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Antibiotic Resistance, Microbial and Morphological Changes of Marketed Bovine Liver at Different Time Interval from Chittagong, Bangladesh: A Public Health Concern

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ABSTRACT

The liver of cattle is commonly eaten as a delicious food item in Bangladesh. A cross-sectional study was conducted to estimate the microbiological and morphological (gross and microscopic) change in liver samples in relation to time elapse in retail meat shops in Chittagong, Bangladesh. Liver samples (N=25) from three beef market (Chittagong Sadar, Jawtola and Pahartali) were collected randomly. For each sample, liver was collected in 6 hours interval [(morning, n=25), (noon, n=25 and afternoon, n=25)] and analyzed by bacteriological and anatomical technique, followed by antimicrobial susceptibility testing (disc diffusion method) during the period from December 2015 to April 2016. The mean total viable count (TVC/g) of morning samples were 27.804 ± 4.34 × 10^6 (CI: 18.84-36.76), in noon 170.032 ± 18.45 × 10^6 (CI: 131.96-208.11) and in afternoon sample 70.4796 ± 13.21 × 10^6 (CI: 43.21-97.75). Gross changes in color of liver; morning sample highest prevalence's was reddish brown (52%), in noon dark red (56%); and in afternoon highly dark red (80%); (P=0.001). This color changes indicates the reduction of shelf life of bovine liver as well as deterioration of consumption quality. Histologically; distortion of central vein in morning, noon, and afternoon samples were 12.5%, 25% and 62.25%, respectively. Changes of hepatic lobule and portal triad were indistinct at afternoon (75%) in comparison to morning (0%); (P=0.001). Moreover, complete degeneration of cellular arrangement in liver was observed at afternoon. Pooled bacteria cultured from direct liver smear were found 100% resistant to Tetracycline, Nalidixic acid, and Oxacillin, followed by Erythromycin (53.33%), Ciprofloxacin (40%), Doxycyclin (26.66%) and Ampicillin (6.67%). The results of this investigation revealed that liver from retail meat shops of Chittagong had noticeable extent of multidrug resistance high microbial contamination with considerable degree of morphological changes in advanced of time, that could be threaten to public health.

INTRODUCTION

Liver is the largest visceral organ in animal and considerably used as food in various countries of the world. Like meat, liver are sold in the meat shop preferably in open and hanging condition to attract the customers. There is bulk chance of contamination of liver from the time of post slaughter evisceration to consumption. This condition would exaggerate in elapse of time due to decomposition of hepatic tissue therefore, degradation of food value together with high microbial load. Now a day, it is known that microbial contamination of beef shop (meat) has been considered very important in sense of public health [1] and

thought that environmental and workers pathogens are a potential source of microbiological contamination in meat products [2], but there is a gap of information regarding marketed liver.

Liver comprises a substantial source of high-quality protein, vitamins and minerals. In Bangladesh, the major retail outlets of meat here are the butcher's shops. Majority of butchers of Bangladesh slaughter cattle in open place with poor hygienic condition which provoke microbial contamination and early spoilage [3]. Likewise, liver could be contaminated by both pathogenic and non-pathogenic organisms which have both economic and zoonotic importance. Liver inspection at meat shop for hygienic quality, involves both ante and postmortem examination which include gross and microbiological (e.g., total viable count) investigation [4]. Liver contain high amounts of fat and iron, and therefore, oxidative deterioration of liver during selling is expected due to long time storage or unsold. This oxidative degeneration may causes histopathological changes of normal liver texture and also marked deterioration of consumption quality of liver.

Antibiotic resistance is an emerging challenge in all over the world and has become a very serious problem in the treatment of disease. The monetary cost of treating antibiotic resistant infections worldwide is estimated to be many billions of dollars per year [5]. Some experts predict that, as resistance to antibiotics is increasing at a faster pace than it can be controlled, the future will resemble the pre-antibiotic era. Thus, possible contamination of liver by multi drug resistance bacteria can eventually affect an entire community.

Considering all the above facts, the present study was conducted to determine the microbiological and histopathological status of marketed liver and there antibiotic resistance pattern in relation to selling time interval in between morning, noon and afternoon in retail meat shops.

