ROYAL CENTRE FOR DISEASE CONTROL

ANNUAL SCIENTIFIC REPORT

2022

3
Referral Hospitals

52
Hospitals

225
Primary Healthcare Centers & Subposts

Royal Centre for Disease Control
Ministry of Health
Royal Government of Bhutan

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Acknowledgement

The Royal Center for Disease Control (RCDC) acknowledges the support of all health workers across the country & officials in Bhutan Food and Drug Authority (BFDA) for actively participating in RCDC's surveillance activities relating to public health in Bhutan. We hope that this annual scientific report of RCDC serves as an evidence to guide public health practice in the country.

Please note that this is the first annual report from RCDC and we are keen to hear your critical feedback for improvement. If you have any suggestions, please email it to rcdc@health.gov.bt.

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National Notifiable Diseases Surveillance through National Early Warning Alert and Response Surveillance (NEWARS), 2022

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Background

Royal Centre for Disease Control (RCDC) has been coordinating and conducting national notifiable disease surveillance (NNDS) since 2010. In 2014, the national notifiable disease surveillance (NNDS) was revised to incorporate event-based surveillance (EBS) to complement and supplement the indicator-based surveillance (IBS). The revised NNDS was renamed as National Early Warning Alert and Response Surveillance (NEWARS). This disease surveillance and response system for infectious diseases or syndromes of public health concern aims to monitor trend, provide early detection and alert of potential threats for swift response to contain event effectively and efficiently.

NEWARS is augmented by additional sentinel surveillance systems that focus on the collection of more information on specific diseases and their etiologic agents including risk factors. These systems are: influenza-like illness (ILI) and severe acute respiratory infections (SARI); food-borne disease sentinel surveillance; acute un-differentiated febrile illness (AUFI); national diarrheal diseases surveillance; and vaccine preventable diseases surveillance.

NEWARS consists of indicator-based surveillance (IBS), immediate reporting and event-based surveillance (EBS).It is a real-time reporting system through web based and mobile SMS.

- Indicator-based surveillance: IBS involves weekly reporting of cases and deaths of national priority diseases /syndromes and conditions as per pre-defined case definitions. The aggregated data is reported weekly through web-based online reporting system or Short Message Service (SMS) through mobile phones.

- Immediate reporting: Immediately reporting any of diseases/syndromes that are of major public health importance as a single case may lead to an outbreak or widespread transmission and requires an immediate action.

- Event-Based Surveillance is ad-hoc. It involves immediate reporting of any 'unusual' events such as unusual cluster of cases or suspected outbreak/events that are of potential risk to public health and requires investigation.

The data obtained is analyzed, interpreted, and disseminated by the disease surveillance and epidemiology unit of RCDC at different levels. This report publishes recent surveillance data on diseases of public health significance with the aim of providing health centers, government agencies, and the general public with important statistical information on national notifiable diseases and conditions.

Methodology

Surveillance Setting

The NEWARS is nationwide surveillance with all health centers across the country serving as surveillance sites. Notifiable diseases are reported from 290 health centers across the country. The NEWARS system uses series of steps for collecting, collating, verifying, and responding to diseases and events of public health concern. Currently, there are 10 weekly and 17 immediately notifiable diseases or syndromes (**Table 1).**

Table 1: List of Notifiable Diseases/ Syndromes in NEWARS

	Weekly Notifiable Disease/Syndrome	Immediately Notifiable Disease/Syndrome
1	Acute Bloody Diarrhea	Anthrax (Suspected)
2	Acute Watery Diarrhea	Acute Flaccid Paralysis (Suspected Poliomyelitis)
3	Acute Jaundice Syndrome	Acute Hemorrhagic Fever Syndrome (Suspected)
4	Acute Respiratory Infection	Avian Influenza (Suspected)
5	Mumps	Bacterial Meningitis (Suspected)
6	Fever with Rash	Cholera (Suspected)
7	Food poisoning	Malaria (Microscopy/RDT Confirmed)
8	Typhoid /Paratyphoid /Enteric Fever	Measles/Rubella (Suspected)
9	Severe Acute Respiratory Infection	Pertussis (Suspected)
10	Rickettsioses	Congenital Rubella Syndrome (Suspected)
11		Rabies (Human)(Suspected)
12		Dengue Hemorrhagic Fever (Suspected)
13		Neonatal Tetanus (Suspected)
14		Diphtheria (Suspected)
15		Acute Encephalitis Syndrome (Suspected)
16		Dengue Fever
17		Monkeypox(suspected)

Weekly notifiable disease

In an effort to effectively monitor and respond to diseases of public health concern, 11 national priority diseases/syndromes have been identified for weekly reporting. This process involves the surveillance focal point of the health center reviewing and collating daily reports from various data sources, including the Outpatient Department (OPD) register, Inpatient Department (IPD), register Maternal and Child Health (MCH) unit, Laboratory unit, and the emergency registers. The resulting report contains detailed information on the number of cases by age group. By the Tuesday of the following week, the reporting focal should submit the data in the NEWARS system, which would be recorded as timely reporting, however system can accept the data for

subsequent four weeks. The National Diseases Surveillance and Epidemiology unit (NDSAE) then verifies the report to ensure accuracy, completeness, and correctness for further analysis.

Immediately notifiable diseases

An efficient system has been established to monitor and respond to diseases that require immediate notification. There are 17 immediately notifiable diseases/syndromes, and any one of them has the potential to cause an outbreak or widespread transmission. When a clinician/nurse detects one of these diseases, they report it immediately. The surveillance officer at NDSAE then verifies the information and provides recommendations. Upon verification, the health worker is then required to complete a case investigation form (CIF) for the disease and send appropriate samples to RCDC if applicable. If no cases are detected, the surveillance focal submit zero report on a weekly basis.

Event reporting

Any health event that poses a potential risk to public health can be reported by health professionals through web-based or SMS reporting. The informal events report can be also reported by the general public, social media, community leaders, and non-governmental organizations. The reports are verified and risks assessed by RCDC. Response for action is recommended accordingly. Events related to the following are to be reported:

1) Unusual cluster of diseases or syndromes

2) Unusual cluster of death

3) Disease or deaths in animals

4) Contaminated food and food products

5) Environment hazards such as chemicals and radio-nuclear

The flow of information for weekly and immediately reports is shown in **Figure 1**. The roles and responsibilities of various healthcare workers are detailed in NEWARS guideline.

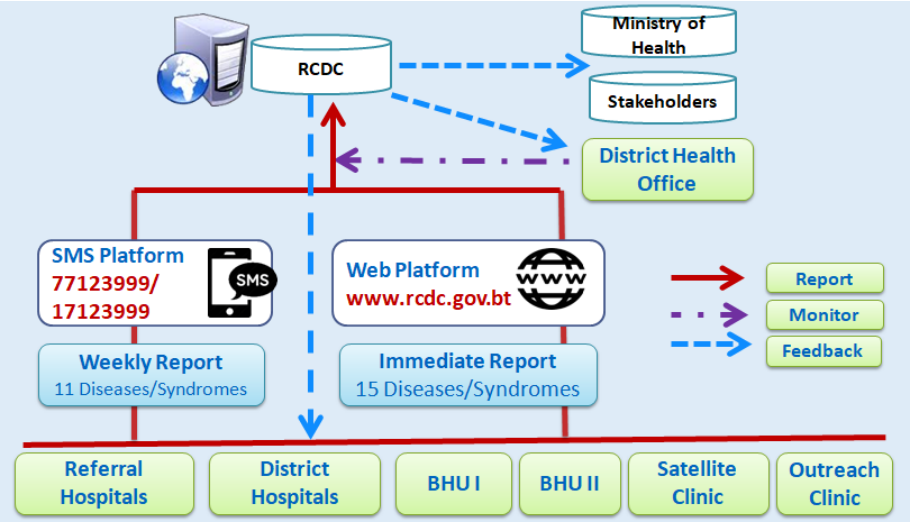

Figure 1: Flowchart for reporting weekly and immediately notifiable diseases (source: NEWARS guideline, RCDC)

Data Analysis

The data from the NEWARS system are exported in excel and analyzed. The top three common diseases are described by time, person and place. The QGIS software 3.16 was used to generate the data by place. The feedback is provided through monthly epidemiology reports and quarterly RCDC bulletin to all the stakeholders through email and hard copy.

