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Food safety surveillance in Bhutan, conducted from 2019 to 2021

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ABSTRACT

Food-borne diseases are caused by a wide range of microbiological and chemical or toxins with different levels of severity, which range from mild sickness to life-threatening illness. The World Health Organization estimates that the global burden of foodborne diseases is 420000 mortality and at least 1 in 10 people falls ill every year from eating contaminated food. African and South-East Asia Regions have the highest burden of foodborne diseases. Several devastating outbreaks of foodborne diseases have been reported in Bhutan. This report presents the food safety surveillance data for food samples collected between June 2019 to December 2021 from five Districts (Paro, Thimphu, Phuentsholing, Gelephu and Monggar). Ready-to-eat food samples were collected by Food inspectors and samples were shipped to Royal Centre for Disease Control (RCDC) maintaining a cold chain during transportation. The results show that 12.36% (n=78) of food samples were non-acceptable due to indicator test organism contamination and 8.71% (n=55) of the RTE food samples were unacceptable due to the presence of the pathogenic organism. The common type of pathogen isolated was *Staphylococcus aureus,* and *Bacillus cereus*, and low detection of *Aeromonas* and *Shigella* spp. The seasonality pattern of food contamination shows that most contamination occurred higher during hot and wet seasons. The findings demonstrate that RTE is likely to cause foodborne illness. Therefore, education on personal hygiene, good manufacturing practices and food safety aspect would improve food quality thereby reducing the incidence of foodborne incidences.

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1. Introduction

Food-borne diseases are caused by a wide range of microbiological and chemical or toxins with different level of severity, which can be mild sickness to life-threatening illness, or both (1).

Moreover, food may be a silent vehicle for microbial, chemical and physical hazards (2).

There is also concern about the transmission of multiple antimicrobial-resistant bacteria via the food chain.

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The World Health Organization (WHO) estimates that the global burden of foodborne diseases to be 420000 mortality and at least 1 in 10 people fall ill every year from eating contaminated food (3). African and South-East Asia Regions have the highest burden of foodborne diseases, with up to 70% of diarrheal cases linked due to food-borne contamination. Several devastating outbreaks of foodborne diseases (FBD) have been reported in Bhutan. For instance, *Campylobacte*r outbreak in Bumthang in 2012 affected about 400 students which was associated with food consumed during sports celebrations (4). *Shigella flexneri* was identified as the probable cause of bloody diarrhea, contaminated through water in acute gastroenteritis (AGE) outbreak that occurred in Monggar 2011 (5). In 2016 food poisoning in Deptsang village Samdrupjongkhar was caused due to *Aeromonas hydrophila* (6). Therefore, in 2014, food poisoning was listed as one of the notifiable diseases in the country and was integrated into National Early Warning, Alert and Response Surveillance System (NEWARS).

The microbiological threshold level is used as criteria for acceptance or non-acceptance of food products. Indicator organisms have been used since 1892 (7). Indicator organism testing may be used to access food safety risk and hygienic indicator of a production facility (8). Indicator organism or surrogacy to safety indicators suggest the presence of conditions associated with increased risk of exposure to a pathogen. The most used indicator organisms include the aerobic plate count/ total plate count, yeasts and molds, the coliform groups, *Escherichia coli, Enterobacteriaceae* and *Listeria*.

The presence of indicator organisms also states the risk that there might have been contamination from sewage and that other pathogen could be present (7). Contamination of ready-to-eat (RTE) by bacterial pathogens can occur during any phase of obtaining raw materials and prior to serving (farm to fork). More often, if the water used during farming or washing of raw materials is done with fecal contaminated water (9).

The Food and Nutrition Laboratory (FNL) at RCDC started conducting foodborne disease surveillance from June 2019 in collaboration with Bhutan Agriculture and Food Regulatory Authority (BAFRA). The objectives of food safety surveillance is to strengthen the reporting of foodborne disease outbreak events as a part of indicator, and event-based reporting systems and provide early warning, and detect outbreaks. Moreover, food safety is a public health concern for both the Ministry of Health (MoH) and Ministry of Agriculture and Forest MoAF).