MATERIALS and METHODS

Study area and sampling design

A cross sectional study was conducted during from the period of December 2015 to April 2016 at three popular meat markets of Chittagong district namely Chittagong Sadar, Pahartali and Jawtola. Randomly collected livers sample of cattle (N=25) of different ages and sexes were taken three times a day (Morning, Noon and afternoon) from the selected market; i.e., the samples from the same liver were collected three times of a day to determine the TVC.

Sample collection

During collection, each respective liver samples were divided into two parts, one part was collected in plastic bags and transferred in an ice box to the laboratory for bacteriological examination (Total viable count), and the second one was immersed in 10% formalin for histopathological examination.

Bacteriological examination (Total viable count)

Just after arrival at laboratory the surface of the liver tissue was sterilized by a hot spatula, incised with a sterile scalpel. Following this, a series of test tubes (n=5), each containing of 9 ml diluents was taken. Then fifty gram/ml food sample was homogenized in 450 ml diluents and making suspension in a beaker. From the original sample, 1 ml was transferred in the test tube no.1 and mixed thoroughly. Transferred 1 ml from 1st test tube to 2nd test tube and continue up to last one and 1 ml was discarded from the last test tube. From each tube 3 Petri dishes was taken containing Plate Count Agar (PCA) media. Then 0.5 ml mixture from each of the test tube was transferred to the corresponding Petri dish separately. Tips of the test tube touched gently to the media and diluted samples was spreaded over the surface of the media using glass spreader or sterilized swab stick. Then the Petri dished was marked (sample no, date etc.) and kept in incubator in inverted position at 37°C for 2/3 days. After 1 day interval up to 3 days after incubation the colonies was observed. In which plate colony counted are 30-300 only this plate was included and others plate was discarded. The three Petri dish colony of each tube was counted and made average to them.

Gross assessment of liver sample

During sample collection at different time interval, each respective gross color of liver sample was recorded by self-evaluation. For estimation of color change there are five different color of liver was recorded like, reddish brown, dark red, highly dark red, yellowish red and white spot.

Histopathological examination

A total of 15 livers [Morning (n=5), Noon (n=5) and Afternoon (n=5) samples] of slaughtered cattle were examined for histopathological changes that may occur due to selling time interval or oxidation of fatty material in liver and microbial contamination or decomposition of liver. Specimens from collected livers were preserved in 10% formalin. After proper fixation, histopathological slides were prepared and stained with hematoxylin and eosin stain for general microscopic examination as described by Bancroft and Gamble [6]. Histopathological study was done in the Department of Anatomy and Histology of Chittagong Veterinary and Animal Sciences University (CVASU).

Antimicrobial sensitivity test

Antimicrobial susceptibility testing of isolates was performed using disk diffusion method according to Clinical Laboratory Standards Institute [7]. The following 13 antimicrobials were tested at the given disc content: Ampicillin (10 µg), Ciprofloxacin (5 µg), Colistin (10 µg), Doxycycline (30 µg), Gentamicin (30 µg), Nalidixic acid (30 µg), Neomycin (30 µg), Tetracycline (30 µg), Erythromycin (15 µg), Oxacillin (5 µg), Kanamycin (30 µg), Amoxicillin (10 µg) and Cefalexin (30 µg), (In accordance with the WHO requirement). The results of susceptibility testing were interpreted according to the criteria described by Wikler [8]. The zones of inhibition were interpreted as susceptible, intermediate and resistant according to the recommendations of the CLSI [7]. An isolate was defined as 'multiple-resistant' if it displayed resistance to ≥3 different classes of antimicrobial [9].

Data analysis

Obtained laboratory data was stored in Microsoft Excell-2007 and imported to the software STAT/IC-11.0 for analysis. Descriptive statistical analysis was done to measure the mean, SE, 95% confidence interval (CI) and p-value of different parameters. The arithmetic means (± SE) of total viable count parameters in different groups (morning, noon and afternoon) were calculated using Paired T-test.

RESULTS

Comparison of number of viable bacteria (in million) in bovine liver at different time interval

The liver samples at noon contained the highest number of bacteria (170.03 ± 18.45 million) in comparison to morning (27.804 ± 4.34 million) and afternoon (70.5 ± 13.2 million) per gram of samples **(Table 1)**. Difference of microbial load at morning, noon and afternoon in the liver samples was statistically significant (P=0.000). The proportions of minimum microbial contamination of liver sample in morning, noon and afternoon is 2.5, 0.7, and 0.19 million respectively. And the proportions of maximum microbial contamination of liver sample in morning, noon and afternoon is 80, 310, and 194 million respectively which is statistically highly significant (P=0.001) **(Table 1)**.

