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Analysis of the effects of storage temperature and contamination on aerobic bacterial culture results of bronchoalveolar lavage fluid

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Abstract

Background: Storage temperature of bronchoalveolar lavage fluid (BALF) impacts cytological evaluation. The effect of storage temperature before bacterial culture has not been evaluated.

Objectives: To assess whether BALF storage temperature alters aerobic bacterial culture results.

Animals: Eight healthy, male, intact, purpose-bred Beagles.

Methods: Prospective, controlled investigation. Samples of BALF were collected sterilely. Half of each sample was reserved for controls, and half was inoculated with 10^4 colony forming units per milliliter (cfu/mL) *Bordetella bronchiseptica* and 10^2 cfu/mL *Escherichia coli*. Control and inoculated samples each were separated into 4 aliquots (1 plated immediately; 3 stored at 4, 24, or 37°C, respectively, for 24 hours before aerobic bacterial culture). Colony counts were compared across treatments for each organism.

Results: In inoculated samples, a statistical difference could not be detected in growth of *E. coli* or *B. bronchiseptica* between the baseline culture and BALF stored at 4°C for 24 hours before culture. However, for *E. coli*, growth in cfu/mL at both 24 and 37°C was higher compared to baseline ($P < .05$) and compared to 4°C ($P < .05$). For *B. bronchiseptica* cfu/mL, growth at 37°C was significantly different ($P = .003$) compared to both baseline and 4°C.

Conclusions and Clinical Importance: Samples of BALF may be stored at 4°C for 24 hours before culture without substantially altering culture results. Inappropriate storage or shipment temperature (room temperature or exposure to heat) can result in overgrowth of *E. coli* or *B. bronchiseptica*, which could alter clinical decisions.

KEYWORDS
aerobic, *Bordetella bronchiseptica*, contaminant, *Escherichia coli*, infection, pneumonia, transport

Abbreviations: ANOVA, analysis of variance; *B. bronchiseptica*, *Bordetella bronchiseptica*; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; cfu, colony forming units; *E. coli*, *Escherichia coli*; PBS, phosphate buffered saline.

1 | INTRODUCTION

Bacterial pneumonia, characterized as inflammation of the lower airways and pulmonary parenchyma associated with bacterial infection, can result from primary or secondary pathogens. Considering that bacterial infection may be introduced through various routes and a wide variety of organisms can be involved, guidance of antimicrobial choices by culture and susceptibility testing is prudent.[1] Appropriate antimicrobial choice leads to an increased chance for a positive outcome.[2] Furthermore, airway sampling and culture allows for antibiotic de-escalation, which decreases cost of treatment, drug-related adverse effects, and antimicrobial resistance selection pressure.[3] Multiple studies have concluded that antimicrobial treatment should be guided by bronchoalveolar lavage fluid (BALF) culture and susceptibility testing, if possible.[4-7] A recent study evaluating bacterial culture and susceptibility in dogs with bacterial pneumonia showed that 26% of cases had at least 1 bacterial isolate that was resistant to empirically selected antimicrobials.[8]

Considering the benefit of BALF culture and susceptibility, it is imperative that results accurately reflect present infections. Inappropriate sample handling could alter the reliability of BALF cultures, leading to inappropriate antimicrobial recommendations. Although the effects of BALF storage temperature on cytological evaluation have been described, studies assessing the effect of canine BALF storage temperature on bacterial culture have not been reported.[9,10]

Bordetella bronchiseptica and *Escherichia coli* are among the most common organisms isolated from BALF and transtracheal wash samples of dogs with lower airway disease, with prevalence ranging from 8 to 71.4% and 14.7 to 50.7%, respectively.[4,5,8,11-13] Two or more bacterial species were isolated from 30.7 to 56% of samples.[4,12] Individual bacterial species respond differently to environmental conditions, which raises concerns for bacterial interactions in culture. In urine specimens, Gram-negative bacilli such as *E. coli* have been shown to overgrow Gram-positive cocci.[14] Microbes in urine samples may grow or die off during storage at room temperature or shipping, resulting in inaccurate cultures.[15,16] It is possible that airway samples behave similarly, but this possibility has not been evaluated in veterinary medicine.

