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Case Report

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Diagnosis of Naturally Occurring *Klebsiella pneumoniae* in Rodent Colonies Using Conventional, PCR and DNA Sequencing Method: A Case Report

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ABSTRACT

Pathogen-free animals are crucial in biomedical research for achieving reliable and reproducible results. Pathogens can influence both physiological and immunological responses in animals. *Klebsiella pneumoniae* is a gram-negative opportunistic pathogen that can colonize the respiratory and intestinal tracts of both humans and animals. During the microbiological quality control, we identified *Klebsiella pneumoniae* in the lung of SD rat and the liver and cecum of a Swiss mouse. A lung sample was collected from a moribund SD rat and the liver and cecum were collected from a sick Swiss mouse. Samples were processed for culture-based identification, which includes colony characterization and biochemical tests. The presence of *Klebsiella pneumoniae* was confirmed by conventional PCR targeting the tyrB gene. The PCR products were validated by Sanger sequencing and the resulting sequences were examined using 'BLAST' to confirm the homology with the reference strain. Culture-based methods help treat animals promptly and reduce the spread of disease. The combination of culture-based and molecular techniques, along with sequence validation, ensured accurate and reliable detection of the pathogen in different species.

Keywords: *Klebsiella pneumoniae*, laboratory rodents, PCR, DNA sequencing.

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INTRODUCTION

Klebsiella pneumoniae is a gram-negative, rod-shaped, non-motile bacterium belonging to the Enterobacteriaceae family (Asokan, 2025). It is a lactose-fermenting, facultative anaerobic bacterium with a prominent polysaccharide capsule (Alsadawi *et al*., 2020). *Klebsiella pneumoniae* is an antibiotic-resistant bacterium as classified by the World Health Organization (Assoni *et al*., 2024)causing high morbidity and mortality, which is worsened by the increasing rates of antibiotic resistance. It is a constituent of the host microbiota of different mucosa, that can invade and cause infections in many different sites. The development of new treatments and prophylaxis against this pathogen rely on animal models to identify potential targets and evaluate the efficacy and possible side effects of therapeutic agents or vaccines. However, the validity of data generated is highly dependable on choosing models that can adequately

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reproduce the hallmarks of human diseases. The present review summarizes the current knowledge on animal models used to investigate K. pneumoniae infections, with a focus on mucosal sites. The advantages and limitations of each model are discussed and compared; the applications, extrapolations to human subjects and future modifications that can improve the current techniques are also presented. While mice are the most widely used species in K. pneumoniae animal studies, they present limitations such as the natural resistance to the pathogen and difficulties in reproducing the main steps of human mucosal infections. Other models, such as Drosophila melanogaster (fruit fly. It is known as an 'ESKAPE' group of organisms, which comprises *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and Enterobacter species, all of which have the ability to resist the effect of antibacterial medications (Osman *et al*., 2020)including Sudan. The aim of this study was to test the accuracy of identification of K. pneumoniae in Khartoum, Sudan. Two hundred and fifty K. pneumoniae isolates were collected and identified using conventional phenotypic methods, biochemically using API 20E and genotypically by amplification of 16S-23S rDNA and sequencing of rpoB, gapA and pgi. Only 139 (55.6%.

Klebsiella pneumoniae asymptomatically colonizes the skin, upper respiratory tract, and digestive tract of healthy individuals. It causes diseases in newborns, adults and immunocompromised humans and animals, such as meningitis, pneumonia, wound infection, urinary tract infections (UTIs) and sepsis (Zhang *et al*., 2021). This bacterium was discovered in a variety of environmental conditions, including soil, plant foliage, mammalian intestine and wastewater (Joseph *et al*., 2021). According to epidemiological research, *Klebsiella pneumoniae* is typically spread by the fecal-oral route (Young *et al*., 2020). Immunocompromised rodents show more severe clinical signs of dyspnea, sneezing, cervical lymphadenopathy, inappetence, hunched posture, and rough hair coat (Stair *et al*, 2022), whereas rats show cervical inguinal abscesses and lung infection with granulomatous pneumonia (Baker, 1998). It is also found in the gastrointestinal tract of mice, rats, and other animals, but is mostly reported in mice and rats as an opportunistic pathogen. This organism is a causative agent of respiratory diseases in rodents; therefore, it should be regularly monitored as part of the health monitoring program. (Jeong *et al*., 2013). Culture techniques are commonly used to detect bacterial and fungal infections. Samples need to be collected from the genital mucosa, large intestine, nasopharynx, lungs and trachea; and other sites as necessary for diagnosis (Mahler *et al*., 2014)2002.

