Original Article

Intradermal Immunization with Heat-Killed *Klebsiella pneumoniae* Leading to the Production of Protective Immunoglobulin G in BALB/c Mice

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Abstract

Introduction: *Klebsiella pneumoniae* superbug is emerging as a serious health concern as resistance to last-resort antibiotics spreads. To bypass the therapeutic molecules used today, the development of an immunoprophylactic safe approach is of great clinical relevance. This study was conducted to determine the protective efficacy of antibodies elicited by killed vaccine against multidrug-resistant (MDR) *K. pneumoniae*. **Materials and Methods:** In this study, heat-killed MDR *K. pneumoniae* isolated from different clinical samples were employed for the intradermal immunization of 10 BALB/c mice. Two weeks after the third dose of immunization, the mice were intraperitoneally challenged with live *K. pneumoniae* and observed for 14 days. Tail blood was collected 7 days after each booster followed by cardiac puncture 14 days postchallenge. Bactericidal activity and antigen-binding capacity of the serum antibody produced by the vaccine were evaluated by serum bactericidal antibody (SBA) assay and ELISA, respectively. **Results:** In this study, 80% survival rates were observed at 14 days postchallenge among the immunized mice. Regarding SBA assay, 100% bactericidal activity of the immunized mouse sera was observed using 50% guinea pig complement at 1:10 serum dilution after 3 h of incubation, and all the pre- and postchallenge immunized serum immunoglobulin G antibody had significantly higher optical density values comparing the control mice in ELISA. **Conclusion:** In our study, intradermal immunization with heat-killed MDR *K. pneumoniae* produced protective antibodies in BALB/c mice. These findings suggest that the use of a first-generation vaccine provides the supply of a larger number of candidate antigens for eliciting required immune response.

Keywords: *Immunization, inactivated vaccine, Klebsiella pneumoniae, protective immunoglobulin G, serum bactericidal antibody*

Introduction

Klebsiella pneumoniae is a Gram-negative pathogen that is evolving in both nosocomial and community-acquired settings.[1] Infections caused by *K. pneumoniae* include pneumonia, urinary tract infection, septicemia,[2] pyogenic liver abscess, meningitis, and endophthalmitis.[3] All of these infections can progress to shock and death if not treated early in an aggressive fashion. The emergence of multi- and pandrug-resistant strains of *K. pneumoniae* renders antibiotic treatment of infected patients very difficult and usually with high mortality rates.[4]

Infections caused by multidrug-resistant (MDR) *K. pneumoniae* are increasingly frequent, and the morbidity, mortality, and financial costs associated with these infections are unacceptably high.[5,6] Meanwhile, the development of novel antibacterial agents remains stagnant causing significant concern.[7] This has directed attention toward controlling *K. pneumoniae* infections through employing alternate strategies to improve immune defenses such as probiotics[8] or more specific means such as vaccination and antibody therapy.[9] By reducing susceptibility to infection, vaccines decrease the necessity for antibiotic and can ultimately limit the environmental pressure leading to the selection of resistant strains.[10] Hence, a vaccine approach against *K. pneumoniae* is appealing and is badly needed. The types of vaccines that have been investigated against *K. pneumoniae* mainly include lipopolysaccharides (LPSs), capsular polysaccharides, and protein-based antigens.[11] The limitation of the first two types is high diversity among

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K. pneumoniae strains along with the potential toxicity of LPS, while the third is more conserved. The use of the whole bacteria for the construction of a vaccine provides a more ample supply of candidates for vaccination resulting in a more suitable inflammatory stimulus for establishing an appropriate immune response.[11]

The dermal layers are easy to access biological sites that have abundant presence of Langerhans cells, dendritic cells, and other antigen-presenting cells (APCs).[12,13] During an inflammatory process, these APCs capture and process the invading antigens followed by rapid migration to secondary lymphoid organs. This configuration makes the dermal tissue a good site for the initiation of immune responses.[12]

Assays are needed to measure functional antibodies elicited by the new vaccines to evaluate their immunogenicities and protective capacities.[14] The serum bactericidal activity as measured by serum bactericidal antibody (SBA) assay has become the "gold-standard" correlate of both infection and successful immunization.[15,16] The SBA assay measures complement-mediated killing of bacteria by vaccine-induced antibody. This assay requires active complement, either intrinsic from the serum being tested or the addition of exogenous complement, either from a human or from another species such as guinea pig, rabbit, goat, and horse.[15,14] Although the measurement of serum antibodies through antigen-binding assays (i.e., ELISA) offers the most practical tool for monitoring the immunogenicity of different immunization schedules, regulatory agencies have urged that the functional capacities of antibodies should also be documented, in addition to their antigen recognition capacities.[14] No assay has been reported to determine the SBA responses induced by killed vaccine against MDR *K. pneumoniae* in BALB/c mice.