The microbial load in liver sample was compared between three beef markets in relation to time interval. In morning sample, the highest bacterial load recorded in Jawtola market was 194 million, 70 million at Chittagong Sadar and 45 million at Pahartali market. Likewise, in noon maximum bacterial load revealed in Jawtola market was 310 million, whereas another two markets contains 270 million TVC respectively. Also in afternoon the proportion of highest bacterial load was recorded in Jawtola market (80 million) in comparison to Chittagong Sadar (190 million) and Pahartali market (156 million). It clearly indicate that in comparison to Sadar and Pahartali beef markets, the Jawtola beef market assessed poor hygienic condition as well as high microbial load in collected sample out of three beef markets.

Table 1. Comparison of number of viable bacteria (in million) in bovine liver at different time interval.

Variable	N	Mean ± SE	95% CI	Std. Dev.	Minimum	Maximum
Morning	25	27.81 ± 4.34	18.84-36.76	21.70	2.5	80
Noon	25	170.03 ± 18.45	131.96-208.11	92.24	0.7	310
After noon	25	70.48 ± 13.21	43.21-97.75	66.06	0.19	194
P–value	0.000					

Alteration of liver color

The present study was also conducted to assess the alteration of color of liver **(Figure 1)** due to long interval of selling time and oxidation of liver fat or microbial contamination. In morning four different colors of liver was recorded. Out of four colors in morning sample highest prevalence's of liver color subjectively assessed is reddish brown 52% (13 out of 25 samples) and dark red, yellowish red, white spot color were 16%. In noon sample highest prevalence's are dark red 56% (14 out of 25 samples) and in afternoon sample highest prevalence's are highly dark red 80% (20 out of 25 samples). The alteration of liver color was markedly denoted in afternoon 3.16 ± 0.15 in comparison to the morning 2.28 ± 0.32 and noon 2.4 ± 0.13 Samples. The proportions of color change in the morning and noon samples have no statistical significance (P=0.700), but in comparison to morning and noon samples the proportions of color change in afternoon sample have statistically significantly different (P=0.000). Overall frequency of color change in morning, noon and afternoon samples of retail meat shops were statistically significant (P=0.001) and it indicates marked alteration of shelf life of bovine liver.

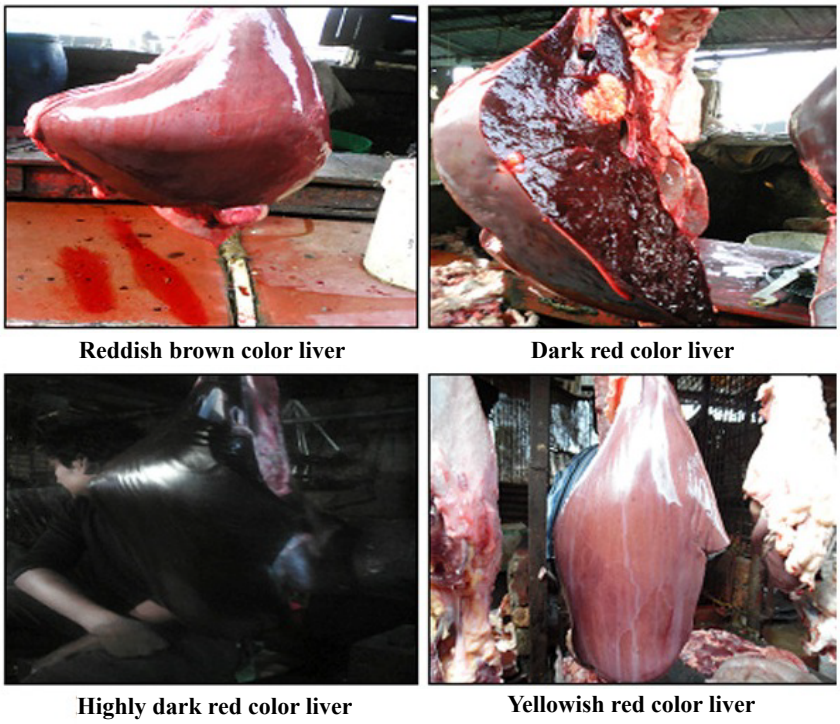

Figure 1. Gross characteristics of marketed cattle liver at different time interval showing different color changes due to change in microbial population and cellular degeneration.