MATERIALS AND METHODS

The animal study proposal for microbiological screening of rodent pathogens was approved by IAEC of ACTREC, Navi Mumbai, vide proposal no. 01/2021. As part of the Laboratory Animal Facility's quality control program at ACTREC, we regularly screen randomly selected and symptomatic/moribund animals for health monitoring to investigate the cause of illness and/or death using microbiological methods. In this case presentation, we used one symptomatic SD rat and one Swiss mouse.

Sample collection

Laboratory Animal Facility (LAF), ACTREC (https://actrec.gov.in/index.php/cri-research-support-facility-detail/70) maintains rodent strains of A/J, BALB/c, B6D2F1, C57BL/6, CD-1, C3H, DBA/2, FVB, ICRC, Swiss, Swiss/ba, Nude mice, SCID mice, SD rats and Golden hamsters in polysulfone-made IVC cages under 12-hour dark and light cycles, with a temperature of 22±2°C and humidity of 50±5% as mentioned in an earlier publication from our lab. (Mathan Raj *et al*., 2022). Animals were dissected under a biosafety hood and organs were observed for abnormality, if any. Lung, liver and cecum samples were collected for microbiological investigation. Samples were collected in aseptic conditions to avoid any contamination. Also, lungs, liver, kidney and cecum were collected for histology in 10% neutral buffered formalin solution.

Conventional microbiology

Tissue samples were inoculated on Nutrient and MacConkey agar and incubated for 18-24 hrs at 37 °C under aerobic conditions. Agar plates were read after 24 hrs for bacterial growth. Colonies grown on agar plates were processed further according to colony characteristics (Osman *et al*., 2020; Alsadawi *et al*., 2020). Biochemical reactions were performed for bacterial identification according to the HiMedia kit (KB003). An antibiotic sensitivity test was performed for ciprofloxacin, enrofloxacin, ofloxacin, norfloxacin, tetracycline and oxytetracycline on Mullen-Hinton agar. Antibiotic susceptibility of isolated bacteria was determined by the Kirby-Bauer disk diffusion method as per the Clinical Laboratory Standards Institute (CLSI) guideline (He *et al*., 2022).

DNA isolation

Isolated bacterial colonies were picked up from overnight bacterial culture. Bacterial colonies were digested in 300 µl

digestion buffer containing sodium dodecyl sulfate (SDS) and 20 µl proteinase K for 2 hrs or until digestion was complete (Wright *et al*., 2017). DNA isolation was done using the Phenol-Chloroform extraction method (Ghatak *et al*., 2013). The lung sample was digested overnight at 50 °C in a shaker incubator. Phenol:chloroform:isoamyl alcohol (25:24:1) was used for phase separation. The supernatant layer containing DNA was precipitated by adding sodium acetate and isopropyl alcohol. In the final step, DNA was recovered via rehydration (Ghatak *et al*., 2013). The quantity of extracted DNA was determined using a NanoDrop analyzer at dual UV light (260/280) and the DNA samples showed a ratio of 1.7-1.9 (Dashti *et al*., 2009). Isolated pure DNA was stored at -20 °C for further usage.

PCR amplification

A PCR reaction was set up using 100 ng of lung DNA (1 µl), PCR master mix, primers, and nuclease-free water. 2X PCR master mix contained 0.25 u/µl Taq DNA polymerase, 2X PCR buffer, 0.4 mM dNTP, 3.2 mM $MgCl_2$, and 0.02% bromophenol blue. The primer concentration was set to 0.4 uM, as specified in the manufacturer's kit. Primer sequences used for this reaction are forward- GGC TGT ACT ACA ACG ATG AC and reverse- TTG AGC AGG TAA TCC ACT TTG, which yielded 931 bp of product size (Jeong *et al*., 2013). PCR was performed on individual samples without pooling. PCR amplification involved the following steps:

Initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 20 sec; annealing at 57 °C for 30 sec; synthesis for 20 sec at 72 °C; and final extension at 72°C for 3 min. The amplified PCR product was separated on 2% agarose gel.

