Detection of Hypervirulent *Klebsiella pneumoniae* in Tanta University Hospital, Egypt

Marwa M. E. Abd-Elmonsef[1*], Haidy S. Khalil[2], Amal Selim[3], Sherief Abd-Elsalam[4], Walaa Elkhalawany[4], Sameh Samir[5], Mohamed S. Abd-Elghafar[6] and Mohamed M. E. Abd-Elmonsef[7]

[1]*Department of Microbiology and Immunology, Faculty of Medicine, Tanta University, Egypt.*
[2]*Department of Microbiology and Immunology, Faculty of Medicine, Helwan University, Egypt.*
[3]*Department of Internal Medicine, Faculty of Medicine, Tanta University, Egypt.*
[4]*Department of Tropical Medicine and Infectious Diseases, Faculty of Medicine, Tanta University, Egypt.*
[5]*Department of Cardiovascular Medicine, Faculty of Medicine, Tanta University, Egypt.*
[6]*Department of Anesthesia and Surgical Intensive Care, Faculty of Medicine, Tanta University, Egypt.*
[7]*Department of Mathematics, Faculty of Science, Tanta University, Egypt.*

Authors' contributions

Article Information

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ABSTRACT

Aims: This study aimed to investigate the frequency of hypervirulent *Klebsiella pneumoniae (K. pneumoniae)* isolated from various hospital-acquired infection cases admitted to Tanta University Hospital, Egypt and to determine the antibiotic resistance profile of these isolates.
Study Design: Retrospective observational study.
Place and Duration of Study: After collection of *K. pneumoniae* isolates from microbiology laboratory of Tanta University Hospital. Further work was carried out in the laboratory of

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*Corresponding author: E-mail: marwa.ezzat@med.tanta.edu.eg;

Microbiology and Immunology Department, Faculty of Medicine, Tanta University, Egypt, from June 2015 to May 2016.

Methodology: A total of 113 *K. pneumoniae* isolates were collected from different hospital-acquired infections and were tested for hypermucoviscosity phenotype by string test. Antibiotic disc diffusion test was performed for all isolates to identify their resistance pattern. Existence of *rmpA* gene was investigated by polymerase chain reaction.

Results: Forty-six out of 113 (40.71%) isolates were string test-positive (HVKP), the remaining 67 (59.29%) negative isolates were CKP. Twenty-six (56.52%) out of 46 HVKP isolates possessed *rmpA* gene. Lower resistance rates were observed in HVKP than CKP.

Conclusion: ESBL production by *rmpA*-positive HVKP isolates in hospital-acquired infections is worrisome, though its rate is still low. Control of the spread of this organism in the hospital environment and the general community is an important concern.

1. INTRODUCTION

Klebsiella pneumoniae (K. pneumoniae) is a Gram-negative bacilli that was first isolated in 1882 [1]. Since then, many studies were performed on *K. pneumoniae*, its virulence, epidemiology and management. One of the important studies was carried out by Liu et al. [2] in 1986 that divided *K. pneumoniae* into classic (CKP) and hypervirulent (hypermucoviscous) (HVKP). This HVKP variant underwent global spread starting from Taiwan, where it was first detected, crossing China, South America, the United States, Canada, Europe, Australia, the Middle East and elsewhere [2-9]. The importance of this variant is associated with its ability to cause invasive, life-threatening infection in young healthy individuals [10]. Importantly, it can spread metastatically from primary sites of infection in immunocompetent hosts, which is an abnormal feature for enteric Gram-negative pathogens [2,11]. Its mortality rate has reached significant levels (up to 42%) [7]. It is involved in both community- and hospital-acquired infections [12].

Certain criteria were used to define HVKP. One notable criterion is the hypermucoviscosity phenotype exhibited by this variant. This was attributed to the increase in the capsular or extra-capsular polysaccharides production which represents an important virulence factor [1,13]. The increase in the surface polysaccharide renders the bacteria resistant to engulfment by phagocytes or to serum bactericidal factors [14,15]. It also has a role in limiting the bactericidal effects of antimicrobial peptides [16]. It is associated with high mortality rates in experimentally infected animals [17,18].