Results

Timeliness of report

In 2022, 66.0% of the health centers reported on time, 23.0% had delayed reporting and 11.0% did not submit a weekly report (**Figure 2).**

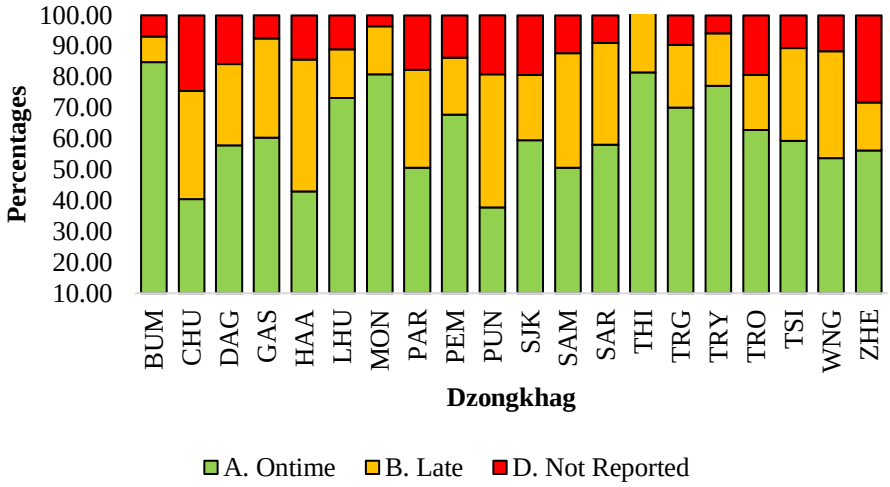

Figure 2: Status of weekly notifiable disease reporting by district

A reduction in the reporting of notifiable diseases by health centers was observed during 2020 and 2021 compared to 2019 and 2022. (**Figure 3)**.

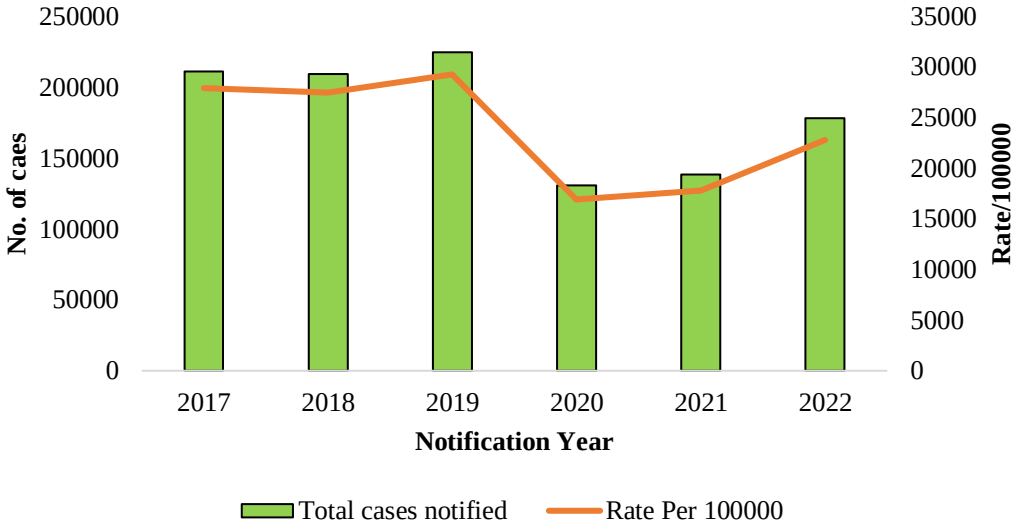

Figure 3: Total number of National Notifiable Diseases reported from 2017 to 2022

Weekly Notifiable Diseases

In 2022, the weekly reporting system recorded a total of 178,349 notified cases. Of the 10 weekly reportable diseases/syndromes, respiratory infections and acute diarrheal diseases were the most frequently reported, accounting for 84.9% (n=151358) and 13.8% (n=24656) of notified cases respectively(**Table 2).**

Table 2: Notifiable diseases/syndromes reported by Dzongkhags 2022

DZONGKHAG	ABD	AWD	AJS	ARI	MUM	FWR	FDI	TPF	SAR	RKS
Bumthang	46	827	0	6616	1	0	3	1	6	3
Chukha	212	869	9	9684	1	6	0	96	25	21
Dagana	21	670	61	7486	0	14	2	2	14	4
Gasa	22	162	0	508	0	1	1	0	0	0
Haa	6	359	0	2214	0	10	11	0	0	0
Lhuntse	65	263	19	3957	1	0	0	2	4	1
Mongar	197	1097	86	10148	1	12	0	1	35	12
Paro	409	2501	7	9037	21	3	0	0	14	5
Pemagatshel	18	637	21	5797	1	3	4	15	8	4
Punakha	66	531	1	1360	3	11	0	0	51	0
Samdrup Jongkhar	68	900	54	9045	1	4	181	6	73	2
Samtse	68	1768	1	17098	5	26	0	11	39	8
Sarpang	124	1014	20	15096	2	226	0	1	37	3
Thimphu	209	3270	45	14992	6	54	2	8	57	1
Trashigang	142	3912	18	7979	7	64	32	34	39	23
Trashiyangtse	164	730	4	6876	2	25	2	1	58	16
Trongsa	78	950	33	5662	0	13	8	4	30	0
Tsirang	62	487	22	5419	0	1	0	0	15	2
Wangduephodrang	124	1281	33	7616	17	6	1	0	27	0
Zhemgang	23	304	0	3143	1	22	1	3	6	9
Total	**2124**	**22532**	**434**	**149733**	**70**	**501**	**248**	**185**	**538**	**114**

Abbreviations: ABD (Acute Bloody Diarrhea), AWD (Acute Watery Diarrhea), AJS (Acute Jaundice Syndrome), ARI (Acute Respiratory Infection), MUM (Mumps), FWR (Fever with Rash), FDP (Food borne Illness), TPF (Typhoid/Paratyphoid fever), SARI (Severe Acute Respiratory Infection), RKS (Rickettsioses).

Descriptive analysis of commonly reported notifiable diseases

Respiratory illnesses (ARI & SARI)

A total of 151,358 cases of respiratory illness were reported (ARI-98.9% and SARI-1.1%) in 2022. The highest cases of ARI & SARI were notified in the week 41 through 44, 2022. The trend of ARI was lower than that of the median of the last three years until week 35 (**Figure 4A & 4B**). The most commonly affected age group for ARI & SARI were pediatric age of ≤ 9 years

respectively (**Figure 4C**). By district, Samtse, Sarpang& Thimphu reported the maximum number of ARI & SARI cases **(Figure 4D).**