Antimicrobial sensitivity test result

A total of 15 individual samples were randomly selected from three different markets in three different time interval (morning, noon and afternoon) to represent the antimicrobial resistance pattern of contaminated liver bacteria. Among the 15 samples, all were sensitive to Colistin sulfate, Kanamycin, Neomycin and all were resistant to Tetracycline, Nalidixic acid and Oxacillin. Antibiotic susceptibility pattern and prevalence of antimicrobial resistance of environmental organisms from samples of retail meat shop in three different beef market has been outlined in **Table 2**. Intermediate sensitivity was only found to seven antibiotics (Cefalexin Erythromycin, Ampicillin, Amoxicillin, Gentamicin, Doxycyclin, and Ciprofloxacin). Specially, Cefalexin and Gentamicin were close to 100% sensitive **(Table 2)**. All of the bacteria showed multiple drug resistance (up to against 7 antibiotics out of 13 used in the test).

Table 2. Prevalence of antimicrobial resistance pattern against environmental organism.

Antibiotics	Pattern			
	Total	Resistance (%)	Intermediate (%)	Sensitive (%)
Tetracycline	15	100	0	0
Nalidixic acid	15	100	0	0
Colistin sulfate	15	0	0	100
Cefalexin	15	0	6.67	93.33
Erythromycin	15	53.33	40	6.67
Ampicillin	15	6.67	86.67	6.67
Oxacillin	15	100	0	0
Kanamycin	15	0	0	100
Amoxicillin	15	0	73.34	26.66
Gentamicin	15	0	6.67	93.33
Neomycin	15	0	0	100
Doxycyclin	15	26.66	73.34	0
Ciprofloxacin	15	40	40	20

Histopathological changes of liver in relation to time interval

A total of 24 individual sample was randomly selected from three different markets in different time elapse (morning, noon and afternoon) to represent the histological changes of liver that occur in relation to time interval after slaughtering.

In case of central vein of liver, there was highest distorted central vein in afternoon samples (62.5%) and lowest in morning samples (12.5%). About 87.5% central vein were intact in the morning samples whereas only 37.5% was intact in the afternoon samples **(Table 3 and Figure 2)**. Regarding hepatic lobule, there was highest well defined hepatic lobule was 87.5% in morning but in afternoon there was no well-defined hepatic lobule. Moderately distinct hepatic lobule was markedly recorded in noon (62.5%) and totally indistinct hepatic lobule was highest recorded in afternoon (75%) **(Table 3)** and mentioned as **Figure 2**. Another variable in case of portal triad, highest well defined portal triad was recorded in morning (87.5%) but in afternoon there was no well-defined portal triad. Moderately distinct portal triad was markedly recoded in noon and afternoon (25%) but totally indistinct portal triad was highest recorded in afternoon (75%) as stated in **Table 3** and demonstrated in **Figure 2**. In case of alteration of cellular arrangement, there was highest well defined cellular arrangement (87.5%) in morning but in afternoon there was no well-defined cellular arrangement. Complete degeneration of cellular arrangement markedly denoted in afternoon **(Table 3)** and demonstrated in **Figure 2**.

Table 3. Histopathological alteration of hepatic lobule, portal triad and cellular arrangement of marketed liver samples at different time intervals.