Another criterion of HVKP is the possessing of a large 200- to 220-kb virulent plasmid, which carries several virulence factors encoded genes, such as the regulator of mucoid phenotype gene (*rmpA*) and siderophores (aerobactin and salmochelin), which are responsible for regulation of iron acquisition by bacteria enhancing their growth, replication and virulence [17,19,20]. As regards *rmpA* gene, it encodes a protein of 15.5 kD. It *is a virulent gene as it* acts as a regulatory gene controlling the mucoid phenotype. It is suggested to be a foreign gene, as it has a sequence with low GC content that is completely different from that of *Klebsiella* Species [13].

Generally, plasmids has a central role not only in the acquisition of virulence factors but also in the dissemination of resistant determinants in *K. pneumoniae* [21]. The acquisition of multidrug resistance by HVKP is one of the critical features that will render it to become the next "superbug" [10]. *K. pneumoniae* prevalence has reached high rates (46.5%) in hospital-acquired infections in Egypt [22]. Besides, as far as we know, no data have been published on the detection of HVKP isolated from human in Egypt. Only one study was carried out on HVKP in Egypt, but their isolates were recovered from buffalos [23]. Therefore, this study was designed to determine the existence of hypermucoviscous phenotype and *rmpA* gene among *K. pneumoniae* isolated from different hospital-acquired infections in Tanta University Hospital, Egypt and to detect the association between hypervirulence and antimicrobial resistance.

2. MATERIALS AND METHODS

2.1 Bacterial Isolates and Growth Conditions

A total of 113 hospital-acquired *K. pneumoniae* isolates were obtained from the

microbiological laboratory of Tanta University Hospital, Egypt, during the period from June 2015 to May 2016. Patient's samples were collected as part of routine management. Isolates data were collected retrospectively from patients fulfilling criteria of Centre for Disease Control (CDC) for Hospital-acquired infections [24]. According to CDC, cases of ventilator-associated pneumonia (VAP) were defined as patients who were under mechanical ventilation for more than 48 h with new or progressive infiltrate on chest X-ray and at least 1 of the following criteria: Fever (38°C) with no other recognized cause, WBC < 4000/mm3 or >12000/mm3; plus at least 2 of the following criteria: new onset of purulent sputum, increase in respiratory secretions or increased need for suctioning, new onset or worsening cough, or dyspnea, or tachypnea, rales or bronchial breath sounds, and worsening gas exchange [25]. Urinary tract infection (UTI) was considered hospital-acquired in patient who had an indwelling urinary catheter in place for >2 calendar days, with day of device placement being Day 1, and at least one of the following criteria: fever (>38°C), suprapubic tenderness, costovertebral angle pain or tenderness (with no other recognized cause) [26].

All isolates were identified by routine microbiological methods, and the species was confirmed using the API 20E system (bioMerieux, France). The agar plates of identified isolates were then, sent immediately to the microbiological laboratory of Microbiology and Immunology Department, Faculty of Medicine for further processing.

2.2 Phenotypic Detection of Hyper-mucoviscosity by String Test

This was done using a bacteriologic loop to stretch the colonies on the agar plates. Any generated viscous string > 5 mm in length was considered positive test. *K. pneumoniae* isolates with positive string test were designated as hypermucoviscous *K. pneumoniae* (HVKP), the remaining negative isolates were designated as classic *K. pneumoniae* (CKP) [1].

2.3 Antibiotic Susceptibility by Disc Diffusion Test

Antibiotic susceptibility was performed for all isolates, using the Kirby-Bauer disc diffusion test and interpreted according to Clinical and Laboratory Standards Institute guidelines [27]. A panel of 14 antimicrobial drugs was tested,

including gentamicin (10 μg), amikacin (30 μg), amoxicillin/clavulanate (30 μg), piperacillin/tazobactam (110 μg), cefoxitin (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), ceftazidime (30 μg), cefepime (30 μg), aztreonam (30 μg), imipenem (10 μg), meropenem (10 μg), ciprofloxacin (5 μg), sulphamethoxazole/trimethoprim (25 μg) [Oxoid, UK]. All included isolates were screened for the production of extended-spectrum beta-lactamases (ESBLs). They confirmed by double-disc synergy test [27]. Phenotypic detection of AmpC was indicated by the resistance to a cefoxitin disc (30 μg) according to Singhal et al. [28]. *K. pneumoniae* ATCC 700603 and *Staphylococcus aureus* ATCC 25923 were included in each test as control strains. All isolates were then subcultured and frozen at -70°C in 25% glycerol for the molecular study.

