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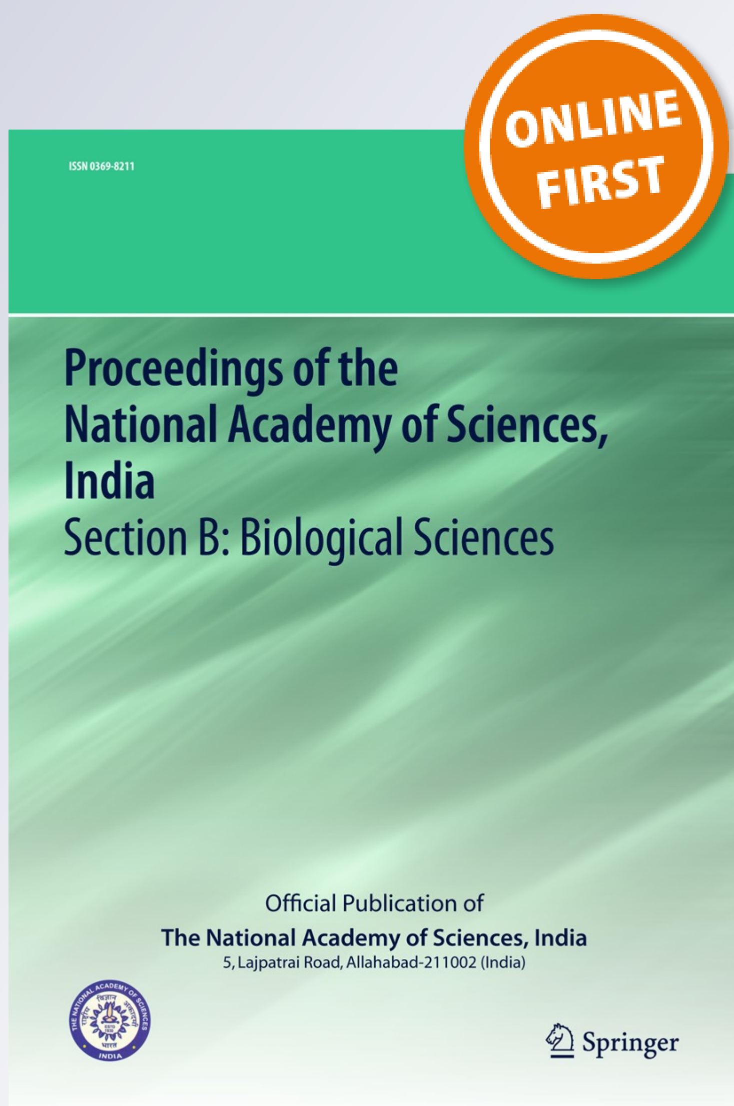

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RESEARCH ARTICLE

A Study on the Occurrence of Non-O157 Shiga Toxin Producing *Escherichia coli* Isolates in Retail Chicken Meats Marketed in North-East India

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Abstract North-eastern region of India is inhabited by different ethnic tribes devoted to livestock farming and agriculture. In recent years, tourism has had a significant development with increase in tourist destinations and holiday resorts resulting in increasing demand for supply of meat in the local market. Chicken meat is one of the predominantly consumed animal originated food item with constant increase in demand, usually met by the local retail markets. To assess the microbiological quality and safety of the retail chicken meat sold across the markets of North-East India, a study was undertaken to survey the prevalence of shiga toxin-producing *Escherichia coli* (STEC). Although there was no *E. coli* O157 recovered from the retail samples, 22 (11.5 %) of the isolates obtained from different samples were STEC positive and 11 (50 %) of the STEC isolates carried the *eae* factor but none of them harbored the EHEC-*hly*A gene. None of the STEC isolates showed s*tx*2 gene amplification. The counts of *E. coli* per g in the STEC positive samples exceeded or were close to the limit recommended by the Meat Standard Committee (Australian Standards 2002) and Microbial Food Safety–Indian Regulations. The higher counts observed were attributed to high temperature, poor hygienic practices and fecal contamination of the dressed meat. The present study suggests that retail meat is potential vehicle for transmitting food-borne diseases in the region and there is an urgent need for increased implementation of hazard analysis of critical control point and consumer food safety education programme.