Variable	Time Interval	N	Well define		Moderately distinct		Totally indistinct	
			Freq.	%	Freq.	%	Freq.	%
Hepatic lobule	Morning	8	7	87.5	1	12.5	0	0
	Noon	8	2	25	5	62.5	1	12.5
	Afternoon	8	0	0	2	25	6	75
Portal triad	Morning	8	7	87.5	1	12.5	0	0
	Noon	8	5	62.5	2	25	1	12.5
	Afternoon	8	0	0	2	25	6	75
Cellular arrangement	Morning	8	7	87.5	1	12.5	0	0
	Noon	8	1	12.5	6	75	1	12.5
	Afternoon	8	0	0	0	0	8	100
Central vein (Intact/ distorted)	Morning	8	7	87.5	1	12.5	8	100
	Noon	8	6	75	2	25	8	100
	Afternoon	8	3	37.5	5	62.5	8	100

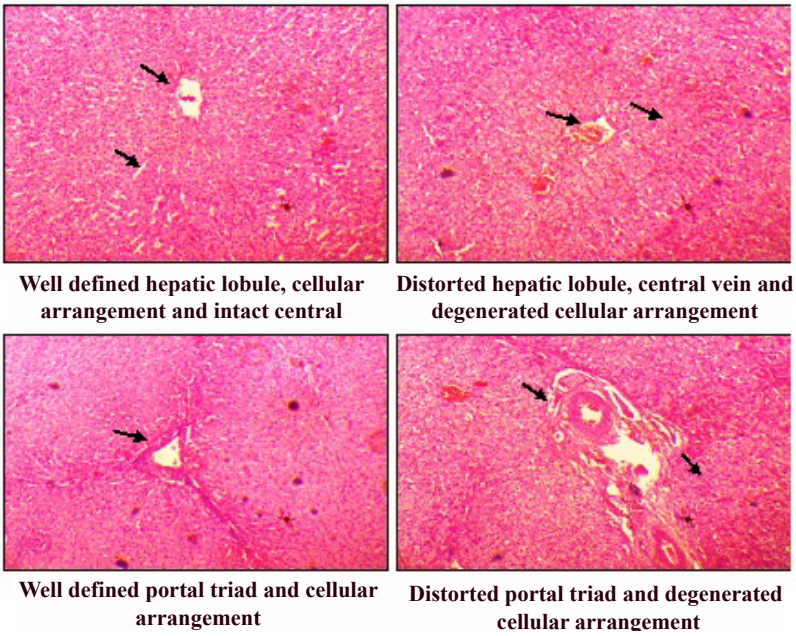

Figure 2. Histopathological changes of marketed cattle liver at different time interval.

DISCUSSION

There are two government registered abattoir in Chittagong, here every day slaughter large number of cattle and buffaloes, but excluding these, others are not scientific or not modern abattoir. After slaughtering of cattle all edible part of carcass send in local beef market like Jawtola, Pahartali, and Chittagong Sadar etc. Here, all the beef and edible byproducts like liver are hanging throughout the day for selling. A great majority of consumers buy meat from butcher's shops at which food hygiene and safety

conditions are not assured. Because, this edible carcass contaminated by environmental or airborne bacteria due to unhygienic handling, transportation and dusty environment of beef market. During handling of liver, this is also contaminated by abattoir instruments and butchers [1]. However, some of the livers are seems to be contaminated by bacteria causing septicemia. On the other hand, risk include disease affected animal treated with antibiotics but animal is not cure properly and does not maintain full antibiotic course or dug withdrawal period disseminate the burden of drug resistance.

Results of investigations in beef market samples do provide an estimate of the prevalence of microbial load in retail meat shops. The high level of microbial load in the liver indicated that contamination did accumulate throughout a day and reached peak levels at the noon due to unhygienic handling and hanging of liver in dusty environment of open market [10]. Favorable ambient temperature enhances rapid multiplication of bacteria and resulting responsible for high microbial load [4]. In our study, we speculate that high microbial load in noon may be due to rapid multiplication of bacteria at noon time.

Total viable mean counts (log10 CFU) of liver ranged from 2.5 to 80 million for morning sample, from 0.7 to 310 million for noon samples and from 0.19 to 194 million for afternoon samples. Such levels are higher than those detected by Bolton et al. [11] in a small scale slaughterhouse registered level accounting between 4.5 and 4.7 log. In Switzerland, Zweifel et al. [12] obtained TVC mean counts that averaged out at 3.3 log. The proportions of total viable count in retail meat shops higher in the noon sample compared to those in the morning and afternoon sample (P=0.001) reflects the spread of contamination throughout a day up to selling within the shops.