2.4 Detection of *rmpA* Gene by Polymerase Chain Reaction (PCR)

The existence of *rmpA* gene was detected in HVKP isolates using PCR as described by Yeh et al. [29]. The used primers were: *rmpAF* 5´-ACTGGGCTACCTCTGCTTCA-3´ and *rmpAR* 5´-CTTGCATGAGCC ATCTT TCA-3´). The reaction mixture was kept at: 95°C for 5 min, followed by 40 temperature cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min, and 72°C for 7 min. The expected PCR product of *rmpA* was 535 bp in length.

2.5 Statistical Analysis

The z- test for proportions was used in the analysis of categorical variables, while Fisher's exact test was used for small samples. A *P* value < 0.05 was considered statistically significant. All statistics were performed using Minitab17.

3. RESULTS

3.1 Clinical Characteristics of HVKP vs. CKP Isolates

A total of 113 hospital-acquired *K. pneumoniae* isolates were collected from different hospital-acquired infections during the period of the study. Of these, only 46 (40.71%) isolates were string test-positive and they were designated as (HVKP), the remaining 67 (59.29%) isolates were designated as (CKP). HVKP was isolated from 23 (50%) cases of VAP, 4 (8.7%) post-operative wound infection, 11(23.91%) cases of UTI and 8(17.39%) bacteremic cases. The distribution of the two variants of *K. pneumoniae* among clinical

cases showed that HVKP was significantly higher in VAP and bacteremia than CKP (P = .04 and .002, respectively). While CKP was significantly higher in post-operative wound infection (P = .002). Both variants presented nearly equal in UTI (P = .08) (Table 1).

3.2 Prevalence of *rmpA* Gene in HVKP Isolates

PCR amplification for the presence of *rmpA* gene (Fig. 1), showed that 26 (56.52%) out of 46 HVKP isolates possessed *rmpA* gene. The gene was presented in all HVKP of bacteremic cases (100%), 73.91% of VAP cases and 25% of post-operative wound infection. None of HVKP isolated from urine carried the gene (Table 2).

3.3 Antimicrobial Resistance Profile of HVKP vs. CKP Isolates

HVKP showed significantly lower resistance rates to all tested antimicrobial drugs than CKP, except to carbapenems. Resistance to carbapenems was observed in 8.96% of CKP isolates, while none of HVKP isolates were resistant to them. The frequencies of ESBL and AmpC production among HVKP were significantly lower than that among CKP (8.7% vs. 55.22% and 2.17% vs. 28.36%, respectively) with (P<.001). None of HVKP isolates produced both ESBL and AmpC together, while 5.97% of CKP isolates were both producers. Highest resistance rates in HVKP were against sulphamethoxazole/trimethoprim (43.48%), followed by amoxicillin/clavulanate (32.61%) and

aztreonam (30.34%). The least resistance was against carbapenems (0%, for each) (Table 3).
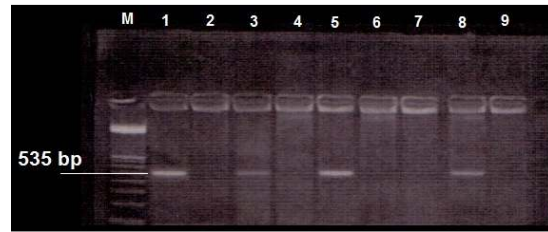

Fig. 1. Gel electrophoresis of the PCR products of *rmpA* gene
First lane, molecular weight marker; Gene Ruler 100bp DNA ladder, lanes numbered 1, 3, 5, 8 show 535 bp bands of PCR products, while lane numbers 2, 4, 6, 7, 9 show negative results

3.4 Antimicrobial Resistance Profile of *rmpA*-positive HVKP Isolates

Among 46 HVKP isolates, *rmpA*-positive isolates had the lesser resistance rates than *rmpA*-negative isolates. Only one isolate out of 26 *rmpA*-positive isolates produced ESBL (3.85%) and no one produced AmpC. While in *rmpA*-negative isolates of HVKP, 3 out of 20 isolates were ESBL producers (15%) and only one isolate produced AmpC (5%). Highest resistance rates in *rmpA*-positive isolates were against sulphamethoxazole/trimethoprim (23.08%), followed by aztreonam (19.23%) and ceftriaxone (15.38%). All *rmpA*-positive isolates were sensitive to amikacin, piperacillin/tazobactam, cefoxitin and carbapenems (Table 4).