Keywords Retail chicken meat · *E. coli* O157 · Shiga toxin-producing *E. coli* · Food-borne contaminants

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Introduction

Escherichia coli is an important component of the intestinal microflora of humans and other warm-blooded mammals. While *E. coli* typically harmlessly colonizes the intestinal tract, many *E. coli* clones have evolved the ability to cause a variety of diseases within the intestinal tract and elsewhere in the host [1]. Those strains that cause enteric infections are generally called diarrheagenic *E. coli*, and their pathogenicity is associated with a number of virulence attributes, which vary according to pathotype [2]. Shiga toxin producing *E. coli* (STEC) is the focus of immense international research effort driven by its recognition as a major cause of large scale epidemics and thousands of sporadic cases of gastrointestinal illness [3]. STEC carry one or more genes encoding genetically related types of cytotoxins, called verotoxins or Shiga toxins (*Stx)*, which are encoded by lysogenic bacteriophages [4, 5]. The ability of STEC strains to cause severe disease is related to the production of one or more Shiga toxins (*stx*1, *stx*2, and variants of *stx*2) [6, 7]. In addition to toxin production, some strains harbor other virulence factors associated with attaching and effacing lesions, such as intimin [8]. Another factor is an enterohemolysin, encoded by EHEC-*hly*A gene [9, 10]. STEC causes a broad spectrum of human illnesses ranging from uncomplicated diarrhea to hemorrhagic colitis (HC) and hemolytic–uremic syndrome (HUS) [11].

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In adult animals, STEC usually are not pathogenic, although some strains cause diarrhea in calves and others cause edema disease in pigs [12, 13]. STEC are normally not present in the feces of healthy humans. However, some STEC originating from healthy animals can behave as human pathogens and cause serious illness in humans [4, 14].

Worldwide, the presence of zoonotic agents such as STEC in food items is a major concern. Their persistence in soil and water combined with agricultural and farming practices indicate the recent increase of STEC outbreaks linked to environmental pathways [15]. Acute diarrheal diseases have been recognized as one of the major causes of mortality and morbidity in developing and underdeveloped countries. The common bacterial pathogens associated with diarrhea are mainly enteric pathogens harbored in the intestine of most of the animals as their reservoir [16, 17]. The nature and prevalence of these microbes would be highly dependent on the processing, storage and retail of meat and meat products.

During the past decade, STEC has evolved from a clinical novelty to a global public health concern. STEC infections have been reported from over 30 countries on six continents, causing a spectrum of human illness ranging from symptom-free carriage to severe bloody diarrhea and even life threatening sequel such as HUS [18]. However, there are very few reports on STEC in India. Wani et al. [19] and Kiranmayi et al. [20] reported the presence of STEC from mainland India while for North-East there is only one report on STEC prevalence [17].

North-East India (21°59′–28°12′N and 89°54′–97°15′E) is a major tourist destination with high rural settings largely inhabited by ethnic tribes practicing livestock farming and agriculture. Traditionally, the region experiences high consumption of meat and meat products processed using indigenous methods. The major meat types consumed by the tribal people of the region include pork, beef, chicken and mutton. However, with the increasing inflow of tourist and their preferential selection of chicken meat in the urban settings has resulted in its higher consumption. Microbial testing of meat samples is an important component of monitoring potential biotic hazards. Understanding the quantum of consumption of locally processed meat, the present study was aimed to screen the pathogenic microflora and to assess the prevalence of *E. coli* especially the STEC strains in the chicken meat sold in the retail markets. Based on the available literature, the present study is the first of its kind on the prevalence of *E. coli* STEC strains in retail market chicken meat of the region.

Material and Methods

Sample Collection and Processing

A total of 336 chicken meat samples comprising meat portions from various body parts of chicken viz. thigh, breasts, wings, tail, gizzard and skin were collected manually over a period of 42 months from December 2007 to June 2011 from retail markets of Shillong, Jowai, Tura, Guwahati, Tezpur, Dibrugarh, Aizawl, Lunglei, Bairabi, Kohima, Dimapur, Mon, Itanagar, Pasighat, Aalong, Agartala, Melaghar, Kallashahar, Imphal, Bishnupur, Ukhrul spreading over seven states of North-East India. The samples were collected in sterile containers and kept at 4 °C until processed within 12 h of collection.