2.Materials and Methods

2.1. Study area

Lying between latitudes 26° and 29 °N and longitudes 88° and 93 °E the Kingdom of Bhutan is a landlocked country (10). It is subdivided into 20 districts with approximately 700,000 live populations. The current surveillance result presents the samples collected between June 2019 to December 2021 from the five sites (Paro, Thimphu, Phuentsholing, Gelephu and Monggar), Fig. 1.

These sites were chosen because these are the towns with higher populations and functional hotels/restaurants.

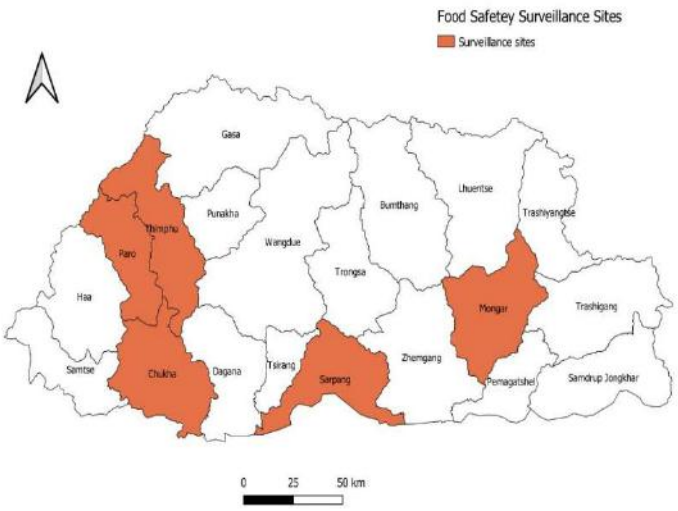

Figure 1. Food safety surveillance sites

2.2. Samples collection

The food samples were aseptically collected fortnightly from randomly selected restaurants/hotels/bakeries by the Food Inspectors (BAFRA). Any ready-to-eat food samples; including both vegetables, types of meat and bakery items were collected. The samples were coded with a unique sample identification number generated through Food Safety Surveillance Information Management System (FoodSIMS). Samples were packed separately and transported in the cold chain to the Food and Nutrition Laboratory, Royal Centre for Disease Control, Thimphu for laboratory analysis.

2.3. Preparation and Laboratory Analysis of Samples

The sample preparation procedure was done in accordance to the Association of Official Analytical Chemist (AOAC) method (11). Briefly 25 g of each sample was homogenized for 30 s at 230 rotation per minute (RPM) by stomacher in 225 ml of sterile buffer water (peptone water) (BD, USA). Furthermore, the serial dilution to three more tubes was prepared. The indicator organism test; total plate count, total *Enterobacteriaceae* count, total coliform, Environmental *Listeria,* and yeast mould count was performed from the diluted samples using specific culture media (3M™ Petrifilm™ Plates). The plates were incubated aerobically for 24 h at 37°C except for yeast mould at 25°C for 48 h. All discrete colonies were counted where possible and expressed as the $\log^{10}$ of colony-forming units per gram (cfu g^{-1}). To improve recovery and detection, pre-enrichment media was used for *Campylobacter, Salmonella, Shigella, Listeria monocytogenes,* the tubes were incubated aerobically at 37°C for 12–24 h, except for *Campylobacter* at 35 ± 2°C for 48 h with 5% CO_2. From the enrichment tube a loop-full of broth was plated on their respective media; *Salmonella/Shigella* (Sigma, India), Mac Conkey (Criterion, USA), Modified Charcoal Cefoperazone Deoxycholate Agar (mCCD) (Sigma, India), Hektone (Sigma, India), Baird Parker media (Sigma, India), Mannitol-egg-yolk-polymyxin agar (Criterion, USA).

The plates were incubated aerobically for 24–48 h at 37°C, except mCCD was incubated at 35 ± 2°C for 48 h with 5 % CO_2.

2.4. Isolate identification

The bacterial colony was identified by performing Gram staining and also based on colony pigmentation and characteristics. Nutrient agar incubated aerobically at 37°C for 24 h was used for isolating pure culture (12). Biochemical identification test and furthermore API 20E were performed on isolated with discordant results.