No approach to develop a protective vaccine for this notorious pathogen has been taken so far in Bangladesh. Hence, this study has been done to evaluate the development of killed vaccine after intradermal immunization of heat-killed MDR *K. pneumoniae* in murine infection model.

Materials and Methods

Animals

Ten 6–8-week-old BALB/c female mice and two 3–4-week-old guinea pigs were kept and cared in the animal house facility of the microbiology department at the affiliated institution. Ten mice were grouped randomly into four. Group 1 and Group 2 were experimental groups containing 3 mice each; Group 3 and Group 4 were control groups containing 2 mice each. Nonmedicated feed and water were provided throughout the experiment. An animal study was approved by the ethical review committee of the corresponding institution.

Immunization of mice

Bacterial culture

All bacterial cultures were maintained at 37°C and for a cultivation time of 24 h before each use to ensure that the bacteria were at the same growth stage in all experimental steps.

Preparation of the dosage of vaccine formulation

Group 1 was immunized with a formulation prepared from *K. pneumoniae* isolated from a blood sample, and for Group 2, a mixed solution of *K. pneumoniae* (in normal saline) isolated from urine, sputum, wound swab, and endotracheal aspirate was prepared. Subculture was done in Mueller-Hinton agar media aerobically at 37°C overnight. Using a sterile wire loop, 8–10 well-isolated colonies of organism were emulsified in 10 ml of sterile normal saline. The acquisition of the colony-forming unit (CFU) was done by spectrophotometry. After obtaining the desired bacterial load (1.5×10^7 CFU/ml), the bacteria were heat inactivated at 60°C for 30 min in a water bath. These heat-killed inoculums or bacterial solutions were used for the immunization of mice and checked for viability by streaking again on the culture plates which was confirmed by observing absence of any growth after overnight incubation at 37°C.

Immunizations schedule

Prior to each immunization, the mice were anesthetized with intraperitoneal injection of ketamine adjusted to the body weight of mice (ketamine 100 mg/kg). Ketamine acts as a muscle relaxant, and chloroform was used to maintain the proper anesthetic condition. Three intradermal immunizations were performed in the alternate ear of mice with 20 µL of bacterial solution (1.5×10^7 CFU/ml in 20 µL sterile normal saline) prepared on the same day for the two experimental groups and 20 µL of sterile saline for the control Groups 3 and 4 on days 0, 14, and 28. The intradermal inoculation was done with an insulin syringe BD Ultra-Fine™ (31G).

Collection of SBA and ELISA

Tail blood was collected 7 days after each booster for the evaluation of the *in vitro* bactericidal activity of the mouse serum and to detect antibody titer by ELISA. The tail was stretched and cleansed with 70% alcohol. With the help of sterile scalpel (22 FR), the tail was cut 2 mm proximal to its blunt end. About 10 µl of fresh blood was collected and taken into microcentrifuge tubes containing 40 µl of phosphate-buffered saline (PBS), thus yielding a dilution of 1:5. The diluted blood was kept upright for 2 h and centrifuged at 3000 g for 10 min; clear sera were taken into a separate microcentrifuge tube.

Intraperitoneal challenge

Two weeks after the last booster, the mice from Groups 1, 2, and 3 were challenged intraperitoneally with 1.5×10^8 CFU/ml in 100 µL normal saline.[9] The inoculums

were prepared using the same strains that were used in vaccine formulations. All mice were observed for 14 days postchallenge for any clinical manifestations such as weight loss, lack of movement, and reluctance to feed.