Enumeration of Total Aerobic Mesophilic Heterotrophic Bacteria

1 g of meat sample was stomached (230 rpm) with 10 ml of 0.85 % NaCl and 1 ml each of decimal dilutions were transferred to sterile petri dishes. 20 ml of plate count agar (PCA, Himedia) medium melted and cooled at 45 °C was poured into the petri dishes. The plates were incubated at 30 °C for 72 h. All the colonies were counted for aerobic mesophilic count (AMC) and the result expressed as colony forming units (CFU) per gram sample.

Enumeration of Total Coliforms and *E. coli*

For total coliform count (TCC), 1 g of the sample was inoculated in 10 ml of buffered peptone water and macerated using a stomacher [21]. The samples were incubated aerobically at 35 °C overnight. For TCC, 1 ml of pre-enriched sample was plated into the violet red brilliant green agar (VRBGA) and incubated at 35 °C for 24 h and the number of dark red colonies was recorded. For *E. coli* enumeration, samples were enriched in EC broth and plated on Chromobrit agar and incubated at 44 °C for 24 h and the number of colonies with a blue color (where 5-bromine-4 chlorine-3 indolil ß-d-glucuronide is hydrolyzed by ß-glucuronidase) and indole positive reaction were recorded. The results were expressed as CFU/g for both coliform and *E. coli* count.

Detection of *E. coli* O157

A selective enrichment medium was used to screen for *E. coli* O157. A homogenate of 1 g of meat sample was prepared in 1 % buffered peptone containing cefixime (0.05 mg/l), cefsulodin (10 mg/l) and vancomycin (8 mg/l) and incubated at 37 °C for 24 h, followed by subculture on to Cefixime-Tellurite Sorbitol MacConkey's Agar (CT-SMAC) [22, 23].

Isolation of non-O157 *E. coli*

10 g samples were incubated in 90 ml *E. coli* broth at 43 °C for 18 h. After the enrichment, isolations were made

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on MacConkey agar (Himedia, India) and Eosin-Methylene Blue agar (EMB agar, Himedia, India) plates incubated at 37 °C for 24 h. Presumptive *E. coli* colonies were re-isolated in PCA and incubated at 37 °C for 24 h. Biochemical characterization of the isolates was carried out using the API 20E identification system (bioMérieux, Lyon, France).

Molecular Characterization of Non-O157 *E. coli*

The isolates, characterized as non-O157 *E. coli* were subjected to DNA extraction with the HiPurA™ Bacterial Genomic DNA Purification kit (Himedia, India). For PCR amplifications of *stx*1/*stx*2 genes, the primers described by Xia et al. [1] were used (Table 1). The PCR program used was set at 94 °C for 5 min, and 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. After the last cycle, the PCR tubes were incubated at 72 °C for 10 min. The *E. coli* isolates that were positive for *stx* genotype were further tested for *eae* and EHEC-*Hly* genes with the PCR conditions and primers as described by Xia et al. [1]. Reaction products were analyzed by agarose gel electrophoresis and visualized by UV trans-illumination after ethidium bromide staining.

Statistical Analysis

The results of colony forming unit including aerobic mesophilic counts, total coliform count and *E. coli* count were reported as mean values ± standard error. Prevalence of *E. coli* and STEC positive samples at different seasons were compared by Chi-square test and Fisher's exact test. A value of $P < 0.05$ was considered statistically significant.