2.5. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) test was performed onto the *E. coli* isolates to study the presence of any pathogenic strains following the method mentioned by Toma et al., (2003) and Waturangi et al., (2019) (13,14). The primers used were manufactured by Macrogen with following primer sequences; *Enterotoxigenic E. coli* (ETTC) F 5′ CAG ACG GAG CTC CTC AGT 3′ and R 5′ CCC CCA GCC TAG CTT AGT TT 3′, *Enterohemorrhagic E. coli* (EHEC) F 5′ CAG TTA ATG TGG TGG CGA AGG 3′ and R 5′ CAC CGA ACA ATG TAA CCG 3′, *Enteroinvasive E. coli* (EIEC) F 5′ TGG AAA AAC TCA GTG CCT CT 3′ and R 5′ CCA GTC CGT AAA TTC ATT CT 3′ and *Enteroaggragative E. coli* (EAEC) F 5′ CTG GCG AAA GAC TGT ATC AT 3′ and R 5′ ACG ACA CCC CTG ATC AAC AA 3′.

2.6. Antibiotic Susceptibility Testing (ABST)

The ABST was conducted by inoculating primary isolates onto Mueller–Hinton agar (MHA) plates and then disc diffusion method (15,16).

The MHA plates with antibiotic disks were incubated for 18–24 h at 37°C. The zone of inhibition (ZoI) was interpreted according to Clinical and Laboratory Standards Institute guidelines.

2.7. Statistical Analysis

The data analysis was done using excel and descriptive analysis are presented as percentages and ratio.

3.Results

Six hundred and thirty one RTE food samples were received from five sites from June 2019 to December 2021 within a period of 30 months. Of the food sample collected 99% were solid and only 1% were liquid or semi-solid foods. The results show that only 0.7% of foods were acidic foods and 99.3% were low acid food. Of the total 631 food samples tested 12.36% (n=78) were non-acceptable due to indicator test organism contamination. Fifty-five samples (8.71%) of the RTE food samples were unacceptable due to the presence of the pathogenic organism. The common type of pathogen isolated was *Staphylococcus aureus* (22.08%), *Bacillus cereus* (22.07%), *Aeromonas* (1.5%) and *Shigella* species (0.56%), as presented in table 1. The indicator organism presence in a different group of food shows that mostly the fast foods were contaminated with indicator organisms as shown in table 2.

Table 1. Percentage of samples with un-acceptable level of indicator and pathogenic organism

	Indicators test				**Pathogenic organism**			
Place	TPC	*E.coli*	*Enterobacter iaceae*	Yeast Mound	*B. cereus*	*S. aureus*	*Aeromonas*	*Shigella*
P/Ling (n=65)	13.85	12.31	12.31	9.23	1.54	0	0.00	0
Paro (n=106)	11.32	5.66	7.55	11.32	8.49	7.55	0.94	0
Thimphu (n=178)	8.99	8.99	6.18	5.06	5.06	5.06	0.56	0.56
Mongar (n=178)	5.62	8.43	5.62	5.06	5.06	5.62	0	0
Sarpang (n=104)	13.46	6.73	4.81	10.58	1.92	3.85	0	0

Table 2. Common types of food group associated with un-acceptable range of indicator and pathogenic organism

	Phuentsholing	**Paro**	**Thimphu**	**Mongar**	**Sarpang**
Percentage of indicator test organism isolated in different food groups					
Vegetable curry and Rice	9.23% (n=6)	1.89% (n=2)	1.12% (n=2)	0.56% (n=1)	4.81% (n=5)
Meat/Fish and Egg items	12.31% (n=8)	6.60% (n=7)	5.62% (n=10)	2.25% (n=4)	4.81% (n=5)
Bakery products	1.54% (n=1)	1.89% (n=2)	1.12% (n=2)	1.12% (n=2)	0.96% (n=1)
Fast foods	9.23% (n=6)	9.43% (n=10)	5.06% (n=9)	9.55% (n=17)	0.65% (n=9)
Percentage of the pathogenic organism isolated in different food groups					
Vegetable curry and Rice		0.94% (n=1)	1.12% (n=2)	0.56% (n=1)	1.92% (n=2)
Meat/Fish and Egg items	6.15 (n=4)	4.72% (n=5)	1.69% (n=3)	1.69% (n=3)	0.96% (n=1)
Bakery products		1.89% (n=2)	1.12% (n=2)	0.56% (n=1)	0.96% (n=1)
Fast foods	3.08% (n=2)	8.49% (n=9)	5.62% (n=10)	6.74% (n=12)	0.96% (n=1)