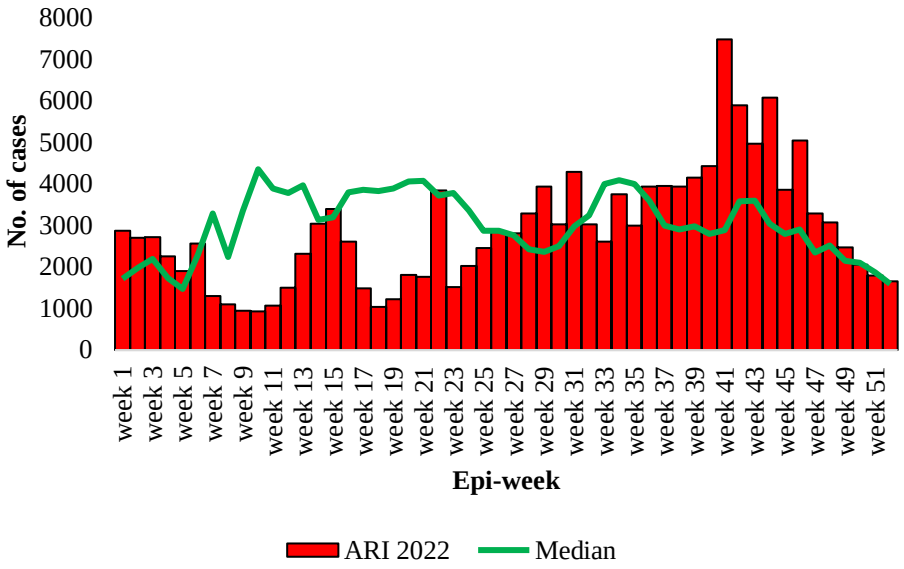

Figure 4A: Incidence of acute respiratory illness by epidemiological week

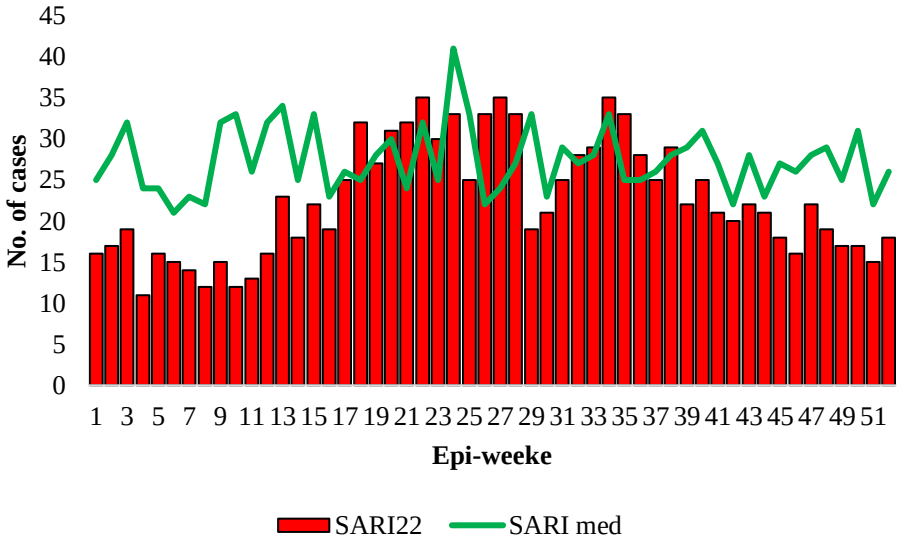

Figure 4B: Incidence of severe acute respiratory illness by epidemiological week

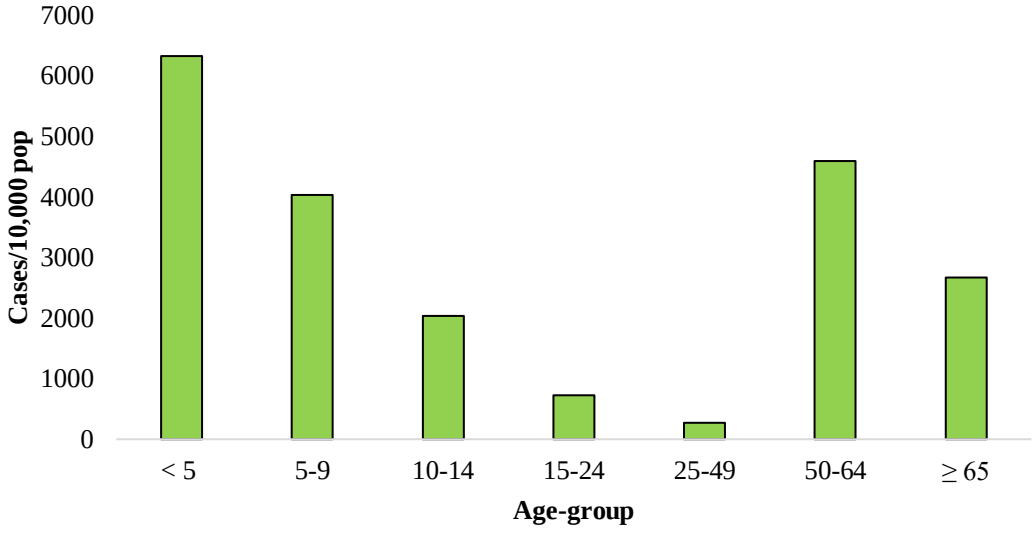

Figure 4C: Distribution of acute respiratory illness and severe acute respiratory illness cases by age group

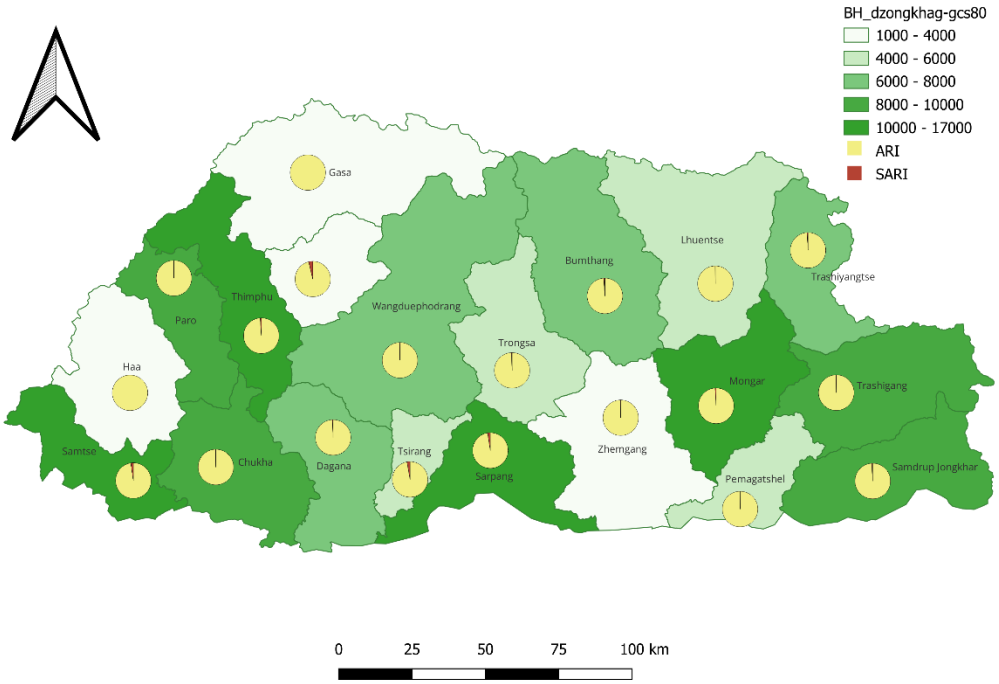

Figure 4D: Distribution of acute respiratory illness and severe acute respiratory illness cases by district

Diarrheal diseases

The second highest incidence of reportable diseases was diarrheal diseases. A total of 24,656 cases (AWD-91.4% & ABD-8.6%) were reported in 2022. The highest cases of AWD were notified in the week 26. The trend of AWD was lower than that of the median of the last three years (**Figure 5A**). The most commonly affected age-group for AWD and ABD was 1-4years (**Figure 5B**). By district, Trashigang, Thimphu & Paro reported the maximum number of AWD while a high number of ABD was reported by Thimphu, Paro and Chhukha**(Figure 5C).**