DNA isolation from gel and sample preparation for DNA sequencing

Bands of interest were cut from the agarose gel and collected in 1.7 ml Eppendorf tubes. DNA was eluted from the bands using the Nucleospin Gel and PCR Clean-up kit. DNA samples, along with primers, were subjected to Sanger sequencing.

RESULTS

Colonies of *Klebsiella pneumoniae* were isolated from a lung sample from an SD rat and liver and cecum samples from a Swiss mouse using MacConkey agar. SD Rat showed hepatic congestion and white spots on the liver surface (Fig. 1 B & 1C, respectively). The Swiss mouse showed a blackish intestine and a pale liver (Fig. 1. E & F, resp.).

Lactose-fermented, pink colored, mucoid colonies were observed on MacConkey agar plates (Fig. 1A & D) after 24 hrs. of incubation. Biochemical reactions were positive for *Klebsiella pneumonia*e (Table 1, Fig. 3). A PCR reaction was performed using lung DNA extracted from SD rat and bacterial colony DNA from the liver and cecum of a Swiss mouse. We selected a primer pair to amplify a specific target sequence of the tyrosine aminotransferase (tyrB) gene from *Klebsiella pneumoniae*. The PCR product was 931 bp, and the primer pair did not yield any product from other pathogens. The results indicated that the primer pair exhibited sufficient specificity to identify its intended DNA sequence during PCR. Sanger sequencing was performed to validate the PCR product. Bacterial and lung DNA were sequenced to confirm the sequences of *Klebsiella pneumoniae*. Sanger sequencing showed 98% homology with *Klebsiella pneumonia*e.

The rat kidney section showed congestion, hemorrhage, and focal tubular necrosis (Fig. 2A). The liver section showed congestion, hemorrhage, focal necrosis, and generalized granular degenerative changes with scarce cytoplasm, altering the architecture of the liver parenchyma (Fig. 2B). The lungs section did not reveal any changes.

The mouse kidney section showed severe congestion, hemorrhages, and focal tubular necrosis. Some of the tubules were almost completely damaged (Fig. 2C). Liver section showed severe congestion, hemorrhages, focal necrosis, and fatty changes (Fig. 2D). The lungs section showed severe congestion and hemorrhages. The rat sample processed for the antibiotic sensitivity test showed that *Klebsiella pneumoniae* were sensitive to ciprofloxacin, norfloxacin, enrofloxacin and ofloxacin and intermediately sensitive to tetracycline and oxytetracycline (Table 2, Fig. 4 A), whereas ciprofloxacin, norfloxacin, enrofloxacin, tetracycline and ofloxacin were sensitive and intermediately sensitive to oxytetracycline for the Swiss mouse sample (Table 3, Fig. 4B).

DISCUSSION

Klebsiella pneumoniae is a gram-negative opportunistic pathogen causing a variety of infections in laboratory animals. It is an important pathogen responsible for respiratory infection in laboratory animals. The presence of *Klebsiella pneumoniae* can cause morbidity and mortality, as well as pose a risk of interference with research results. In this case report, SD rat and a Swiss mouse were received for microbiological investigation. The SD rat was suffering from respiratory distress, and the Swiss mouse was physically weak.