Studies comparing immediate aerobic bacterial culture of canine BALF with storage for 24 hours at room temperature, refrigeration, and heat (simulating a sample inappropriately packaged for shipment) are lacking. Our objectives were to evaluate the effect of BALF storage at various temperatures before aerobic bacterial culture. We hypothesized that storage at 25 and 37°C for 24 hours before culture would alter culture results.

2 | MATERIALS AND METHODS

This study was conducted as a prospective, controlled investigation. Bronchoalveolar lavage fluid was collected from 8, intact, male, purpose-bred Beagles that were undergoing general anesthesia. The study was approved by the appropriate Institutional Animal Care and Use Committee. Animals were cared for in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were housed in an Association for Assessment and Accreditation of Laboratory Animal Care approved facility. Animals were excluded if clinical signs suggestive of lower respiratory tract disease were present based on observation and physical examination or if a Gram stain of the BALF was positive.

Dogs were pre-medicated with .05 mg/kg acepromazine IV (PromAce 10 mg/mL injection, Boehringer Ingelheim Vetmedica, Inc, St. Joseph, Missouri) and .1 mg/kg hydromorphone IV (40 mg/20 mL injection, West-Ward Pharmaceuticals Corporation, Eatontown, New Jersey) and induced with 6 mg/kg propofol IV (PropoFlo 10 mg/mL injection, Abbott Laboratories, North Chicago, Illinois). Dogs were placed in lateral recumbency, and bronchoalveolar lavage (BAL) was performed using a blind technique as previously described.[17]

Briefly, a sterile 8-French ×42-inch plastic feeding tube (C. R. Bard Inc, Covington, Georgia) was passed through the sterile endotracheal tube and down the airway until gentle resistance was met. Two milliliters of sterile 0.9% saline per kilogram of body weight were instilled through the feeding tube. The dog's chest was coupaged as suction was applied to a sterile syringe attached to the feeding tube. If <50% of the infused sample fluid was recovered, a second aliquot of equal volume was instilled. If a second aliquot was used, the BALF from the 2 attempts was combined. Samples were capped to prevent contamination, labeled, and stored on ice until all samples were collected (maximum storage time of 15 minutes).

Each sample was gently mixed, and 500 μL of sample was centrifuged and subjected to Gram staining. Samples with positive Gram stains were excluded from the study. A 4 mL aliquot of BALF was removed from each sample. The remainder of the BALF from each dog was divided into 4 1-mL aliquots. These acted as controls and were reserved for culture as described below without further manipulation.

The 4 mL aliquot was inoculated with *B. bronchiseptica* (wild-type obtained from a clinical patient) to achieve a final concentration of approximately 10^4 cfu/mL to simulate a primary bacterial pathogen. This cfu count was chosen based on a previous study that found a threshold concentration of 1.7×10^3 cfu/mL for defining clinically relevant bacterial growth.[18] Additionally, this aliquot was inoculated with *E. coli* (wild-type obtained from a clinical patient) to achieve a final concentration of approximately 1×10^2 cfu/mL to represent a contaminant bacterial strain.[18] The inoculated sample then was split into 4 1-mL aliquots, which were placed into each of 4 sterile tubes without additive.

One aliquot of the control BALF sample was immediately plated for aerobic bacterial culture (baseline). Of the 4 inoculated samples, 1 was plated immediately (baseline). One of each of the 3 remaining inoculated aliquots and the 3 remaining reserved aliquots were stored at 4, 25, or 37°C, respectively, for 24 hours, and then plated for aerobic bacterial culture. Temperatures during shipping can vary widely, and 37°C was chosen to simulate exposure to mid-summer temperatures in the southeastern United States. For plating, a .01 mL aliquot of BALF was inoculated onto blood agar and MacConkey agar using a calibrated loop.[19] Plates were incubated at 37°C for 18-24 hours on blood agar in 5% CO_2 and MacConkey agar in ambient air. Plates were examined and counts obtained for all organisms present. Organisms were identified by standard conventional methods. All bacterial cultures were incubated and examined in the same manner.