Table 1. Distribution of *K. pneumoniae* isolates from different sites of infection

Clinical samples n=113	HVKP n=46	CKP n=67	*P value*
VAP (endotracheal aspirates)	23/46 (50%)*	21/67(31.34%)	.04
Post-operative wound infection (wound discharge)	4/46 (8.7%)	20/67 (29.85%)*	.002
UTI (urine)	11/46 (23.91%)	26/67 (38.81%)	.08
Bacteremia (blood)	8/46 (17.39%)*	0/67(0%)	.002

*HVKP, hypermucoviscous Klebsiella pneumoniae; CKP, classic Klebsiella pneumoniae; VAP, ventilator-associated pneumonia; UTI, urinary tract infection; *Statistically significant*

Table 2. Distribution of HVKP isolates from different sites of infection

Clinical samples n=46	*rmpA*-positive HVKP n=26	*rmpA*-negative HVKP n=20	*P* value
VAP (endotracheal aspirates)	17/23 (73.91%)*	6/23 (26.09%)	< .001
Post-operative wounds (wound discharge)	1/4 (25%)	3/4 (75%)	.49
UTI (urine)	0/11 (0%)	11/11 (100%)*	< .001
Bacteremia (blood)	8/8 (100%)*	0/8 (0%)	< .001

*HVKP, hypermucoviscous Klebsiella pneumoniae; CKP, classic Klebsiella pneumoniae; VAP, ventilator-associated pneumonia; UTI, urinary tract infection; * Statistically Significant*

Table 3. Antimicrobial resistance among *K. pneumoniae* isolates

Antimicrobial drugs	HVKP n=46	CKP n= 67	*P* value
Gentamicin	12/46 (26.09%)	33/67 (49.25%)*	.009
Amikacin	7/46 (15.22%)	22/67 (32.84%)*	.024
Amoxicillin/clavulanate	15/46 (32.61%)	46/67 (68.66%)*	< .001
Piperacillin/tazobactam	4/46 (8.7%)	15/67 (22.39%)*	.04
Cefoxitin	3/46 (6.52%)	19/67 (28.36%)*	.001
Cefotaxime	13/46 (28.26%)	38/67 (56.72%)*	.002
Ceftriaxone	13/46 (28.26%)	40/67 (59.7%)*	< .001
Ceftazidime	9/46 (19.66%)	33/67 (49.25%)*	< .001
Cefepime	4/46 (8.7%)	20/67 (29.95%)*	.002
Aztreonam	14/46 (30.34%)	47/67 (70.15%)*	< .001
Imipenem	0/46 (0%)	2/67 (2.99%)	.51
Meropenem	0/46 (0%)	4/67 (5.97%)	.14
Ciprofloxacin	11/46 (28.26%)	33/67 (49.25%)*	.004
Sulfa/trimethoprim	20/46 (43.48%)	58/67 (86.57%)*	< .001
ESBL only	4/46 (8.7%)	33/67 (49.25%)*	< .001
AmpC only	1/46 (2.17%)	15/67 (22.39%)*	< .001
ESBL+ AmpC	0/46 (0%)	4/67 (5.97%)	.14

*HVKP, hypermucoviscous Klebsiella pneumoniae; CKP, classic Klebsiella pneumoniae; ESBL, extended-spectrum beta-lactamase; *Statistically Significant*