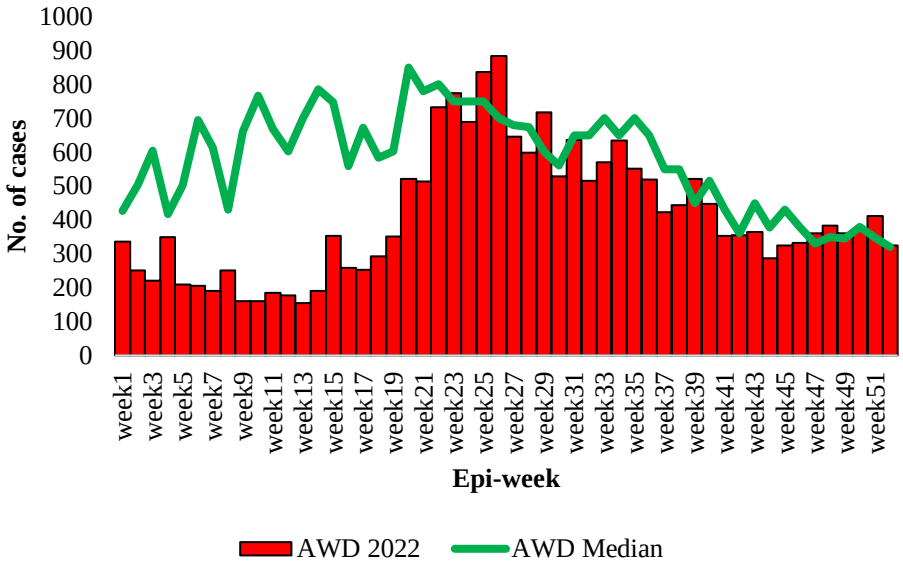

Figure 5A: Incidence of acute watery diarrhea cases by epidemiological week

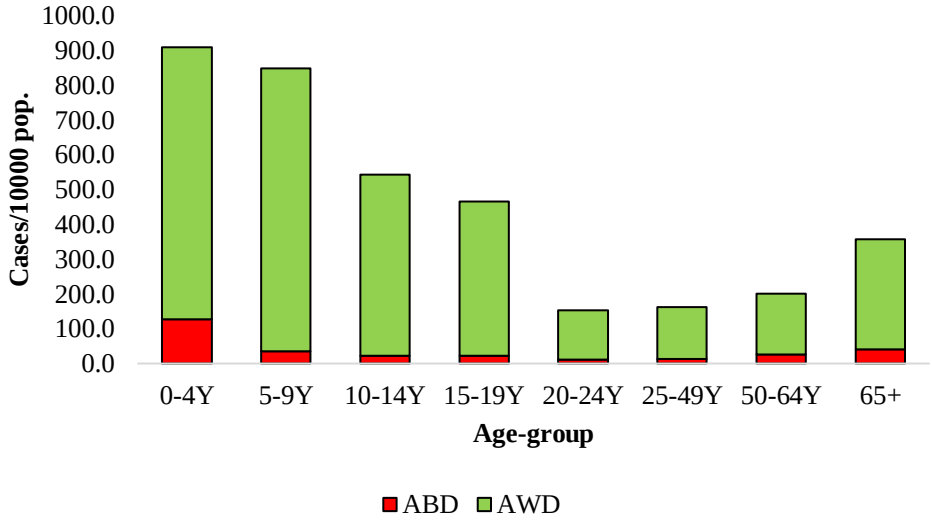

Figure 5B: Distribution of acute watery diarrhea and acute bloody diarrhea cases by age group

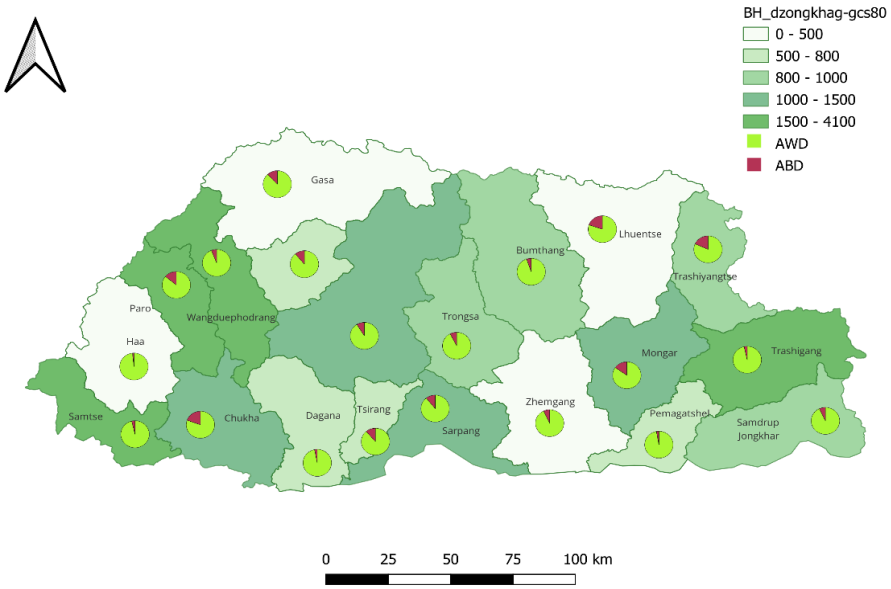

Figure 5C: Distribution of cases by district acute watery diarrhea and acute bloody diarrhea

Fever with Rashes syndrome

A total of 501 cases of fever with rash (FWR) syndrome were reported in the quarter (**Figure 6A**).The trend of FWR was found lower compared with the previous year (650 vs 501). The majority of FWR cases occurred among children under 9 years (**Figure 6B**), with Sarpang and Trashigang reporting the highest number of cases (**Figure 6C**).