2.1 | Statistical analysis

Sample size calculation was performed using a repeated measures analysis of variance (ANOVA) based on 2 groups (inoculated and control) with 4 measurements (immediate culture, storage at 4, 25, and 37°C) per group. Means were based on data from a previous study, with minimum clinically relevant bacterial growth deemed as 1.7×10^3 cfu/mL and the majority of BAL samples cultured in dogs with clinical respiratory disease being 3×10^4 cfu/mL.[20] Standard deviation was estimated at 1.0×10^3 cfu/mL (α = .05). Calculations indicated that a sample size of 6 would yield a power of 90%.

Bacterial counts estimated at $>10^4$ cfu/mL were assigned a value of 1.5×10^4 cfu/mL for statistical calculations. Statistical analysis was performed utilizing SigmaPlot 12.0 (Systat Software, San Jose, California). Results of statistical analysis are shown as median and range. We compared the different temperatures (4, 25, and 37°C) as well as the baseline culture for each microbe using Friedman Repeated Measures ANOVA on Ranks. Pairwise multiple comparison was completed using the Student-Newman-Keuls Method. Statistical significance was set as $P < .05$.

3 | RESULTS

The median age of the enrolled population was .78 years (range .68-.86). No animal showed clinical signs suggestive of lower respiratory tract disease based on observation and physical examination. The BALF samples from all 8 dogs had negative Gram stains.

In all inoculated samples, colony counts for *E. coli* at baseline were less than the previously defined threshold concentration for defining clinically relevant bacterial growth.[20] For *E. coli* in inoculated samples (Figure 1A), a significant difference in growth was found as measured by cfu/mL among the various storage conditions ($P < .001$).