A higher prevalence of TVC was found in Jawtola beef market with subjectively assessed poor hygiene compared to those with Sadar and Pahartali market (P=0.001). A higher prevalence of TVC in open shops might be due to easy access of flies and dust compared to closed shops [1]. The widespread contamination of the different samples in retail meat shops demonstrates that the shops create ample opportunities for the entry and spread of contaminations. Bacteria could be present in environment, animal feed, offal and vegetables [13,14]. In general guideline, the microbial load or TVC/g for liver that is acceptable for human consumption categorized into 4 grade like Excellent (<1000), Good (1000-10000), Acceptable (10000-100000), Marginal (action required) (100000-1000000) [15]. But in our study high proportion of sample TVC count cross the “Marginal grade” that indicate this product is not suitable for human consumption as it may create potential public health hazard. So, necessary action should be taken against all the meat shop.

Shelf-life evaluation was based on color, microbial counts, and sensory assessment of odor and color. Samples keep under air exposure showed the highest lipid and hemo-pigment oxidation rate, resulting alteration of color. The present study also identified that significant color alteration (p=0.001) from morning to afternoon which is similar to a previous study by Fernández-López et al. [16]. Cell damage can occur as a result of long time storage which disrupts the normal texture and arrangement of cell. In our study, in afternoon sample found marked alteration of cellular arrangements. Alison and Sarraf [17] stated, due to anaerobic glycolysis and enzymatic process damage may occur in liver lobular structure and hepatic cellular arrangements. Additionally, decomposition of bovine liver may also due to anaerobic bacteria residing in the liver.

In our study there was no isolation of any specific bacteria for AMR test, because we assume public come in contact with diverse bacterial population present in each liver sample. Multiple antimicrobial resistances might happened in bovine liver due to indiscriminate use of antibiotics, chemotherapeutics and or disperse of drug resistant microorganism in the environment [2,13,14]. Resistances that observed against Tetracycline was closed with Islam et al. [18], they showed 96.6% resistance to Tetracycline of *Escherichia coli* isolated from poultry farm at Chittagong District in Bangladesh. But in our study Tetracycline found 100% resistant against nonspecific pooled bacterial samples. The resistance of non-specific bacteria against Doxycycline was 27% which agreed with Raum et al. [19] who stated 29–58% resistance of *E. coli* to Doxycycline isolated from stool sample in a study in Germany. Nalidixic acid susceptibility was a good marker for fluoroquinolones susceptibility but NA resistance had a poor predictive value for ciprofloxacin resistance [20]. In previous study nalidixic acid was (71.4%) resistant toward the environmental organisms it is not closely similar with our study because 100% are resistant in our result. *E. coli* isolates were found resistant (100%) to Ciprofloxacin which is higher than the earlier report [21] but in our present study revealed that ciprofloxacin are 40% sensitive 40% intermediate sensitive toward the nonspecific environmental organism. Resistance that was observed to Kanamycin (69.24%) agreed with Akond et al. [22] in a study on chicken collected from different poultry markets of Dhaka, Bangladesh (76%).

It was revealed that 100% sensitive to Colistin and this finding have similarity with Catchpole et al. [23] who observed Colistin is active against most strains of *E. coli* in a study on reassessment of the *in vitro* activity of Colistin sulfate. Schroeder et al. [24] observed O100: H–STEC strains isolated from healthy slaughter pigs were resistant to neomycin. In this study it was observed that all the isolates were sensitive to gentamicin and neomycin this finding is in agreement with Alam et al. [25] who reported that most of the environmental strains were (97%) sensitive to Gentamicin.

CONCLUSION

Mean levels total viable count of retail meat shop liver samples appeared to be critical. The high contamination levels reflect an inadequate application of hygienic practices. Histopathological and color changes of liver seems to be linked with oxidative

processes and high microbial counts or load. Therefore, before purchase it is very important to inspect the morphology (gross change) in liver as possible. We would also like to suggest the buyers' try to purchase the liver preferably in the morning. Our study emphasizes the need to monitor total viable count of liver from slaughtering to selling and find out antibiotic resistance of contaminating bacteria as well as histopathological changes of liver in relation to time interval. However, control measures with better hygienic practices at the meat shops are suggesting reducing the risk of contamination of liver.

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AUTHORS' CONTRIBUTIONS

EAR and MA conducted the research and actively prepared the manuscript. MA designed the work and provided advice during the research work. MSB and MA participated in the manuscript preparation and data analysis. All the authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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