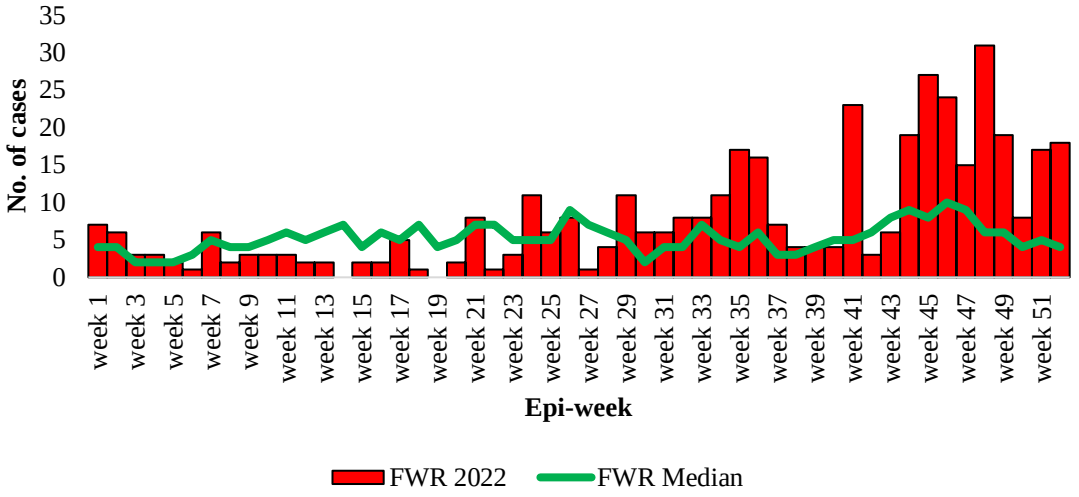

Figure 6A: Incidence by Epi-week

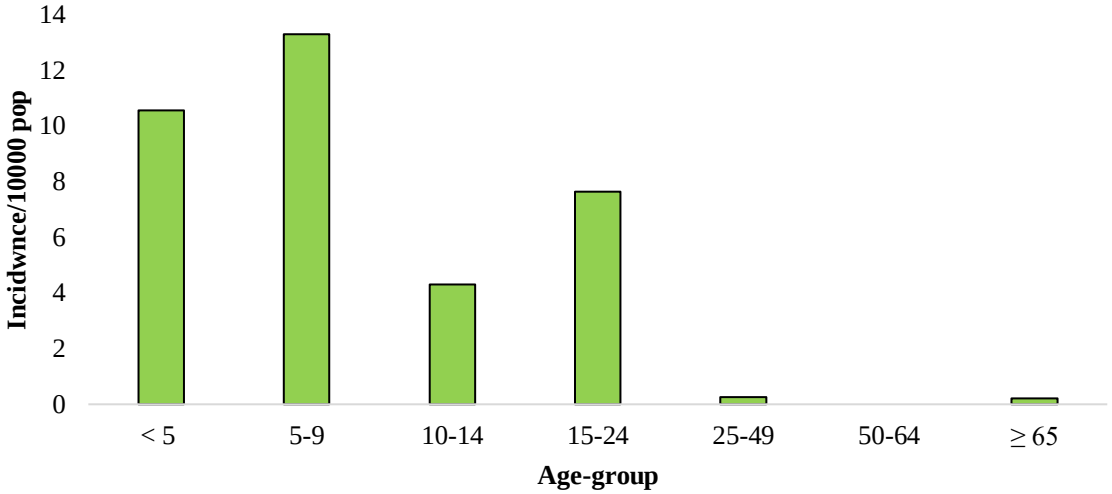

Figure 6B: Incidence by age groups

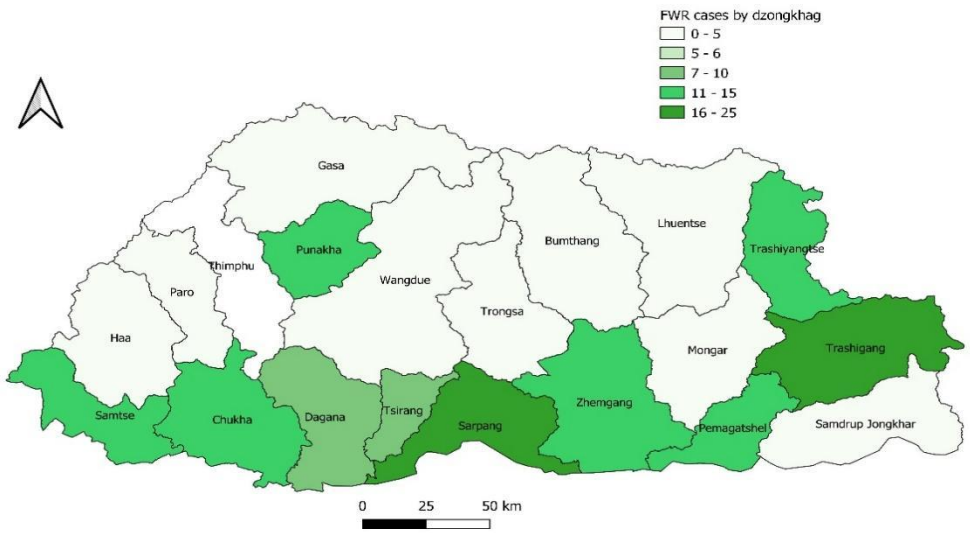

Figure 6C: Incidence by district

Other weekly reportable diseases/syndromes reported in 2022 were Mumps (260), Typhoid fever (285), Acute jaundice syndrome (501) and Rickettsiosis (114). All the diseases/syndrome that are in the weekly notifiable list were detected by the health centers.

Immediately Notifiable Diseases

There are 17 diseases or syndromes that require immediate notification. Of these 10 were detected by the reporting centers in 2022. Dengue fever, measles, acute encephalitis, and bacterial meningitis were the most commonly immediately notifiable diseases or syndromes.Out of the 93 blood samples from suspected dengue cases, 24 (25.8%) were confirmed as positive by the ELISA at RCDC (**Figure 7**). Sarpang and Chhukha districts accounted for the majority of the dengue cases. Only Sarpang reported malaria cases, while the other districts had none. Two suspected cases of Acute Flaccid Paralysis (AFP) were reported to NEWARS but tested negative for poliovirus at a reference laboratory in Thailand. Similarly, seven cases of measles and four rubella cases were confirmed for IgM antibody at RCDC. The distribution of immediately notifiable diseases reported by districts is shown in **Figure 8**.

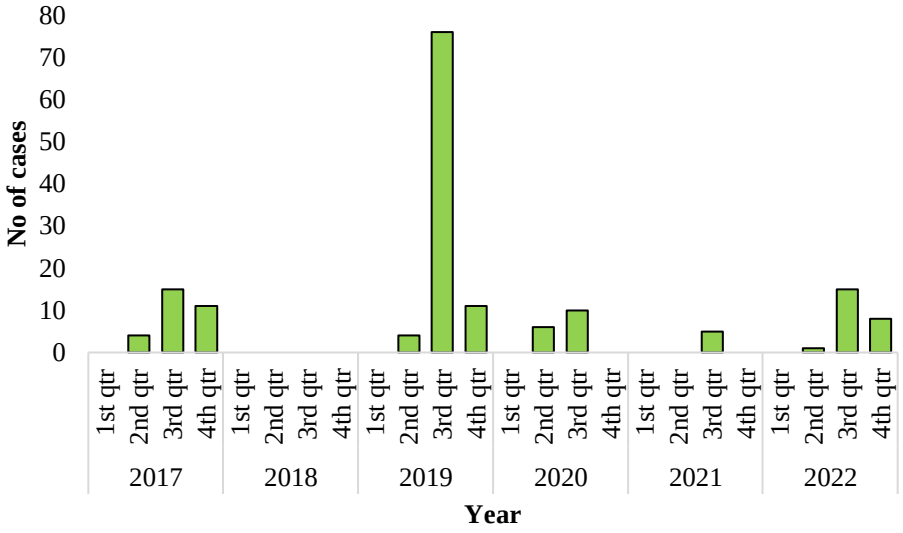

Figure 7: Number of dengue positive by ELISA

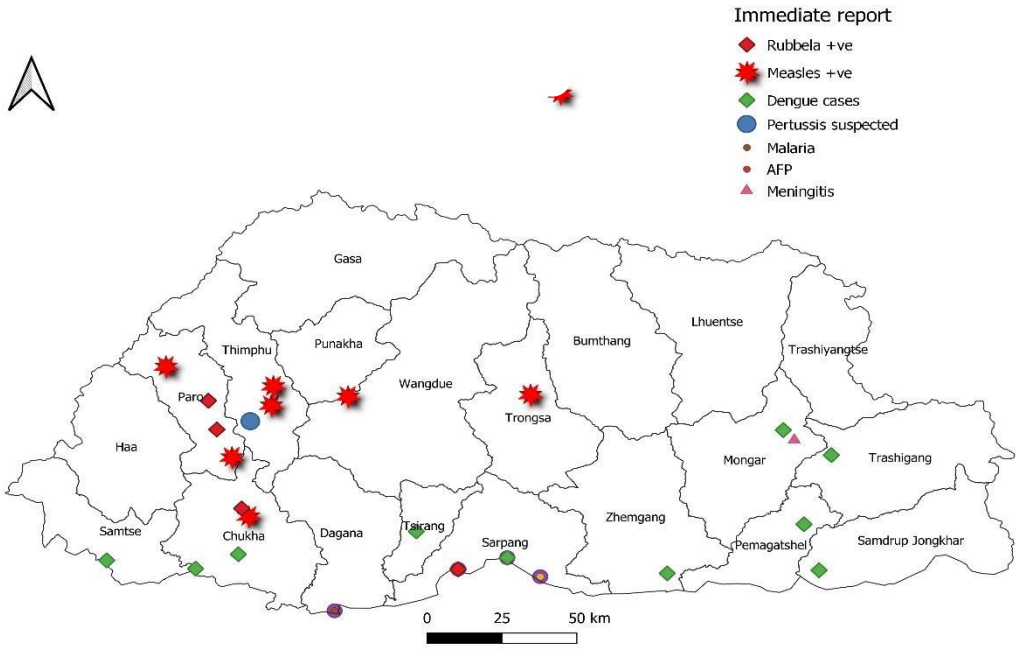

Figure 8: Distribution of immediately reportable cases by districts

Events

A total of 80 events were reported in 2022. Among the total 49 events were classified as low risk and 31 as moderate-risk. The highest number of outbreaks reported in 2022 was of Hand Food &