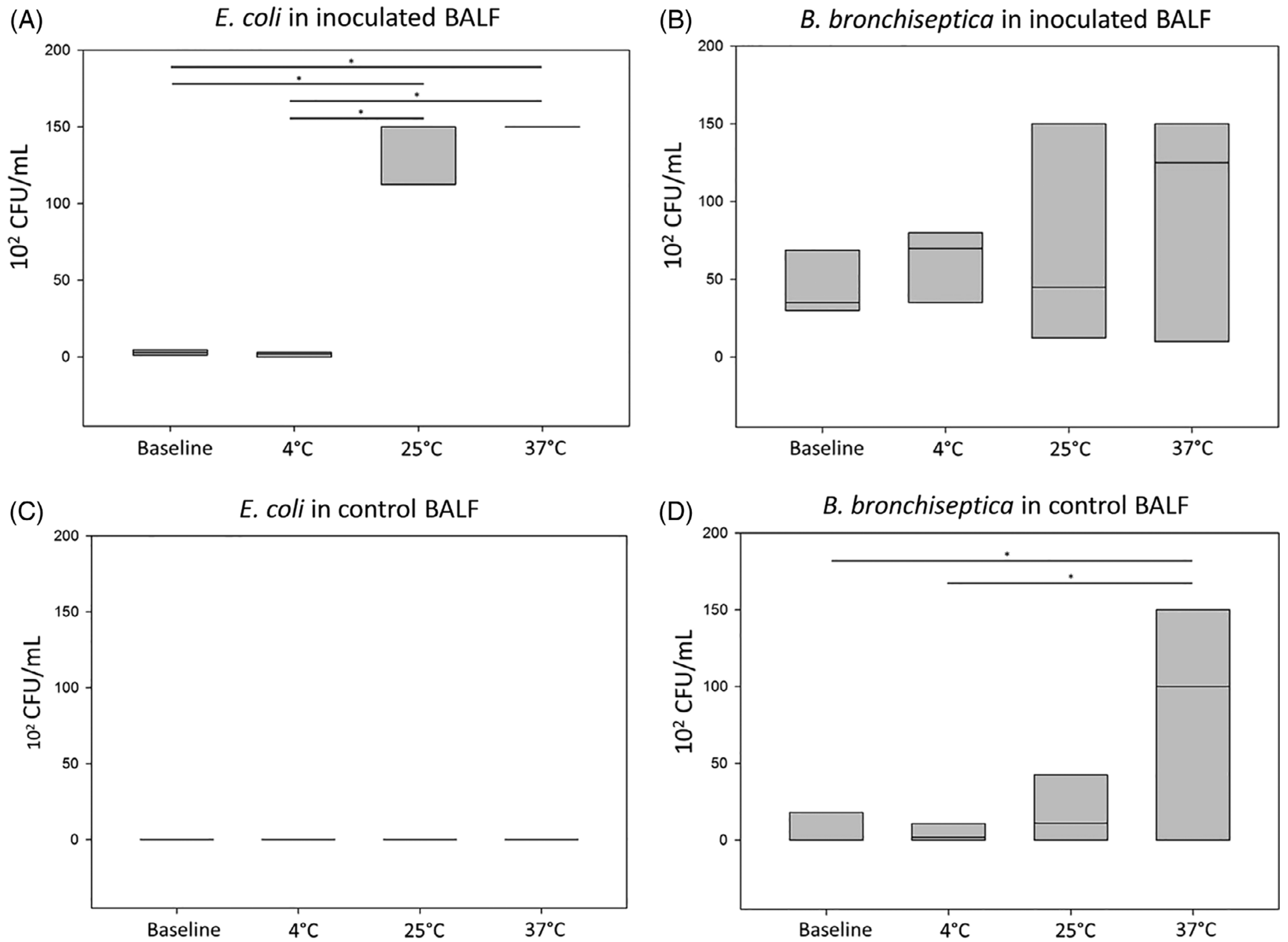

FIGURE 1 Box and whisker plots showing colony counts of bacteria grown in BALF samples when cultured immediately after sample procurement (baseline) or after storage at 4, 25, or 37°C for 24 hours prior to culture. A, *Escherichia coli* grown in inoculated BALF; B, *Bordetella bronchiseptica* grown in inoculated BALF; C, *Escherichia coli* grown in control BALF; D, *Bordetella bronchiseptica* grown in control BALF. Boxes represent the interquartile range (IQR) from the 25th to 75th percentile. The whiskers extend to the minimum and maximum values. The horizontal bar in each box represents the median value. Significant differences between treatment groups are marked with stars (*$P < .05$). BALF, bronchoalveolar lavage fluid; CFU, colony forming units; mL, milliliter

In all inoculated samples, baseline colony concentrations for *B. bronchiseptica* were >1.7 × 10^3 cfu/mL (consistent with clinical relevance). For *B. bronchiseptica* in the inoculated samples (Figure 1B), no significant differences in cfu/mL were found between any combinations of storage conditions (*P* = .54). However, a large amount of variation was noted in *B. bronchiseptica* growth in inoculated samples from all dogs across the various storage treatments. After storage for 24 hours at 37°C, *B. bronchiseptica* growth in 5 of the 8 samples (62.5%) was ≥10^4 cfu/mL. One of the 8 samples (12.5%) had similar growth to baseline, with 2.5 × 10^3 cfu/mL at baseline and 4 × 10^3 cfu/mL after storage for 24 hours at 37°C. Two of the 8 cases (25%) showed no growth after storage for 24 hours at 37°C.

Three of 8 control samples grew *B. bronchiseptica* at baseline (Figure 1C). One additional sample showed *B. bronchiseptica* growth after storage at 4°C, and 2 additional samples showed *B. bronchiseptica* growth after storage at 25 and 37°C. A significant difference was found among the various storage conditions (*P* = .003).

Escherichia coli grew in only 1 of 8 control samples (Figure 1D). For this case, there was no *E. coli* growth at baseline, but after storage at 25 and 37°C growth was >1.7 × 10^3 cfu/mL (consistent with clinical relevance). For *E. coli* found in control samples, no statistically significant differences were found in cfu/mL between any combinations of storage conditions (*P* = .39).

No organisms other than *B. bronchiseptica* and *E. coli* were identified in any sample or after any storage condition.

