PCR, it can be said that the existence of *Brucella* spp. in the dromedary aborted camels were confirmed. Fig. 2 shows the gel (2%) of RT-PCR products with 1st lane is

50bp ladder, 2nd lane is control positive, and third to last lane shows the tissues samples i.e. liver, lungs, spleen, stomach (content fluid), and uterine swab.

It should be also noticed that in many incidences, pathogenic organisms and antibiotic resistance bacteria are transmitted to humans from other sources such as food animals, poultries, plants, fish, and other industries, in which antibiotics are used for different purposes and may lead to emerging resistant strains [34,35]. In developing countries, a combination of molecular methods like PCR with one of the commonly used serological tests can be applied to detect brucellosis in cattle [36]. There are numerous researches were published on the detection of *Brucella* DNA by PCR, both from pure culture [37,38] and from field samples, mostly of the cattle origin [39,40].

Table 4. Number of positive results per serological test and real-time PCR used for the detection of brucellosis in milk samples of dromedary camels

Tests	Total number of samples	Number of positive samples	Number of negative samples	Correlation (%)	
				Positive	Negative
Real-time PCR	50	5	45	10	90
MRT	50	6	44	12	88

Table 5. Number of positive results per serological test and real-time PCR used for the detection of brucellosis in uterine swab samples of dromedary camels

Tests	Total number of samples	Number of positive samples	Number of negative samples	Correlation (%)	
				Positive	Negative
Real-time PCR	30	5	25	16.66	83.33

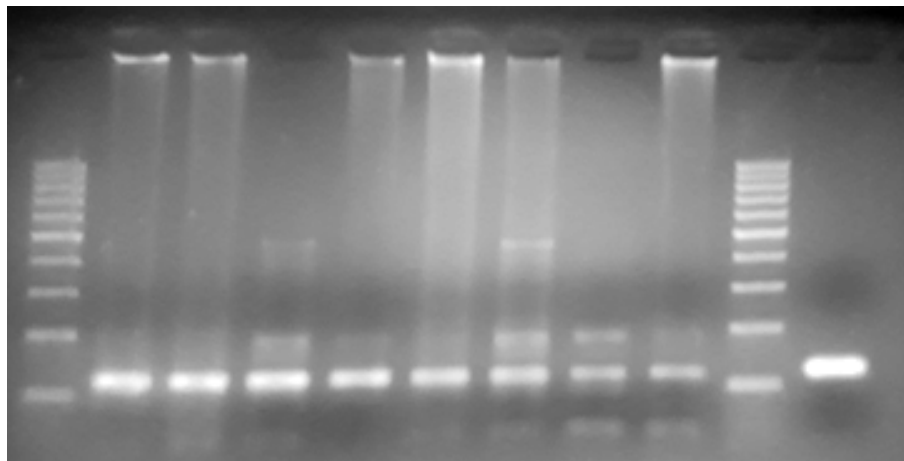


Fig. 2. Agarose gel electrophoresis of PCR products

Speedy and precise diagnostic techniques are fundamental for control and eradication of brucellosis. Culture techniques provide the definitive diagnosis of brucellosis and it is considered the gold standard method for the identification of brucellosis [41]. Due to the difficulty of performing culture in the field, it's consuming for the time, its health hazard and lack sensitivity of the most culture procedures, the serological tests are the main tools used for detection of *Brucella* infection in animal's herds in diagnostic laboratories [42].

Real-time PCR assay evidenced to be a valued diagnostic tool when culture fails or serological results are indecisive in human brucellosis. It is faster and more sensitive than culture methods. The risk of transmission of brucellosis to laboratory workers can be minimized [43]. Interestingly, in this research work we noticed that the real-time PCR targeting the genus specific bcspp31 was positive in 153 (75.36%) out of 203 samples demonstrating the presence of the agent within the animal.

4. CONCLUSION

Efficient methods for genomic DNA preparation are crucial for the PCR-based detection of an organism or pathogen in the different samples of dromedary camels. Rose Bengal test and ELISA assay demonstrated the higher incidence of brucellosis in the camels, which was further confirmed by PCR-based detection. Also it cannot be concluded that these camels are permanently infected, although brucellosis tends to have a chronic course. However, these animals may be asymptomatic carriers and shedders. They pose a permanent risk to other animals and humans and have to be removed from the herd. Therefore, it is concluded that the incidence of the brucellosis in the camels is prevalent in Qatar region and there is an urgent need to control the spread of the disease from camels to camels as well as from camels to humans. In this research work we presented the evidences of possible presence of *Brucella* species in the dromedary camels of Qatar. However, the further studies still needed in the future to confirm the presence of brucellosis in the dromedary camels of Qatar.

ETHICAL APPROVAL

Animal Ethic committee approval has been taken to carry out this study.

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DISCLAIMER

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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