Results and Discussion

In the present study, 190 non-O157 *E. coli* isolates were identified and confirmed by biochemical tests. Skin and tail samples were more frequently contaminated as compared to thigh, breasts, wings, gizzard portions of the meat samples. There was considerable variation in the average total counts of bacteriological hygienic indicators during different seasons with highest in summer followed by spring, autumn and winter season (Fig. 1). Aerobic plate count is a widely accepted measure of the general degree of microbial contamination and the hygienic conditions of processing plants. Highest STEC percentage was observed during the summer season with 11 out of 93 samples (11.8 %) showing STEC positives. The least STEC were observed in winter season with 2 out of 88 samples (2.3 %) showing STEC positives. Sample wise prevalence of

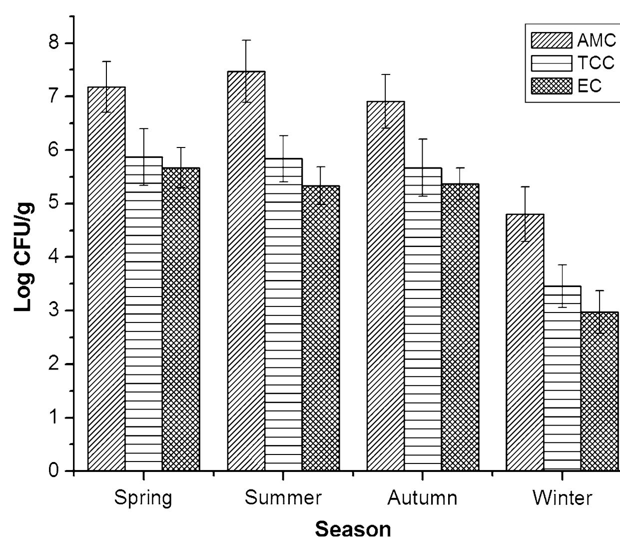

Fig. 1 Average bacterial indicator count at different sampling seasons. (AMC-Total Aerobic Mesophilic Count (CFU/g), TCC-Total Coliform Count (CFU/g), and EC-*E. coli* Count (CFU/g))

Table 2 Prevalence of *E. coli* and STEC positive samples in different seasons

Season	Number of isolates (%)*	
	E. coli	STEC
Spring	83.33 (70/84)	5.95 (5/84)
Summer	65.59 (61/93)	11.82 (11/93)
Autumn	49.29 (35/71)	5.63 (4/71)
Winter	2.27 (2/88)	2.27 (2/88)

* Differences in the isolation rate were considered statistically significant when $p < 0.05$. (Figures within the parentheses indicate the number of positive sample/number of sample examined)

Table 1 Oligonucleotide primers used for amplification of the target genes

Assay target gene or primer name	Forward primer	Reverse primer	Product size (bp)	References
stx1	GTGGCATTAATACTGAATTGTCATCA	GCGTAATCCCACGGACTCTTC	109	Jinneman et al. [32]
stx2	GGCACTGTCTGAAACTGCTCC	TCGCCAGTTATCTGACATTCTG	255	Schroeder et al. [35]
eae	CTGAACCAGATCGTAACGGC	TGATAAGCTGCAGTCGAATCC	229	Moyo et al. [34]
EHEC-*hlyA*	AGCCGGAACAGTTCTCTCAG	CCAGCATAACAGCCGATGT	526	Meng et al. [33]

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E. coli was higher in spring season followed by summer, autumn and winter seasons (Table 2). In the present study, AMC count was found to be significantly higher ($P < 0.05$) in all the seasons. AMC, TCC and EC obtained especially in the warm months reveal the poor sanitary quality of the studied meat samples. It is noteworthy that the samples obtained from all the sites in one occasion or more, produced the counts that reached or exceeded the limit recommended by the Meat Standards Committee [24] and Microbial Food Safety–Indian Regulations [25]. Although no *E. coli* O157 was recovered from any sample in the present study, the STEC strains showed high values for retail meat hygienic indicators.

The centers for disease control foodborne diseases active surveillance network (FoodNet) data indicate that outbreaks and clusters of food-borne infections peak during the warmest months of the year [26]. The reasons for this seasonal pattern are not known, but they may include (i) increased prevalence of the pathogens in livestock or vehicles of transmission during the summer; (ii) greater human exposure to contaminated foods during the cook-out months; and/or (iii) more improper handling (e.g. temperature abuse) or incomplete cooking of products during warm months. Some studies also have shown that the rate of microbial contamination of food products follows the same seasonal trend [27–29].