Table 4. Antimicrobial resistance among HVKP isolates

Antimicrobial drugs	*rmpA*-positive HVKP n=26	*rmpA*-negative HVKP n=20	*P* value
Gentamicin	3/26 (11.54%)	9/20 (45%)*	.009
Amikacin	0/26 (0%)	7/20 (35%)*	.001
Amoxicillin/clavulanate	2/26 (7.69%)	13/20 (65%)*	< .001
Piperacillin/tazobactam	0/26 (0%)	4/20 (20%)*	.03
Cefoxitin	0/26 (0%)	3/20 (15%)	.07
Cefotaxime	2/26 (7.69%)	11/20 (55%)*	< .001
Ceftriaxone	4/26 (15.38%)	9/20 (45%)*	.03
Ceftazidime	2/26 (7.69%)	7/20 (35%)*	.02
Cefepime	0/26 (3.85%)	4/20 (20%)*	.03
Aztreonam	5/26 (19.23%)	9/20 (45%)	.06
Imipenem	0/26 (0%)	0/20 (0%)	-------
Meropenem	0/26 (0%)	0/20 (0%)	-------
Ciprofloxacin	2/26 (7.69%)	9/20 (45%)*	.002
Sulfa/trimethoprim	6/26 (23.08%)	14/20 (70%)*	< .001
ESBL only	1/26 (3.85%)	3/20 (15%)	.3
AmpC only	0/26 (0%)	1/20 (5%)	.44
ESBL+ AmpC	0/26 (0%)	0 (0%)	-------

*HVKP, hypermucoviscous Klebsiella pneumoniae; CKP, classic Klebsiella pneumoniae; ESBL, extended-spectrum beta-lactamase; *Statistically significant*

4. DISCUSSION

In the past 3 decades, HVKP has appeared as a cause of community-acquired invasive infections [30,31]. In this study, HVKP was isolated from hospital-acquired infections in a rate of 40.71% of all tested *K. pneumoniae* isolates. HVKP induced hospital-acquired infection has been reported by other studies in different rates; 37.93% [3], 28.57% [12], 15.38% [32] and 3.13% [33]. These variations in rates could be explained by many factors, including the geographical

distribution, the immunity status of the included patients, and the level of infection control measures practiced by different hospitals. This could not be explained by the difference in the sample types, because the similar samples yielded different rates of HVKP as denoted by two previous studies, in which both had used blood samples [32,33].

In the majority of studies, HVKP isolates were associated with highly invasive diseases, including,1ry liver abscess [34], peritonitis [35], necrotizing fasciitis [36] and endophthalmitis [37], but the low incidence of hospital-acquirement of such infections, rendered their involvement in our study difficult. Although HVKP is known to be a common blood isolate [2,30,31], few studies detected it in other samples [3,18]. The distribution of our HVKP isolates among different samples was as: 50% from endotracheal aspirates, 23.91% from urine, 17.39% from blood and 8.7% from wound discharge. Quite similar results were obtained by Wen-Liang et al; their HVKP isolates were mainly from sputum (65.62%), blood (26.04%), abscess pus (4.17%), urine (2.08%), wound discharge (0%) and others (2.09%) [18]. However, the type of infection in their study is not clear, whether community- or hospital-acquired. On the contrary in Li et al. study, the higher presentation of their hospital-acquired HVKP was in ascites and blood samples (28%, for each), followed by sputum and bile (14%, for each), then abscess discharge (7%) and lastly urine (0%) [3]. The lower presentation of blood HVKP isolates in our study, could be explained by the small size of blood samples to the whole samples (8/113). Across our study period, only 8 *K. pneumoniae* could be isolated from blood, though all of them were hypermucoviscous.

Besides hypermucoviscosity, a number of virulence factors was suggested to contribute to the pathogenesis of HVKP. To date, *rmpA* gene is the most frequently reported factor that has direct correlation with HVKP virulence [35,38-41]. *RmpA* gene (regulator of the mucoid phenotype) was documented to be the main factor that mediated the expression of the hypermucoviscous phenotype [17,42]. Although the mechanism by which it mediates mucoviscosity is still unrevealed, it is suggested to be through its binding to the regulatory 5' region of capsule genes, causing increase in the production of a capsular material [20,43]. Whether this increased material is the constitutive capsule [20,42], or it is an extracapsular polysaccharide, remains obscure [44].