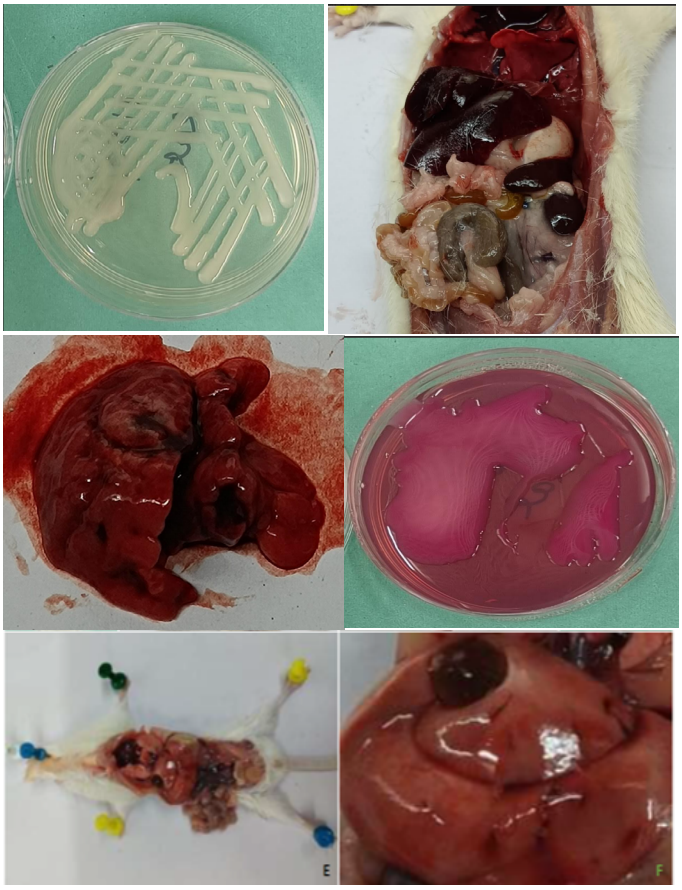


Fig. 1. (A). Representative colonies of *Klebsiella pneumonia* grown on Nutrient and (D) on MacConkey agar from lung samples of SD rat and liver and caecum of Swiss mouse, respectively. (B) & (C). SD Rat showing liver congestion and white spots on the liver surfaces. (E) & (F) Swiss mouse showing blackish intestine and pale liver.

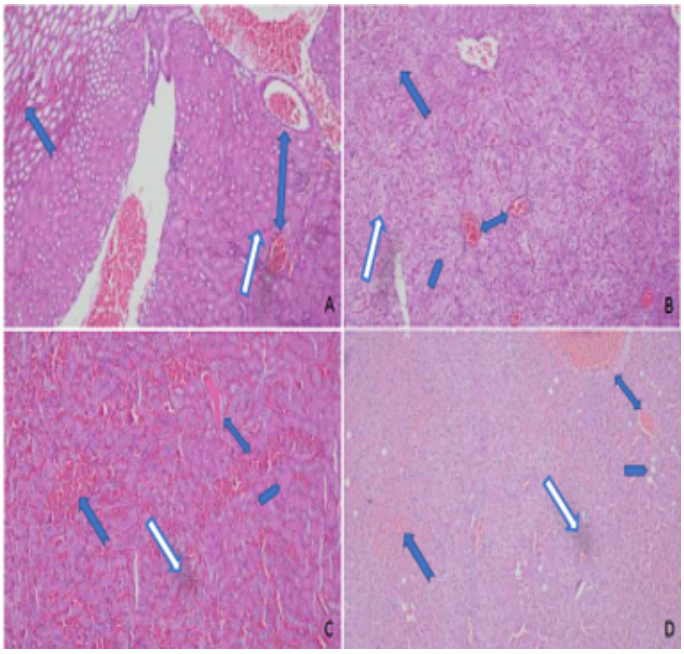


Fig. 2. A. Rat kidney section showing congestion (double arrowheads), hemorrhages (thick arrow) and focal tubular necrosis (empty arrow). B. Rat liver section showing congestion (double arrowhead), hemorrhages (thick arrow), focal necrosis (empty arrow), and generalized granular degenerative changes with scarce cytoplasm (pentagon arrow). C. Mouse kidney section showing severe congestion (double arrowhead), hemorrhages (thick arrow), focal tubular necrosis (empty arrow) and damaged tubules (pentagon arrow). D. Mouse liver section showing congestion (double arrowhead), hemorrhages (thick arrow), focal necrosis (empty arrow), fatty changes (pentagon arrow).

Fig. 3.

Fig .4 .