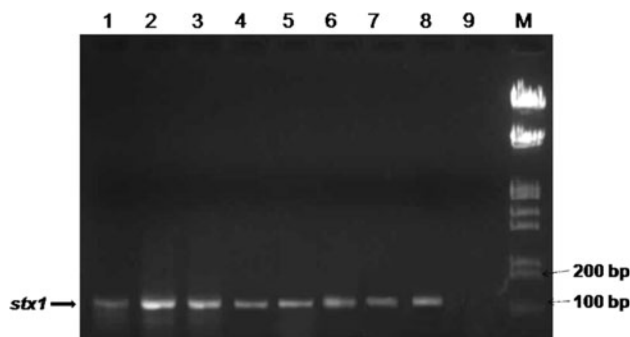

Fig. 2 PCR amplified bands of *stx1* (109 bp) gene on agarose gel (2.2 %). *Lane M* 100 bp Marker, *Lanes 1 to 8 E. coli* isolates, *Lane 9* negative control (MTCC723)

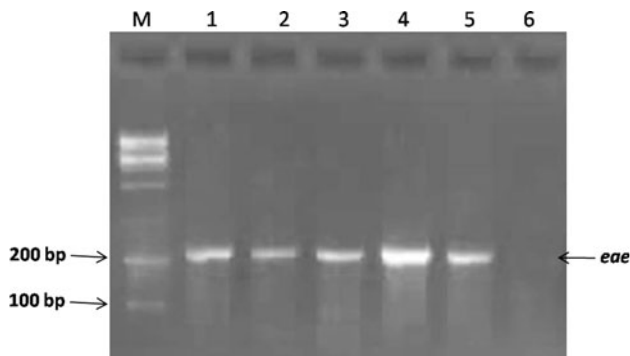

Fig. 3 PCR amplified bands of *eae* (229 bp) gene on agarose gel (2.2 %). *Lane M* 100 bp Marker, *Lanes 1 to 5* STEC isolates, *Lane 6* negative control (MTCC723)

In this study, from among the total 190 isolates, 22 (11.5 %) isolates of *E. coli* obtained from different samples showed the amplification of *stx*1 gene and 11 (50 %) of the STEC positive isolates carried the *eae* factor but none of them harbored the EHEC-*hly*A gene (Figs. 2, 3). None of the STEC isolates showed s*tx*2 gene amplification. The data obtained in the study are consistent with those of Samadpour et al. [30] who recovered 4 STEC isolates from 33 chicken breasts and 1 STEC isolate from 15 turkey samples. Kiranmayi et al. [20] reported similar findings for STEC positive isolates from chicken meat samples while Wani et al. [19] reported the presence of 2.74 % *eae* gene from STEC positive chicken samples. Although *eae* is considered important for attaching and effacing lesion in human intestinal epithelial cells, it may not be essential for STEC pathogenicity since *eae* negative STEC have also been reported to cause severe human infections [31–35]. In the present study, skin and tail samples were found to be more contaminated with STEC as compared to other portions. Both *E. coli* and STEC were found to be present at higher percentage in summer season in all the states (Figs. 4, 5). STEC were found to be higher in Nagaland followed by Meghalaya, Mizoram and Arunachal Pradesh but were not recorded from the samples collected from the states of Manipur, Assam and Tripura (Fig. 5). This could be attributed to the cross-contamination from other meats as beef and pork which were observed to be simultaneously sold along with chicken in the same retail outlets, primarily in unhygienic conditions. However, the rate of presence of both *E. coli* and STEC was comparatively lower in Mizoram which could be attributed to pleasant climate

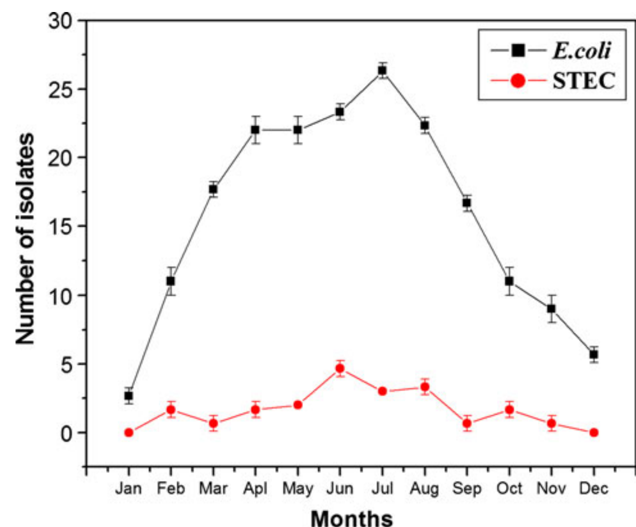

Fig. 4 Seasonal counts of *E. coli* isolated from samples collected from different states