4 | DISCUSSION

Aerobic bacterial culture results from BALF that had been refrigerated at 4°C for 24 hours before culture were representative of results from samples that were immediately cultured. This was true for both control and inoculated samples. Inoculated samples that were exposed to room temperature (25°C) or heat (37°C) for 24 hours showed overgrowth of contaminant *E. coli*. Control samples with zero or subclinical growth of *B. bronchiseptica* at baseline that were exposed to heat for 24 hours showed overgrowth of *B. bronchiseptica*, leading to growth that could be misinterpreted as a clinically relevant concentration (≥1.7 × 10^3 cfu/mL). Samples inoculated with clinical concentrations of *B. bronchiseptica* showed variable results after storage at 25 and 37°C, with some cfu concentrations decreasing below the limit of clinical importance and some remaining above it. In contrast, with 1 exception, samples inoculated with clinical concentrations of *B. bronchiseptica* had similar cfu concentrations when cultured at baseline and after 24 hours of storage at 4°C. Thus, although samples can be held up to 24 hours at 4°C before aerobic bacterial culture (eg, while awaiting cytology results before submitting a culture for financial purposes), samples stored in an uncontrolled environment may not give reliable aerobic bacterial culture results.

Many practitioners must send BALF samples to external laboratories for culture. These samples may be held for courier pickup or shipped overnight. Anecdotally, it is common for these laboratories to receive samples that have not been packaged appropriately (eg, ice packs, etc), or that have been packaged with ice packs that have subsequently melted. This leaves the samples exposed to, at minimum, ambient temperatures. However, temperatures inside a vehicle can increase rapidly. Over an ambient temperature range of 22 to 35°C, internal temperatures in a car increase an average of 23°C in just 1 hour.[21] Our study showed that exposure to both room temperature and heating could lead to false culture results. For this reason, proper packaging and sample handling in transit to a microbiological laboratory is imperative.

We used 1.7 × 10^3 cfu/mL as a distinction between clinically relevant and unimportant bacterial concentrations based on a previous study.[20] For analytical purposes, it was necessary to have a strict cutoff. However, some of the bacterial concentrations were very close to this cutoff. For example, 2 samples (1 control and 1 inoculated) had *B. bronchiseptica* concentrations of 1.3 × 10^3 cfu/mL after storage at 4°C for 24 hours. Both of these samples had baseline aerobic cultures with *B. bronchiseptica* counts in the clinical range (2.2 × 10^3 and 7.5 × 10^3 cfu/mL, respectively). Using our cutoff, the post-storage cultures were determined to be consistent with subclinical infections. Given the baseline culture results, it is apparent that these both were initially clinically relevant infections. It is possible that the recommended cutoff for clinical relevance should be different in samples that have been refrigerated before aerobic bacterial culture rather than immediately cultured.

We elected to use *B. bronchiseptica* and *E. coli* as representative bacterial agents in BALF samples. We chose these bacteria because of their high prevalence in lower airway disease as well as their variable biologic behavior. In recent studies, *B. bronchiseptica* has been among the most common bacterial isolates in BALF and transtracheal wash samples from dogs with lower airway disease, ranging from 8 to 71.4% prevalence.[4,5,8,11-13] Likewise, *E. coli* is the most commonly isolated enterobacteriaceae, at between 14.7 and 50.7% prevalence.[4,5,8] As many as 30.7-56% of samples have ≥2 bacterial species isolated, although *B. bronchiseptica* was found to be the organism most commonly isolated in pure culture.[4,12] Although *E. coli* may be present in the lower airways as a clinical infection, such as secondary to aspiration pneumonia, it also may be present in the lower airways of healthy dogs. A recent study showed the presence of bacteria from the family enterobacteriaceae in both nasal swabs and BALF of healthy dogs.[22] Another study found *E. coli* in the pharynx of clinically healthy dogs.[23] Thus, *E. coli* isolated from a BALF aerobic bacterial culture may be evidence of clinical infection, colonization, or contamination from the upper respiratory tract. Using the previously mentioned cutoff of 1.7 × 10^3 cfu/mL as a distinction between clinically relevant and unimportant bacterial concentrations can help determine the clinical relevance of *E. coli* grown on BALF culture. However, this is only applicable if bacterial concentrations on culture are accurate. With a doubling time of 22-44 minutes for *E. coli* and 1.8 ± 0.02 hours for *B. bronchiseptica*, it is possible for *E. coli* to outgrow the *B. bronchiseptica*, leading to a false culture interpretation.[24,25]