Collection of blood by cardiac puncture

Fourteen days after the lethal challenge, sera were separated from blood collected by cardiac puncture from Groups 1, 2, and 4. The chest area of mice was shaved and washed with povidone-iodine and 70% alcohol. Cardiac pulsation was felt by finger and blood was drawn from heart by insulin syringe introduced at an angle of 45°. About 1 ml of blood was collected and was kept undiluted in an sterile test tube, and serum was collected as described above and kept at −20°C for further use.

Antibody detection by ELISA

Mouse sera were evaluated for the presence of immunoglobulin G (IgG) specific for *K. pneumoniae* antigen by determining the optical density (OD) value by ELISA.

Procedure

Sonication of whole-cell Klebsiella pneumoniae

Bacterial pellets were resuspended in 100 µL of distilled water, kept on ice for 30 min, sonicated at 20 kHz for 2 × 10 s (depending on samples and viscosity of the samples), and again kept on ice for 5 min followed by centrifugation at 10,000 g for 20 min to pellet debris (debris may contain unlysed cells, nuclei, or unlysed organelles). Then, supernatants were transferred to a new microcentrifuge tube and were stored at −20°C for use as antigen. Optimization of the antigen was done by checkerboard titration, and 10 µg of antigen was used in ELISA.

Optimized ELISA

ELISA plates were coated with 100 µl/well of antigen (10 µg/ml) in a bicarbonate-coating buffer (pH 9.6). After overnight incubation at room temperature, plates were washed twice with PBS and subsequently blocked with 200 µl/well of 5% w/v skimmed milk in PBS, incubated at 37°C for 30 min, and washed thrice with PBS-tween (0.05% tween 20) and once with PBS. Then, the serum samples were added at different dilutions (100 µl/well) and incubated at 37°C for 90 min and then at 4°C overnight. After washing the plates as described above, diluted (1:5000) conjugate, horseradish peroxidase-labeled anti-mouse IgG antibody (Thermo Fisher Scientific, USA) in PBS-Tween was added (100 µl/well) and incubated at 37°C for 90 min. Again after washing, 100 µl/well tetramethylbenzidine (50 µl) and urea peroxide (50 µl) substrate were added. Thereafter, 50 µl of 1M sulfuric acid was added to stop the reaction. Absorbance was measured at 450 nm using ELISA plate reader (BioTek Inc., USA). Cutoff value of OD was calculated by the following formula:

OD = M (mean) +2 × Standard deviation

Serum bactericidal antibody assay

Bacterial strains, media, and growth kinetics

A MDR hypermucoid *K. pneumoniae* strain isolated from wound swab sample was grown at 37°C in Mueller-Hinton agar media for 18–20 h overnight. 0.5 McFarland standard bacterial suspension was made (1.5×10^8 CFU/ml). The suspension was serially diluted in PBS to achieve different concentrations ~1×10^3 CFU/ml.

Complement

Pooled guinea pig sera served as a source of complement. With all aseptic precaution, blood was collected in sterile test tubes after cardiac puncture of two guinea pigs, centrifuged at 3000 g for 10 min; serum was separated, collected in sterile cryotubes, and stored at −20°C. To determine the serum resistance of the target strain, survival of the *K. pneumoniae* was tested in the presence of complement alone at a concentration of 50%, 25%, and 12.5% (diluted in PBS). Survival of the *K. pneumoniae* strain was also tested in the presence of immune and nonimmune serum alone without complement.

Optimization of serum bactericidal antibody assay to measure antibody-dependent complement-mediated killing

10 µl of heat-inactivated (at 56°C for 30 min) and serially diluted (1:10, 1:20, 1:40, 1:80, and 1:160 in PBS) immune and control serum samples of mice were placed in a 96-well round-bottom microtiter plate. Then, 10 µl of bacterial suspension (of different concentrations from 1.5×10^8 CFU/ml till 1.5×10^3 CFU/ml) was added to the duplicate wells. Finally, 30 µl of pooled guinea pig sera (50%, 25%, and 12.5%) was added to each well for each serum dilution. Incubation was done at 37°C for 30 min, 1 h, 2 h, 3 h, and 4 h.[14]

In each well, 50 µl of final reaction volume was achieved from which 1 µl of reaction mixture was inoculated in Mueller-Hinton agar media. After overnight incubation at 37°C aerobically, viability of the bacteria was determined by observing the growth on agar plates. Each of the procedures was performed in triplicate.[17]