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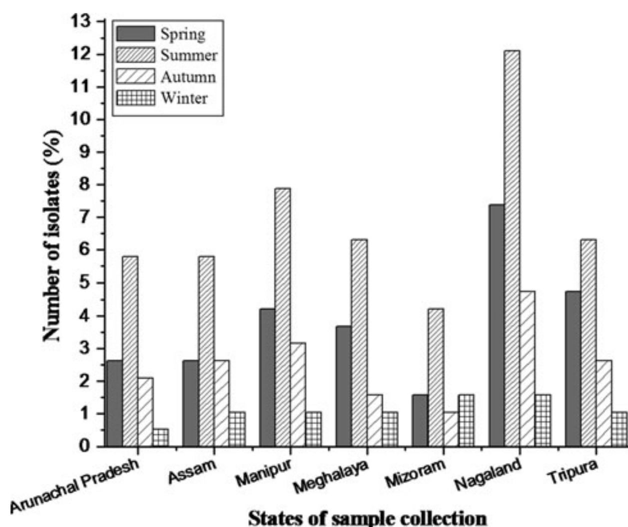

Fig. 5 Seasonal counts of STEC isolated from samples collected from different states

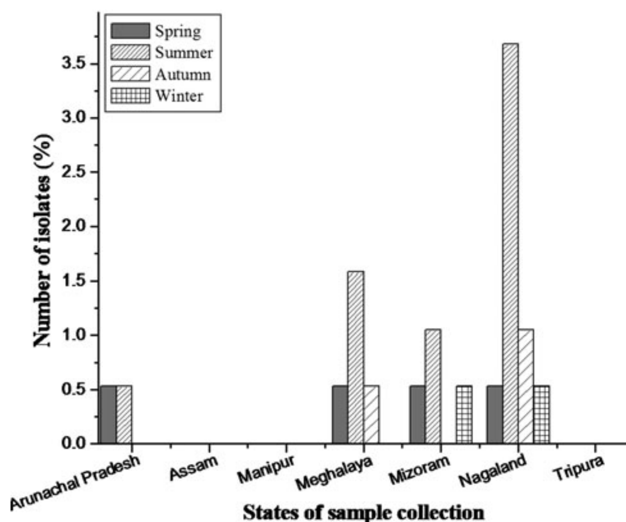

Fig. 6 Average month wise prevalence of *E. coli* and STEC in retail market chicken meat

throughout the year. Statistical analysis showed that there were significant differences ($P < 0.001$) of *E. coli* prevalence in different seasons (Table 2) with the highest prevalence in samples collected during summer months (Fig. 6). There was significant difference in prevalence rate of *E. coli* and STEC ($P < 0.05$) with highest prevalence of 83.3 and 11.8 % in spring and summer months respectively.

Conclusion

During the warm season, majority of samples were found to be beyond the safety limits for the counted bacteria. This might be due to poor hygienic conditions, contamination with Enterobacteriaceae from the intestinal contents, and congenial temperature prevalent during slaughtering and storage adopted in the traditional methods. These data confirm that raw retail chicken meat is an important vehicle for transmitting food-borne diseases influenced by poor hygienic conditions in the slaughter houses and retail markets. To diminish Enterobacteriaceae contamination rates in retail meat, it is suggested that risk reduction strategies are used throughout the food chain. These strategies may include on-farm practices that reduce pathogen carriage, increased hygiene at both slaughter and meat processing, continued implementation of HACCP systems, and increased consumer education efforts. Additionally, cross-contamination during food handling and preparation must be avoided to ensure food safety. Although not at alarming frequency, retail chicken meat was contaminated with STEC, some of which contained haemolytic genes with potential to cause severe human disease, which calls for immediate attention of health experts in implementation of safer practices and standards.

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