Mouth Disease (HFMD) (47) of which 90.0% (43) were categorized as low-risk outbreak with minimum numbers. Influenza outbreaks alone accounted for 54.0% of the moderate-risk events (**Figure 9**) with 88.0% occurring at schools. Laboratory confirmation of representative samples shown subtype A/H3 as the causative agent. An outbreak of dengue fever was reported by Phuntsholing town during the month of September 2022. A total of 43 samples linked to the Dengue outbreak was referred to RCDC of which 21 samples were confirmed positive by ELISA at RCDC. The outbreaks were reported from all most all the districts (**Figure 10)**.All outbreaks were responded to by the respective health centers and the District Health Rapid Response Team (DHRRT) upon the recommendations of RCDC. Details of event reporting by the type and the number of people affected are shown in

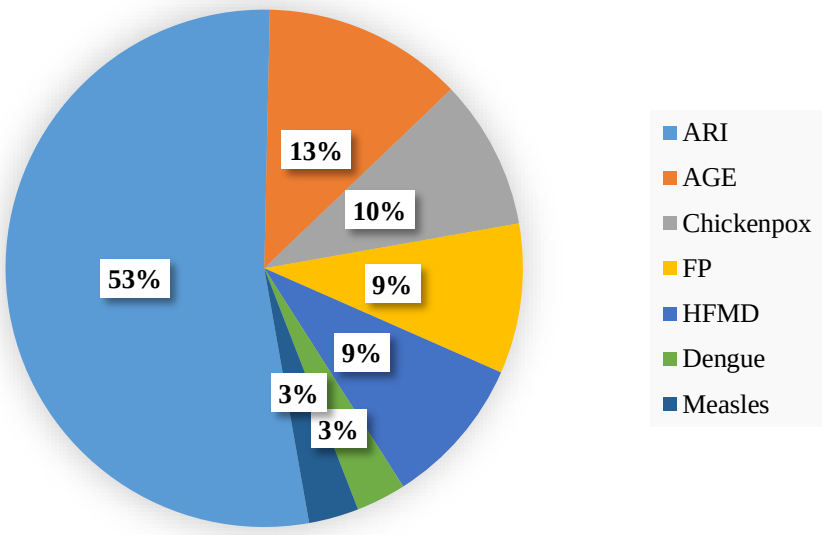

Figure 9: Distribution of events reported by type

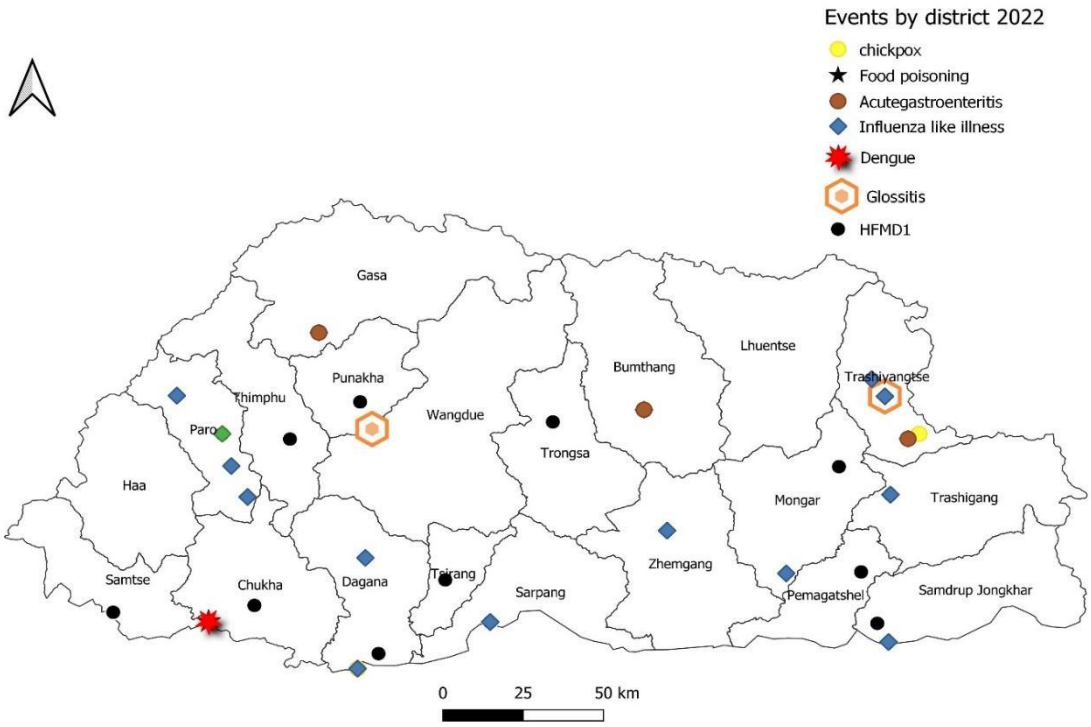

Figure 10: Distribution of the events by districts

Discussion and conclusion

The overall timeliness of reporting of notifiable diseases (63.0% vs. 57.0%) and completeness (89.0% vs. 81.0%) has improved compared to 2020 and 2021. The underreporting in 2020 and 2021 was due to the closer of health centers and remobilized of focal persons to respond SARS-CoV-2 pandemic.

While comparing the number of cases reported by year, we observed that the total number of notifiable diseases reported by reporting centers in 2020 and 2021 (**Figure 2**) dropped by about 40.0% in 2020 and 2021 as compared to 2019. This suggests a decline in the utilization of health services by the population during the COVID-19 pandemic. The reporting of notifiable diseases may have also been influenced by the suspension of some Out-Patient Department (OPD) services amid the lockdown measures in 2020 and 2021. Therefore, it is possible that some notifiable disease cases were not reported or detected during this period, which could pose challenges for disease surveillance and control.

Respiratory illnesses continued to lead to the highest reported cases of notifiable diseases. The increased trend of respiratory illness observed during the epi-week 41 through 44 was attributed by the frequent outbreak of seasonal Influenza-like illness.

Similarly, the high number of dengue cases notified to NEWARS through the immediate reporting system was due to an outbreak of dengue fever at Phuntsholing. The dengue cases reported through the immediate reporting system had alerted the Dengue outbreak at Phuntsholing and the response was initiated in time.

Overall the number of respiratory diseases outbreak had dropped in the past three years. There was a huge reduction of outbreaks reported in 2020, 2021 and the first half year of 2022. While the cases of respiratory diseases have declined in general over the past yearscould be attributed to the strict implementation of covid-19 protocols such as the use of facemasks, physical distancing, and hand washing. Though the highest number of outbreaks reported in the system was of HFMD, however, there was a reporting bias because HFMD is not a common illness in the community, as well as it is not in the list of weekly and immediately reportable diseases, the reporting center had reported even a single case as an event.

Another interesting finding was the variation in disease incidence by age group and district. The most commonly affected age group affected for the notifiable disease was pediatric cases, Similarly, some districts such as Samtse, Sarpang, and Thimphu reported higher numbers of respiratory and diarrheal diseases than others. This could indicate differences in local environmental or socio-economic factors that affect disease transmission and susceptibility.

In conclusion the overall notifiable diseases reporting status improved after a pandemic, this could be because of strengthening communication with healthcare workers on the revival of diseases notification. Therefore, continuous monitoring and follow-up must be carried out with healthcare centers with low reporting rates.

Awareness especially ahead of peak seasons do help in reducing the disease morbidity and mortality through timely response to any diseases outbreak. There is also a need to regularly train and update healthcare workers on the list of diseases that needs to be notified and the system updates. Integration of NEWARS with another laboratory-based surveillance system of RCDC

will help in linking the epidemiological profile of the patient to their laboratory reports thereby further strengthening the surveillance system.

Further, analysis to understand the implementation and impact of the surveillance system in the healthcare setup will be helpful in improvising the relevancy and timeliness of the early warning system to reduce morbidity and mortality.