Optimized serum bactericidal antibody assay

Log-phase cultures (bacteria grown to an OD 600 of 0.4 or 1.5×10^4 CFU/ml) of *K. pneumoniae* were prepared in normal saline. Serum samples from immunized and control mice were serially diluted (1:10 and 1:20). For the *K. pneumoniae* strains, optimal SBA results were obtained by combining 30 µl of guinea pig sera (50% final concentration) and 10 µl of diluted mouse serum; these were then incubated with 10 µl of diluted

bacteria (1.5×10^4 CFU/ml) at 37°C for 2 h. Viable CFU counts were determined. The negative control contained bacteria and complement only.[18,19]

Data processing

All data were compiled and edited meticulously by thorough checking and rechecking. All omissions and inconsistencies were corrected and were removed methodically.

Data analysis

The results of the study were recorded systematically. Data were analyzed and compared by *Z* test. All statistical analysis was done by SPSS version 16, SPSS Inc (SPSS for Windows, Version 16.0. Chicago, SPSS Inc; 2007). P =0.05 was taken as a minimal level of significance.

Results

Regarding the survival rates of immunized and unimmunized mice after lethal *K. pneumoniae* challenge, 50% of Group 1 and 100% of the Group 2 mice survived the 14 days observation period after challenge was seen. All Group 3 control mice died within 24 h of challenge, and all Group 4 control mice (those were not challenged) survived the 14 days observation period. One mouse from Group 1 died during the anesthetization procedure after the first booster immunization due to chloroform intoxication and another mouse from the same group died 24 h after lethal challenge.

OD of anti-*K. pneumoniae* antibodies in 6 serum samples (3 were from Group 1 and 3 were from Group 2 mice) collected 7 days after the first booster immunization [Figure 1] and in 5 serum samples (2 were from Group 1 and 3 were from Group 2 mice) collected 7 days after the second booster [Figure 2] was determined. All the serum samples from immunized mice had OD of anti-*K. pneumoniae* IgG polyclonal antibody above the cutoff value (0.77). There was a statistically significant difference between the OD values of experimental and control mouse sera (P = 0.0001 after the first booster and P = 0.0011 after the second booster).

OD of anti-*K. pneumoniae* antibodies in serum samples collected after the lethal challenge is shown in Figure 3. Among 5 serum samples, 2 were from Group 1 and 3 were from Group 2 mice. All the serum samples had OD of anti-*K. pneumoniae* IgG polyclonal antibody above the cutoff value (0.57). The difference between the OD values of experimental and control mouse sera was statistically significant (P = 0.0099).

SBA assay using the prechallenge [Figure 4] and postchallenge sera [Figure 5] with bacterial inoculum of 1.5×10^4 CFU/ml after heat inactivation resulted in the killing of bacteria by immunized mouse sera from both the experimental groups has been shown.

Discussion

Vaccination is considered to be the most effective and cost-effective method to prevent infectious diseases. Approaches to enhance host immunity and for reducing bacterial infection such as adenoviral gene therapy for overproduction of interleukin-2, using tumor necrosis factor-α pro-inflammatory cytokine, and cyclic di-Guanosine monophosphate (GMP) have shown promise in boosting immunity and reduced bacterial infections. However, none of these molecules could reach market due to their high price, being nonspecific, and no clinical study support. In these difficult times, promising alternatives to antibiotics seem to be pathogen-specific active vaccination and passive antibody therapy.[20]

In the present study, the survival proportion among the experimental mice was 80% at 14 days postchallenge. A study in Korea reported an 80%–100% survival rate (dose dependent) 5 days postchallenge following immunization of mice with *K. pneumoniae*-derived extracellular vesicles.[9] A study by Kurupati *et al*. found