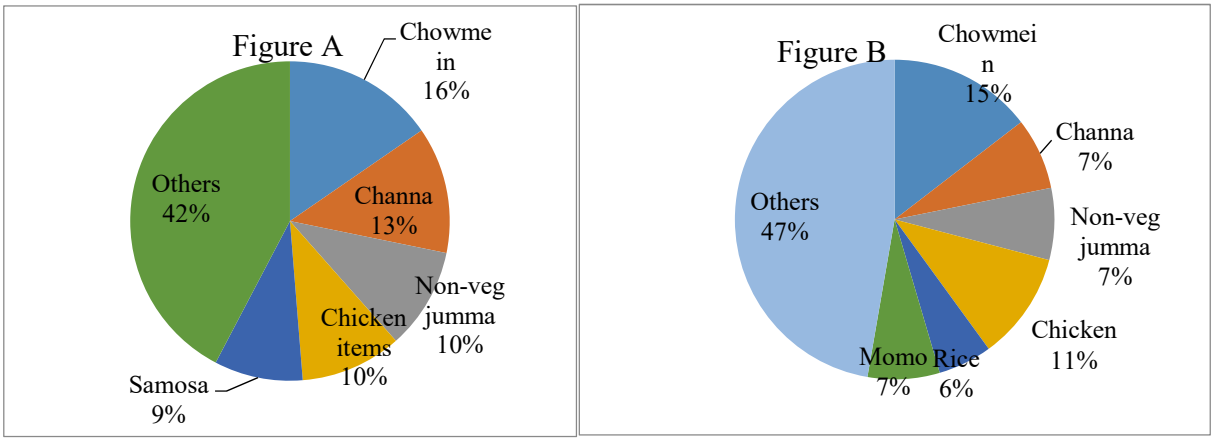

Figure 2. Percentage of common RTE foods non-acceptable A. due to indicator test organism, B. due to pathogenic organism

Amongst the fast food the common food types that atorscwere found to have unsatisfactory levels of indi organism were Chowmein (16%, n=12) followed by Channa (13%, n=10) and non-veg Jumma (10.26%, e ofpn=8), as shown in Fig. 2A. The most common ty reefood in which pathogenic organisms isolated w from Chowmein (14.55%, n=8) followed by Chicken items (10.91%, n=6), Channa, Non-veg Jumma and Momo (7.27%, n=4, each) and Rice .(n=3), respectively (Fig. 2B ,5.45%)

The district wise detection of pathogenic organism d *S.*nshows Paro has higher range of *Bacillus* (8.49%), a ET*aureus* (7.55%) pathogenic organism isolated from R food samples. The food samples collected from Mongar and Thimphu had the presence of both the *Bacillus* and *S. aureus* with no significant detection differences between common pathogens.

The least pathogenic isolates were detected in the samples collected from Phuentsholing this could be due to low samples tested from Phuentsholing. Thus there is a need for more samples to be collected and tested from Phuentsholing to confirm and validate the findings.

The isolates of *E. coli* were collected and stored at -40° C in nutrient broth until the PCR was conducted (Fig. 3). The multiplex PCR was conducted for four pathogenic strains of *E. coli* (*Enteropathogenic E. coli*, *Enterotoxigenic E. coli*, *Enteroinvasive E. coli* and *Enterohemorrhagic E. coli*) but none of the samples were positive for any of the pathogenic *E. coli* strains.