Reference

1. NEWARS guideline, RCDC, accessed from http://www.rcdc.gov.bt/web/wp-content/uploads/2022/08/NEWARS-Guideline-V2-2018.pdf

Sentinel Surveillance for Diarrheal Etiological Agents, 2022

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Background

Diarrheal disease is the second leading cause of child mortality and morbidity in the world resulting mostly from contaminated food and water. Globally, there are nearly 1.7 billion cases of childhood diarrheal disease every year. Each year diarrhea kills around 525,000 children under five (1). It is estimated that there are 800,000 fatalities from diarrhea globally every year, and most diarrhea cases exist in South Asia and Sub-Saharan Africa (2). Diarrhea can occur from a variety of causes, but the most common causes are the consumption of food or drinks that are contaminated with pathogens such as bacteria, viruses, and parasitic organisms or toxins.Rotavirus and diarrheagenic *Escherichia coli* are the two most common etiological agents of moderate-to-severe diarrhea in low-income countries.

Diarrhea is the second-highest notified disease in Bhutan. A total of 23,272 acute watery diarrhea (AWD) and 2112 Acute Bloody Diarrhea (ABD) cases were reported in 2022. Among them, 1175 cases were reported in children less than 5 years of age (3). The Enteric and Invasive Disease Laboratory (EIDL) in the Royal Center for Disease Control (RCDC) (4) conducts and performs diarrheal sentinel surveillance aimed at elucidating the prevalence of bacterial, viral, and parasitic pathogens associated with diarrhea; monitoring the AMR pattern; detecting enteric outbreaks and provide scientific information on intestinal enteric pathogens of public health concern.

Methodology

Sentinel setting

The diarrheal sentinel surveillance is a laboratory-based passive surveillance program designed to identify, serogroup/serotype, and molecular subtype (by pulsed-field gel electrophoresis (PFGE) and detect antimicrobial resistance in enteric bacterial pathogens detected. There were 12 sentinel sampling sites involved in this surveillance **(Figure 1)**. The sentinel sites were

expected to ship approximately 100 samples each in a year, including samples from both the outpatient and in-patient department.

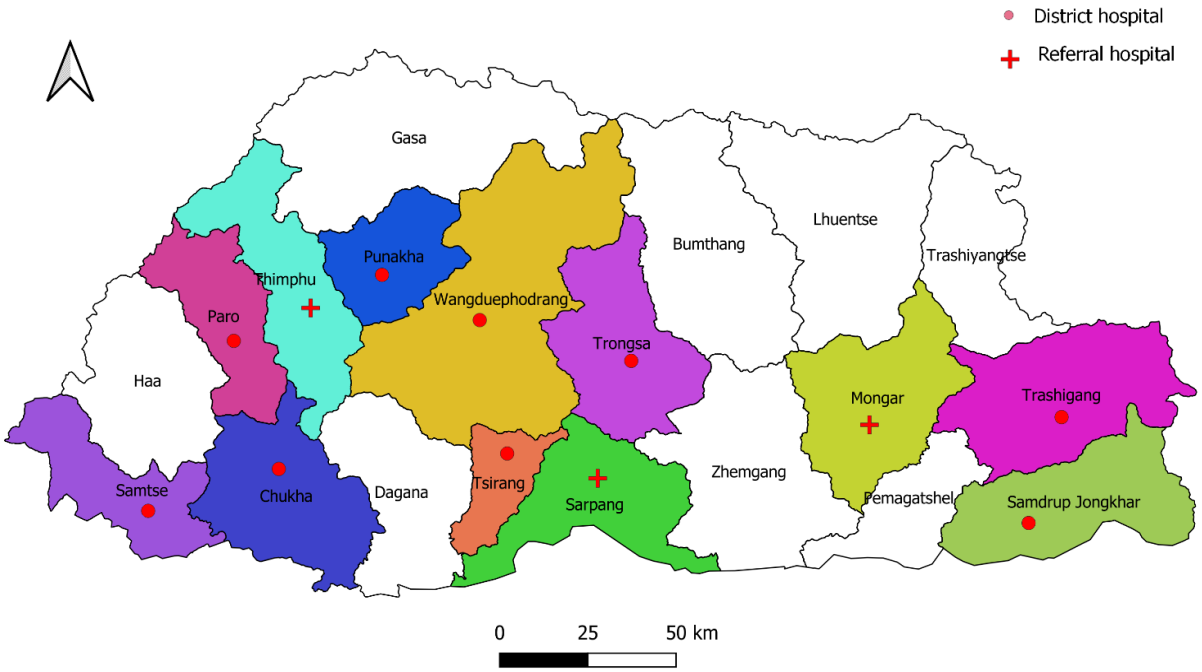

Figure1: Sampling sites

Case definition

Any patient with the passage of three or more loose stools in the past 24 hours were enrolled in the surveillance.

Sampling collection and shipment

Two aliquots (one sample divided into two) of fecal specimens were collected from each patient by a laboratory staff or a nurse at each of the sentinel sites. The sample was aliquoted in cryovials without preservatives and stored at -20 to -40˚C prior to shipping and processing for virological testing. For bacteriological testing, the fresh whole stool was collected in a sterile container and was placed in Cary-Blair Medium at 2 - 4˚C for transportation to RCDC within one week or every weekend. Patients who had taken antibiotics before the sample collection were excluded to be enrolled for the surveillance. Collected specimens were then packed and transported in ice boxes to RCDC in triple packages as prescribed by IATA.

Sample testing

Enteropathogen testing

A stool wet mount was prepared for microscopic examination and simultaneously, inoculated for culture at the sites having microbiology facilities. Upon receiving at the EIDL, all the samples that have not been microscopically examined or cultured were subjected to examination for bacterial, viral, and parasitic agents by culture, serology, and polymerase chain reaction (PCR).

Bacterial pathogens such as *Shigella, Salmonella, Vibrio, Aeromonas, Plesiomonas,* and *Campylobacter*were identified by standard microbiological techniques whereas *E-coli* isolates were then subjected to multiplex PCR to determine the pathotype of *E-coli*. Common intestinal parasites: *Giardia,Cryptosporidium,* and *Entamoeba histolytica,* and common enteric viruses: *rotavirus, and astrovirus,* were identified using commercial Enzyme-Linked Immunosorbent Assay (ELISA) kits.

Antibiotic Susceptibility Testing

The antibiotic susceptibility of the bacterial isolates was performed following the modified Kirby-Bauer disc diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI) for the zone interpretation of the susceptibility disc [5]. The zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimeters (mm). The susceptibility of each antibiotic was semi-quantified by comparing the size of the zone of inhibition and reported as susceptible (S), Intermediate (I), and Resistant (R).

Results

Of the 1200 expected case enrollment and sample collection with diarrhea, 382 (32.0%) sampleswere enrolled from January 2022 to December 2022 and analyzed. Among the sentinel sites, CRRH and JDWNRH enrolled the highest number of patients for the surveillance (**Figure 2**). Overall, 71.0 % of the patients who were enrolled were from OPD and 30.0% were from the IPD, of which 54.5% were males and 44.5% were females. The majority of the patients were from the age group of < 9 years(57.0%) followed by elderly patients > than 50 years.Of the total specimens, 67.0% constituted loose stool, 32.0% watery stool with mucus positive only for 19.0%, and 3.0% samples represented bloody stool. Among the cases, 2.4% (9/382) reported having consumed suspected contaminated food (**Table 1 &2**).

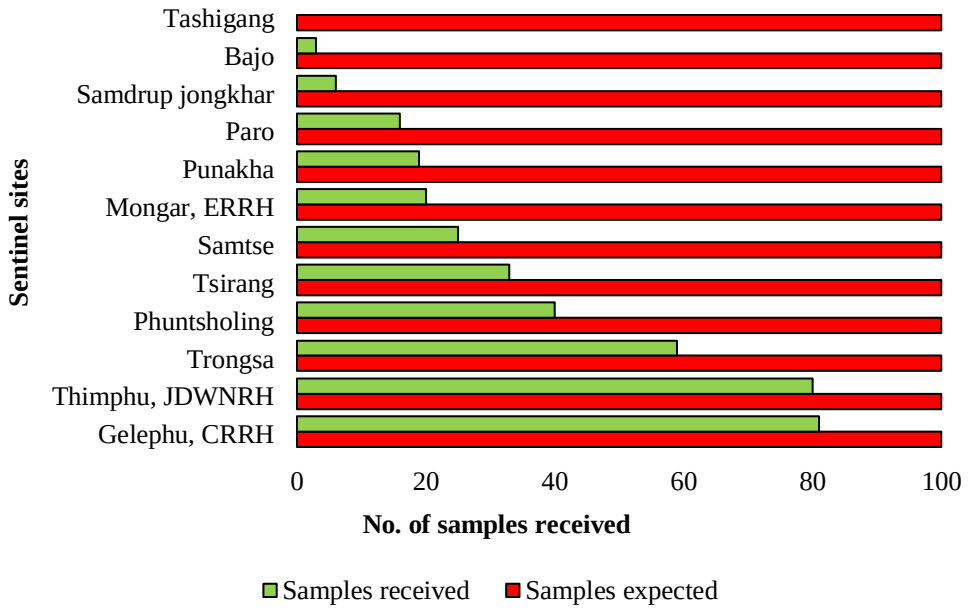

Figure 2: Number of samples received compared to the expected samples

Table 1: Socio-demographic characteristics of the surveillance participants (n=382).