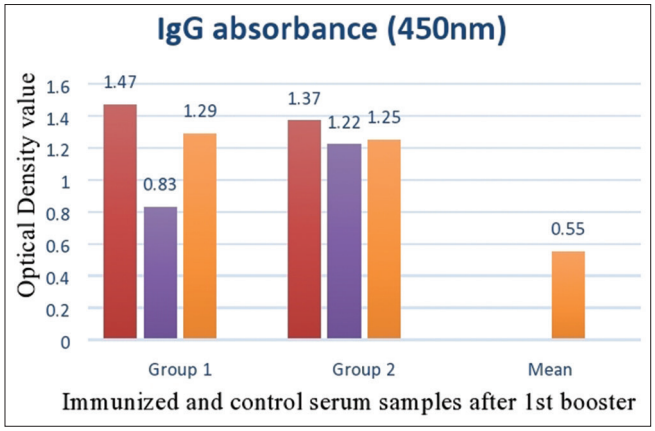

Figure 1: Optical density of serum samples after the first booster by ELISA. Here, mean of negative control 0.55, standard deviation 0.112, cutoff value for prechallenge sera 0.77, range after the first booster 0.83–1.47, P = 0.0001

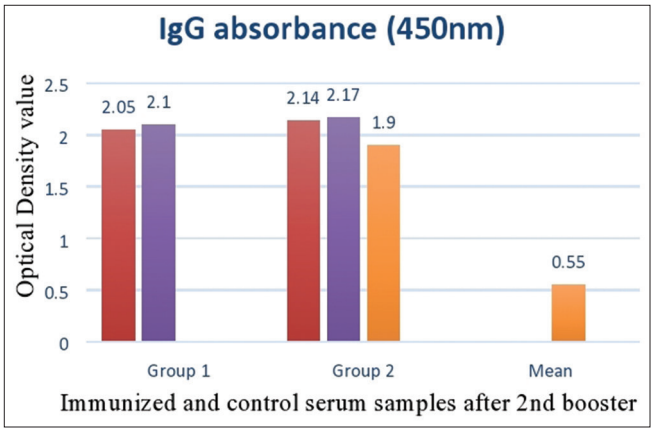

Figure 2: Optical density of serum samples after the second booster by ELISA. Here, mean of negative control 0.55, standard deviation 0.112, cutoff value for prechallenge 0.77, range after the second booster 1.90–2.17, P = 0.0011

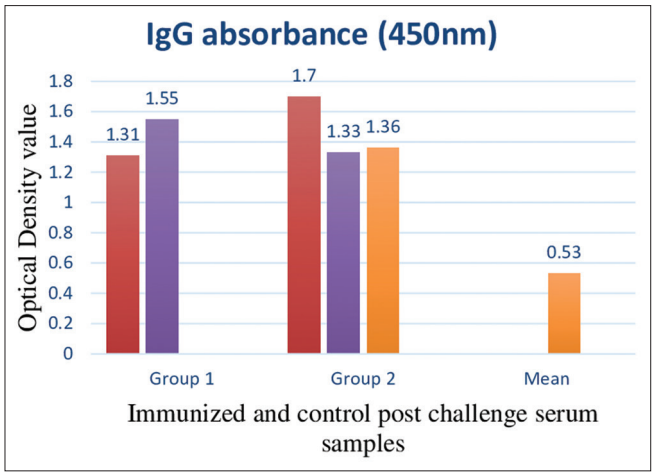

Figure 3: Optical density of serum samples after lethal challenge by ELISA. Here, mean of negative control 0.53, standard deviation 0.0214, cutoff value for prechallenge 0.57, range postchallenge 1.31–1.7, *P* = 0.0099

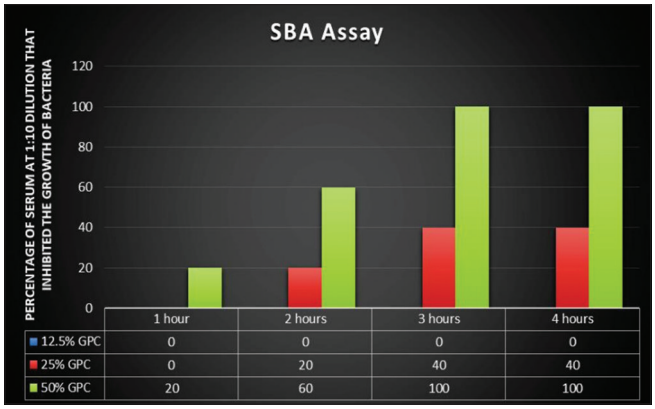

Figure 4: Serum bactericidal antibody assay using the prechallenge sera with bacterial inoculum of 1.5 × 10⁴ colony-forming unit/ml after heat inactivation. Bacterial growth was inhibited with immunized sera at 1:10 dilution and 25% and 50% guinea pig complement