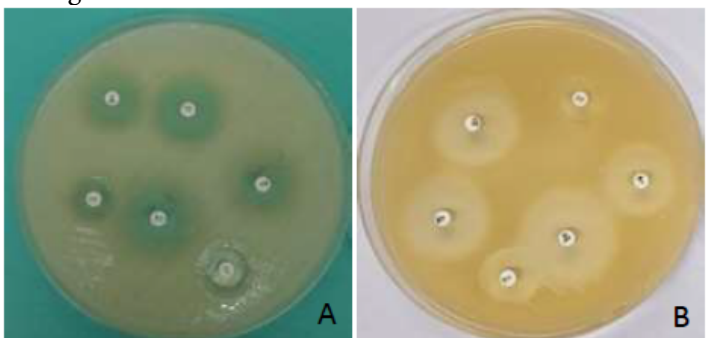


Fig. 3. Biochemical reaction for identification of *Klebsiella pneumoniae.*

Fig.4. Antibiotic Susceptibility Zone for rat and mouse samples .

Complete genome phylogenetic tree of *Klebsiella pneumoniae* isolates compared with representative *Klebsiella pneumoniae* species. The genomes of the *Klebsiella pneumoniae* isolates are closely similar to the standard *Klebsiella pneu moniae* strains. Symptoms were suspected to be due to a bacterial infection.

Samples were processed using conventional methods to detect pathogens. It is easy to cultivate pathogenic bacteria on culture agar, but it takes more time to culture after sample collection.

Table. 1. Biochemical reaction for identification of Klebsiella pneumoniae.

Biochemical test	Result
ONPG	Positive
Ornithine	Negative
Urease	Positive
Citrate	Positive
VP	Positive
Indole	Negative
Malonate	Positive
Carbohydrates	Positive
TSI	Yellow slant, yellow butt with gas

Table 2. Antibiotic Susceptibility Test for SD Rat sample for different antibiotics.

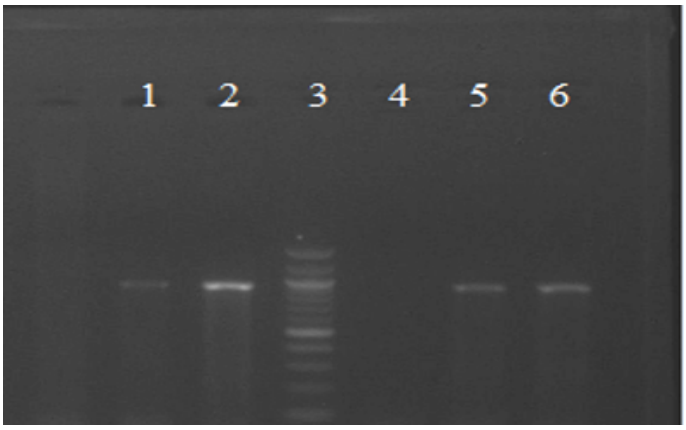
Antibiotics	Zone of Inhibition (mm)	**Interpretation**
Ciprofloxacin	26	Sensitive
Norfloxacin	20	Sensitive
Enrofloxacin	27	Sensitive
Tetracycline	15	Intermediate
Oxytetracycline	15	Intermediate
Ofloxacin	22	Sensitive

Table 3. Antibiotic Susceptibility Test for the Swiss mouse sample for different antibiotics

Antibiotics	**Zone of Inhibition (mm)**	**Interpretation**
Ciprofloxacin	34	Sensitive
Norfloxacin	29	Sensitive
Enrofloxacin	34	Sensitive
Tetracycline	20	Sensitive
Oxytetracycline	21	Intermediate
Ofloxacin	29	Sensitive

Traditional methods for bacterial culture may not always be effective because the conditions and techniques used may not be suitable for the specific bacterial species *(Jeong et al.*, 2013). Also, the conventional method is expensive and laborious. Nucleic acid amplification helps to overcome these problems and improve the diagnostic process to precision.

Fig. 5. Gel picture of *Klebsiella pneumonia* detection with 931 bp product in the lungs sample of the SD rat and the liver and caecum samples of the Swiss mouse.



Lane 1: Lung DNA sample of SD Rat; lane 2: Positive control; lane 3: 100bp DNA ladder; lane 4: Negative control; lane 5-6: Liver and caecum bacterial colony DNA samples of Swiss mouse.

Fig. 6. Sanger Sequencing result of *Klebsiella pneumoniae*.