In the current study, *rmpA* gene was detected in 26 (56.52%) out of 46 HVKP isolates. This agreed with the results obtained by previous studies [3,34], in which *rmpA* gene was detected in (55.17% and 50%, respectively) of their HVKP isolates. Higher rates were reported by other studies (93.75%) [18] and (88.89%) [38], but both studies were carried out on community-acquired isolates. While the tested isolates in the two former studies were collected from both community- and hospital-acquired infections. This could be explained by the data supporting that *rmpA* gene was more frequently detected in community-acquired HVKP, than those acquired from hospitals [35]. Wen-Liang et al. reported that hypermucoviscous isolates lacking *rmpA* exhibited low virulence as observed by mouse lethality [18]. Although HVKP was documented to be strongly associated with *rmpA* gene, our HVKP isolates that did not carry *rmpA* gene may reflect the presumption that other undiscovered regulatory genes are more related to the expression of the hypermucoviscosity phenotype [35].

Among all clinical infections included in this study, *rmpA* gene was strongly related to bacteremia and VAP (P < .001, for each). Lower incidence of *rmpA* gene was detected in post-operative wound infection, although this was not statistically significant (P = .49). While, it was completely absent in UTI. In accordance with a number of previous reports [32-34], *rmpA* gene was observed in 100% of bacteremic isolates. It was detected in 73.91% of our VAP isolates, whereas Yan et al. found it in 100% of their HVKP isolates causing VAP [12]. Also, it was detected in 25% of our post-operative wound isolates. On the contrary, Chang et al. could not detect this gene in any of their wound isolates [34]. The complete absence of the gene in our urine isolates was agreed with other studies [18,34]. Unfortunately, only little data were provided about the prevalence of *rmpA* gene in HVKP isolated from non-blood samples. More studies would be helpful to clarify the association between *rmpA* gene and HVKP related clinical infections.

When we compared the drug resistance rates between HVKP and CKP, we noticed that HVKP isolates were significantly less resistant than CKP to most (11 of 14) antimicrobial agents tested. Whereas resistance to piperacillin/

tazobactam, imipenem and meropenem was nearly equal between the two *K. pneumoniae* variants. Low resistance rates in HVKP were reported by previous studies [3,12,32,33].

In this study, ESBL-production was detected in 8.7% of our HVKP isolates. This rate was quite similar to that detected by Liu et al. (9.09%) [33], Yan et al. (7.1%) [12] and Wen-Liang et al. (5.21%) [18]. Moreover, Li et al. reported an increasing in ESBL production among the HVKP isolates over time [3]. This differed from the rate reported by Jung et al. (0%) [32]. This complete absence of their ESBL producing isolates may be due to their relatively low number of HVKP isolates tested (14 isolates). AmpC was produced by 2.17% of our HVKP isolates. None of HVKP isolates produced both ESBL and AmpC together. Also, none of our HVKP isolates was resistance to carbapenems, that agreed with other reports [3,12,32,33]. On the contrary, carbapenem resistance has emerged in HVKP in China in a rate of (17.86%), they suppose that their HVKP may carry carbapenem-resistance genes [45].

Concerning the association between *rmpA* gene and the antimicrobial resistance, *rmpA*-positive isolates were significantly less resistance to all drugs except for cefoxitin and carbapenems. ESBL was produced by 3.85% of *rmpA*-positive isolates, while AmpC was not produced by any of these isolates, with no significant association between either ESBL or AmpC production and the *rmpA* gene existence. We could find only one study that had investigated this association [18]. In their study, ESBL was produced by 4 out of 95 *rmpA*-positive HVKP isolates (4.21%). Therefore, further studies are required to confirm this finding.

Actually, there is no clear explanation of the lower resistance rates associated with *rmpA*-positive HVKP. It could be explained by the fact that in virulent isolates carrying large plasmids, another resistance carrying plasmids could not maintain stability [46,47], or that drug resistance genes might be lost when these isolates had acquired virulence genes [3]. Another explanation is that, ESBL producing isolates may reduce the genetic function of *rmpA* when they need to amplify ESBL genes under the antibiotic selective pressure [18].

5. CONCLUSION

K. pneumoniae is one of the causes of hospital-acquired infectionI in Tanta University Hospital

and HVKP accounted for a significant proportion among *K. pneumoniae* isolates. The emergence of such virulent strains is not the only fear, but also acquiring antimicrobial resistance which leads to the emergence of hypervirulent pandrug resistant variant of *K. pneumoniae*. Employment of an objective diagnostic test in the clinical microbiology laboratory for rapid and simple identification of HVKP strains should be taken into consideration to detect these strains.

ETHICAL APPROVAL

This study was approved by the local ethical committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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