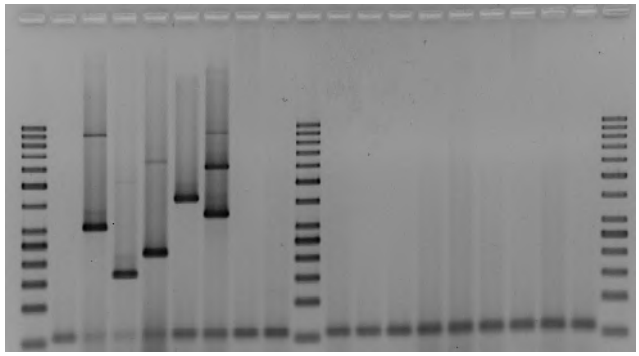

Figure 3. PCR for *E. coli* isolated from food samples

4.Discussion

Royal Centre for Disease Control recorded a total of 61 FBD outbreaks from 2011 to 2020 of which 38.8% were reported as diarrhea, 26.53% as food poisoning, 10.20% AGE, 6.12% as mushroom poisoning and rest 18.37% by disease code. Therefore, the need for effective foodborne disease surveillance was stated in the country.

The pH is often used as an indicator for quality and it can also provide an indication of whether the food was properly stored. The pH of the food is important for consumer acceptability and health. Since food is a complex network of biological and chemical ingredients, the unstable interactions between these ingredients make it challenging to get the best estimate when measurements are made. In our current surveillance water activity (a_w) in samples was not measured, though a_w is a good tool to access the food stability and predict the microbial activity (17,18).

4.1. Indicator test

According to Food classification, food is considered acceptable if the total bacterial count or total plate count was ≤ 10^5 cfu/g and Yeast mould of <10^2 cfu/g (19). The results show that the common violation of indicators tests are TPC (10^5 cfu/g), yeast mould count (>100 cfu/g), Total *Enterobacteriaceae* count (>10^2 cfu/g) and *E.coli* count (>10^2 cfu/g) (12,20). Total Plate count of aerobic microorganisms and yeast mould count are used as indicators to access the food quality and also used as a hygiene indicator (21). The presence of aerobic organisms >10^5 cfu/g indicates poor hygiene during processing or after cooking. Hence those foods considered un-acceptable due to indicator organism violation are considered high risk in transmitting enteric pathogens too. The use of unclean or unsafe water can also be associated with foodborne outbreaks; they may be contaminated either in the farm with human feces or sewage, and from the open irrigation water channel contaminated by animal feces. Also, the total coliform count and the *Enterobacteriaceae* count is assessed as indicator test (22).

During the current food safety surveillance period it was found that indicator test (total plate count and total *E.coli* count) un-acceptability was slightly higher (table 1) and the polymerase chain reaction was performed with the *E. coli* isolates. This presence of Coliform indicates the possibility of using unsafe water for cooking, poor standards of cleaning of cooking utensils and lack of general hand hygiene. The comparison on different indicator test organism contamination among the five sites found that food samples collected from Phuentsholing had highest indicator test organism contamination [TPC (13.85%), *E. coli* (12.31%) and *Enterobacteriaceae* (12.31%), respectively]. On the other hand, samples collected from Paro presented highest contamination due to Yeast/mould (11.32%). This could be due to the temperature differences between the districts and favorable growth temperature differences for bacterial and yeast/mould. Though yeasts and moulds are not considered pathogens it can produce toxins, affecting the quality and taste of food and may affect human health (23).

The fast food such as Chowmein, Channa and non-veg Jumma were mostly contaminated with indicator organism. This could be due long storage after preparation and frequent handling. Moreover, non-veg jumma is prepared from animal intestine which are at risk of contamination by gut micro-organisms.

4.2. Pathogenic Organism

The finding is similar to the earlier study conducted at Sikkim, India (24) with isolation of *S. aureus*, *B. cereus, Shigella* and *Aeromonas* spp.

The percentage of different food groups un-acceptable due to pathogenic organism is presented in table 2.