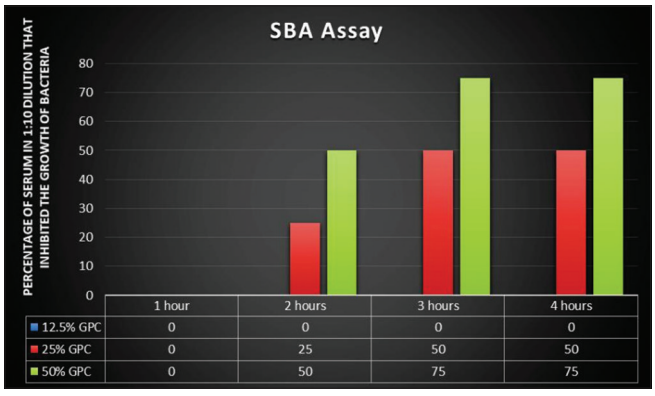

Figure 5: Serum bactericidal antibody assay using the postchallenge sera after heat inactivation with bacterial inoculum of 1.5 × 10⁴ colony-forming unit/ml. Bacterial growth was inhibited with immunized sera at 1:10 dilution and 25% and 50% guinea pig complement. Sera at other dilutions and 12.5% guinea pig complement did not inhibit the bacterial growth

that mouse intradermal injection of four doses of a plasmid containing the OmpK36 gene resulted in a mixed Th1/Th2 response and protected against lethal bacterial challenge.[21] Another study reported that subcutaneous Incomplete Freund's Adjuvant (IFA)-adjuvanted antigens, the percentage survival was 50%, 60%, and 50% for the groups immunized with OmpK17, OmpK36, and F36/17 fusion protein, respectively.[22] No data are available regarding survival rates of mouse after lethal challenge following intradermal immunization with heat-killed MDR *K. pneumoniae*. In the present study, heat-killed whole-cell bacteria were used due to abundance of surface antigen to stimulate broader immune response. Intradermal route was selected because of ample presence of APCs.

Gram-negative bacterium owing to their cell complexity are predominantly killed by complement cascade and less through opsonophagocytosis.[17] An assay to determine the serum bactericidal activity of antibody against *K. pneumoniae* in mice immunized with heat-killed vaccine strains was established in the present study. Pulled guinea pig serum was used as the source of complement. One previous study compared guinea pig complement (GPC) and baby rabbit complement (BRC) for *Salmonella* SBA assay and reported that in contrast to GPC, BRC was unable to kill multiple *Salmonella* serovars[14] which is in accordance with the present study. There are no data available regarding SBA assay for evaluation of functional activity of serum immunized with *K. pneumoniae* to compare with the present study.

In the current study, the immunization protocol led to the production of *K. pneumoniae* recognizing IgG polyclonal antibody in all the immunized mouse sera after the first booster, second booster, and lethal challenge measured by ELISA. One previous study showed immunization with IFA-adjuvanted antigens elicited significant levels of specific IgG1 but not IgG2a antibodies when compared with the negative control group.[22] Another study by Pereira *et al*. reported that intradermal immunization with heat-killed *K. pneumoniae* strains led to the production of titers of anti-*K. pneumoniae* IgG1 and IgG2a antibody in the sera of immunized BALB/c mice.[23] These antibodies in the serum can be crucial to the activation of the classical pathway of the complement system in the early stages of infection, thus the efficient depuration of the bacteria. In the current study, the OD values of the IgG antibody were highest for prechallenge mouse sera after the second booster. This might be due to the fact that more IgG antibody was produced by the memory cells after the second booster than after the first booster immunization and following lethal challenge as antibodies were used up clearing the offending pathogen from the body.

Conclusion

Developing an effective vaccine against this serious pathogen is an appealing approach which deserves intensive investigation. Although an immunological correlate of protection still needs to be established for the killed *K. pneumoniae* vaccine, the present study provides

new insight for a novel strategy of vaccine development using whole-cell heat-killed Gram-negative bacteria and its evaluation by SBA and ELISA to protect against MDR bacterial infections. Taken together, our results suggest that intradermal immunization with heat-killed MDR *K. pneumoniae* produced protective antibodies in BALB/c mice.

Ethical clearance

Ethical permission for this study was obtained from the Institutional Review board.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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