Gram-negative bacilli, such as *E. coli*, can overgrow Gram-positive cocci in urine specimens.[14] To evaluate whether this overgrowth could occur in BALF, such that a contaminant species could appear as the primary pathogen, we inoculated *E. coli* at a concentration consistent with contamination and *B. bronchiseptica* at a concentration consistent with clinically relevant growth.[20] In the samples held at 25 or 37°C for 2 of 8 dogs, the *E. coli* grew to a concentration that was consistent with a clinically relevant infection, whereas the *B. bronchiseptica* counts decreased to either zero or low enough to be considered contaminants. The increased growth of *E. coli* alongside the severely decreased growth of *B. bronchiseptica* in the same samples indicates that, in up to 25% of samples exposed to room temperature or heat, contaminant species may overgrow pathogens, altering culture interpretation. In contrast, *E. coli* inoculated at contaminant concentrations remained at contaminant concentrations after 24 hours of storage at 4°C in all samples.

One of the striking findings in our study was the variability in the growth of inoculated *B. bronchiseptica*. Although most samples showed increased bacterial growth after storage, a substantial proportion showed either decreased growth or no change in growth. Running samples in duplicate or triplicate would have helped to confirm this variability. Because all inoculated *B. bronchiseptica* came from the same laboratory strain, differences among inoculated strains should not have played a role. Additionally, the possibility that microbes present in the samples utilized all available nutrients, leading to bacterial starvation and a decrease in growth, was considered. Because every sample was able to support strong growth of *E. coli* after storage at 37°C, this possibility was considered unlikely. This same observation makes the possibility of a toxic metabolite or BALF component suppressing bacterial growth unlikely.

Three of the 8 control samples grew *B. bronchiseptica* at baseline. *Bordetella bronchiseptica* may be isolated from asymptomatic infected animals, convalescent carriers, and transiently colonized animals.[26] Based on history and physical examination, a clinical infection with *B. bronchiseptica* was not suspected but neither BALF cytology nor thoracic imaging was performed for any patient in our study. We consider this omission to be a limitation of the study.

All 3 of the control samples that grew *B. bronchiseptica* at baseline had a decrease in *B. bronchiseptica* growth after storage at 4°C. Isolation rates of *Bordetella pertussis* and *Bordetella parapertussis*, 2 organisms closely related to *B. bronchiseptica*, decrease when transport occurs at 4°C.[27] *Bordetella bronchiseptica*, like *B. pertussis* and *B. parapertussis*, is a mesophile, meaning that it grows best in moderate temperatures, typically 20°C-45°C, with the optimal temperature being 37°C.[28] At a temperature of 4°C, growth would be expected to be impaired.

A limitation of our study is that only 2 bacteria were assessed, and neither was assessed individually. Future studies are needed to evaluate if these results apply to other bacterial species. Additionally, only an aerobic bacterial culture was performed; no diagnostic tests to evaluate for mycoplasma were performed. Another limitation is that bacteria used for inoculation were in the exponential growth phase, which may not be the case in clinical patients. Only purpose-bred, male, Beagle dogs were used which is not representative of all clinical populations. Blinded BALF samples were used. Although the possibility of subclinical infection cannot be definitively ruled out, there were no indications to suggest that a clinical infection was present in any dog based on history and physical examination. Therefore, we believed that targeted sampling of a particular lung lobe (ie, using a bronchoscope) was not indicated.

In conclusion, aerobic bacterial cultures of BALF after storage for 24 hours at 4°C adequately represent samples that have been cultured immediately after sample procurement. *Escherichia coli* present in contaminant concentrations at baseline may grow to concentrations consistent with infection after storage at 25 or 37°C for 24 hours. *Bordetella bronchiseptica* present in contaminant concentrations at baseline may grow to concentrations consistent with infection after storage at 37°C for 24 hours. Our findings indicate that BALF can be stored at 4°C for up to 24 hours before being submitted for aerobic bacterial culture.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The protocol was approved by the Auburn University IACUC (PRN No: 2017-3201).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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