The correlation between indicator organism and pathogenic organism shows that 48% of the food samples presented with pathogenic organism had the contamination with indicator organism. *Staphylococcus* species are present in the skin and respiratory tract as a normal flora in the healthy people and widespread in the nature (25). The toxins produced from the *S. aureus* being acid and heat stable could cause food intoxication (26). Therefore, foods that are handled frequently during preparation or after preparation with unhygienic practices targets for *Staphylococci* contamination (27). Similarly, *Bacillus* are also widely present in the environment and survive in the form of spores, temperature abuse during food storage after preparation adds to the higher risk of food being contaminated with *Bacillus* (24,28). *Bacillus cereus* consumed at low concentration does not present a public health risk. However, foodborne *B. cereus* illness occurs and may be underreported due to the mild nature and short duration of symptoms (29).

The seasonal variation of food contamination was analyzed and the results show that the samples collected during monsoon season presented with the highest number of food samples being unacceptable due to either indicator or pathogenic organism. This indicates that the hot and humid environment favors the growth of microorganisms and the probability of foodborne disease to be higher during this season.

The comparison was made to the baseline record 2011-sto020 (Fig. 4) of foodborne illness outbreaks, since m2 f the FBD outbreaks were reported during the hot ando umid months the co-relation between the FBDh utbreak record and food safety surveillance come too oncordance. Moreover, a study by Wangdi andc Clements (2017) stated that the diarrheal cases in Bhutan were seasonal with most of the cases recorded during hot and wet seasons (30). Thus, this indicates that food handlers must take extra caution and follow good hygiene practices to prevent foodborne diseases during hot and wet seasons.

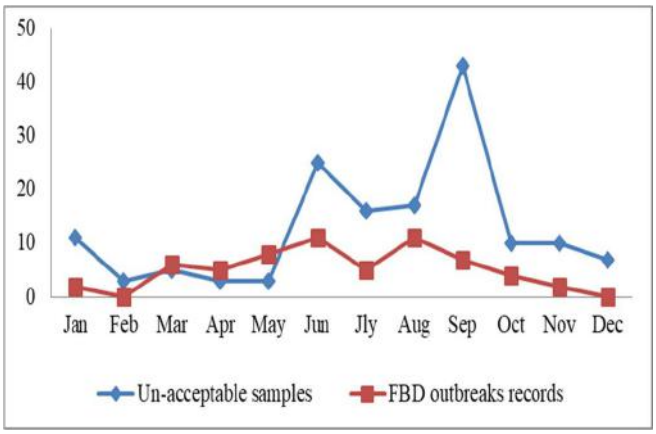

Figure 4. Monthly trend of Foodborne disease outbreaks (2011 to 2020), recorded at Royal Centre for Disease Control and monthly average un-acceptable samples during food safety surveillance (2019 to 2021)

On the other hands, though *Salmonella,* and *Campylobacter,* were common food pathogens identified during the foodborne disease outbreaks, however, these pathogens were not isolated during this surveillance study period and with very low detection of *Aeromonas* and *Shigella*.

4.3. Antibiotic Susceptibility pattern

To rule out the antibiotic resistance pattern to the pathogenic organism isolated from the food samples, the antibiotic susceptibility test was performed with the *S. aureus* isolates. The *S. aureus* isolates were found to be susceptible to penicillin, tetracycline, cefoxitin, erythromycin, but resistance with only to one antibiogram (trimethoprim-sulfamethoxazole). The main limitation of the study is test for detection of any virus and toxins in the food were not conducted.

5.Conclusion

The global food system not only the trade commodity but has also become a public health concern. Food safety is a shared responsibility that involves all the stake holders, including the producer, consumer and distributor. Therefore, it is of paramount importance that food safety along with the nutrition policies forms essential component in health system. The findings from the study shows that the RTE foods can likely be hazardous as 12.36% of samples were contaminated with indicator organisms. Also 8.71% of the samples were unacceptable due to pathogenic organism contamination. The presence of both the indicator and pathogenic organism in RTE foods is a good indication for the need of food safety education. Thus, awareness campaign to the vendors about importance of personal hygiene and good manufacturing practices, food safety and proper disposal of waste would improve food quality thereby reducing food borne incidences.

Our food is diverse and therefore to ensure it is safe for consumption it requires a systematic, proactive method to reduce contamination. Moreover, access to safe running water plays a paramount role in food safety.

Conflict of Interest

Declared none.

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