The PCR method is important for those pathogens that cannot be easily cultured by conventional methods. The PCR method is also useful for detecting rodent pathogens in immunocompromised mice, where the ELISA method failed to detect them due to insufficient antibody titer (Ingle & Shinde, 2019). Standardizing the conventional methods for regular health assessments is challenging, as media, protocol and culturing conditions can differ significantly across various laboratories. To confirm the diagnosis in the present case, PCR was performed. PCR has good sensitivity and specificity for detecting pathogens (Jeong *et al.*, 2013). 100 ng/µl of DNA was sufficient to amplify the PCR product (Fig. 5). PCR provides rapid and reliable identification of pathogens, even in mixed bacterial populations or *samples* with low bacterial loads. Samples were successfully analyzed for *Klebsiella pneumoniae* using BLAST and sequencing. An antibiotic sensitivity test helps determine the appropriate treatment for the infection. In the present study, we observed that Klebsiella pneumoniae was sensitive to some antibiotics but intermediate or resistant to others. *Klebsiella pneumoniae* has been reported to be resistant to many antibiotics (Stair *et al*, 2022). When antibiotic treatment is not required, molecular techniques are the best choice for detecting rodent pathogens. A previous study demonstrated that *Klebsiella pneumoniae* was positively identified in lung, liver, caecum, spleen and stool samples by using a conventional method (Ingle & Shinde., 2019).

Histological examination of kidney and liver lesions in mice and rats infected with Klebsiella pneumoniae revealed cellular changes, including congestion, hemorrhage, focal necrosis, and granular degenerative changes. The cecum section showed no changes. Similar histological changes observed here are also reported by Alsadawi *et al. (2020) and Assoni et al. (2024)* in the liver, kidney, caecum, and lungs. According to reports, lung tissue stimulation may trigger the release of caspase-11, which is necessary to inhibit bacterial growth in the lung parenchyma and trigger the respiratory response against *Klebsiella pneumoniae* infection. Lung tissue stimulation also alters cytokine levels and changes immune cell populations (Jassim & Mohammed Jwad, 2023). Since the Swiss mice colony was showing some degree of morbidity and weakness, they were treated with ciprofloxacin through drinking water at a concentration of 100 mg/kg body weight. In the rat colony, there were no visible signs of illness, so they were not treated. We diagnosed *Klebsiella pneumoniae* in the lungs of SD rats and the liver and caecum of Swiss mice. Conventional and molecular technique results correlated with the tissue histological morphology, helping confirm the pathogens.

CONCLUSION

Klebsiella pneumonia was detected by correlating culture and PCR results. *Klebsiella pneumoniae* can spread the infection in the respiratory and gastrointestinal tracts and also in the liver of rat and mice. The conventional method provided a preliminary diagnosis. Molecular technique helps to confirm the pathogen, and sequencing validated the PCR product. It also helps to treat animals promptly, and rapid pathogen identification reduces the spread of infectious diseases. The combination of culture-based and molecular techniques along with sequence validation, ensured accurate and reliable detection of the pathogen in different species. This case report suggests that regular health monitoring is essential in any facility. It is important to maintain physical barriers, such as positively pressurized rooms and individually ventilated cages (IVCs), to prevent the entry of pathogens. To avoid infections, materials such as food, bedding, water, and equipment must be autoclaved or irradiated. The use of personal protective equipment and restricting access to unauthorized persons in the animal facility helps maintain sterile conditions. Newly imported animals should be quarantined before entering the animal room and tested for pathogens. Regular testing of sentinel animals or environmental samples using conventional microbiology, serology, PCR and histopathology is essential to detect pathogens and prevent the spread of disease. Health monitoring also allows for early detection and effective management of disease outbreaks. Although a single case of a moribund rat and mouse is presented here, screening additional samples from different species using all the screening modalities described here will yield more scientific data on incidence. However, the present case gains importance as it helps to detect, confirm the identified organism and decide the antibiotic sensitivity to the organisms as the *Klebsiella pneumonia* are known for their antibiotic resistance.

IAEC APPROVAL

The study was undertaken under animal study proposal no. 01/2021 approved by the IAEC of ACTREC, Navi Mumbai.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENT

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AUTHORS' CONTRIBUTIONS

Aarti Shinde and Shashi Ahire contributed to the sample collection, sample processing, data analysis, and writing the manuscript. Arvind Ingle contributed to initiating the animal study proposal, H&E slides reading, imaging and interpretation, data analysis, writing, and finalizing the manuscript.

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