Characteristics	Numbers (%)
Gender	
Male	106 (56.0)
Female	83 (44.0)
Age Group (Years)	
0 – 9	94 (50.0)
10-19	14 (7.0)
20 - 29	17 (9.0)
30 - 39	18 (10.0)
40 – 49	4 (2.0)
50 and above	42 (22.0)
Mean ± SD age	1.7 ± 2.3
Visit status	
IPD	80 (42.0)
OPD	109 (58.0)
Occupation	
Civil servant	12 (3.1)
Private	89 (23.3)
Student	59 (15.4)
Farmers	31 (8.1)
Dependent*	146 (38.2)
Housewife	45 (11.8)

*Dependent indicates small children <5 years of age

Table 2: Clinical characteristics of the sample (n=382)

Characteristics	Numbers (%)
Consistency	
Loose	258 (67.0)
Watery	121 (32.0)
Bloody	3 (1.0)
Color	
Yellow	203 (53.0)
Brown	81 (21.0)
Green	70 (18.0)
Black	18 (5.0)
Red	2 (0.5)
Clay	2 (0.5)
Other	6 (2.0)
Mucus	
Positive	73 (19.0)
Negative	309 (81.0)
Blood	
Yes	10 (3.0)
No	372 (97.0)

Cultures showed growth of Diarrheagenic *E. coli* (DEC) in 58 cases, *Salmonella* species in nine cases, *Aeromonas* species in eight cases, and Shigella species in 10 cases. Two cases of *Giardia lamblia* were reported from a total of 69 samples tested. *Rotavirus* (38/275) was the common enteric virus detected followed by *Norovirus* (7/71) and *Astrovirus* (1/69). A single case of co-infection with bacteria-virus: *shigella* and *rotavirus* was identified. Among 327 of the identified *E-coli*, 58 cases were positive for DEC. The most frequent pathotype of DEC was *EAEC* 39 (12.0%), followed by *ETEC* 10 (3.0%), EPEC six (2.0%), and EHEC three (1.0%). Children nine years and below were the most affected age group irrespective of the agent identified (**Figure 3)**

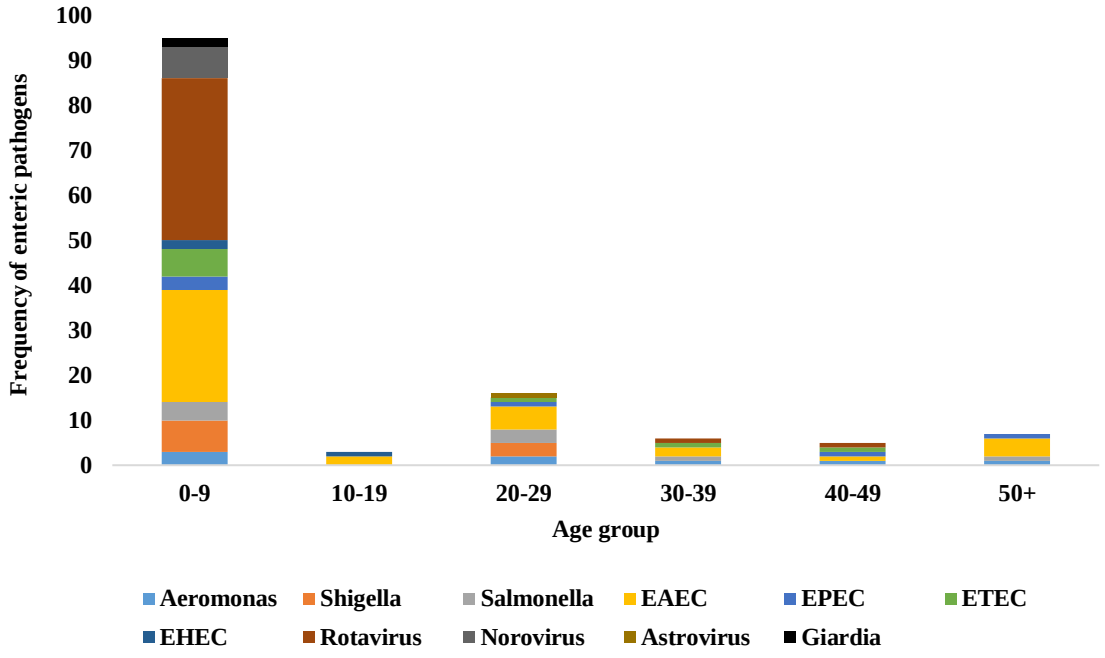

Figure 3: Agent distribution by age-group

Seasonal distribution of agents

Enteric bacteria were a common cause of diarrhea throughout the year, with the incidence peaking during summer and monsoon seasons. A hike in the incidence of enteric viruses was seen in autumn continuing into the early winter. Moreover, two cases of the enteric parasite were reported in the same season (**Figure 4**).

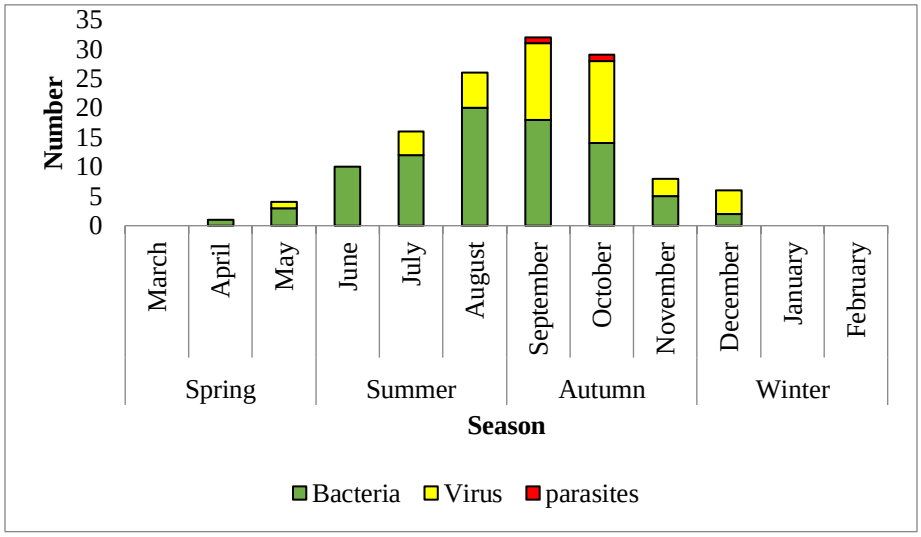

Figure 4: Seasonality of the enteric pathogens

Antibiotic susceptibility

The antimicrobial susceptibility tests showed that the enteric bacteria exhibited high levels (above 60.0%) of resistance to Amoxycillin/Ampicillin and Cephazolin type antibiotics. However, the resistance rates to the newer generation antimicrobials as detected in our surveillance are still very low. Enteric pathogens showed a high level (90.0%) of susceptibility to Gentamycin, Chloramphenicol, and Ceftriaxone antibiotics. Ciprofloxacin exhibits more than 50.0% intermediate results to the isolates tested **(Figure 5)**

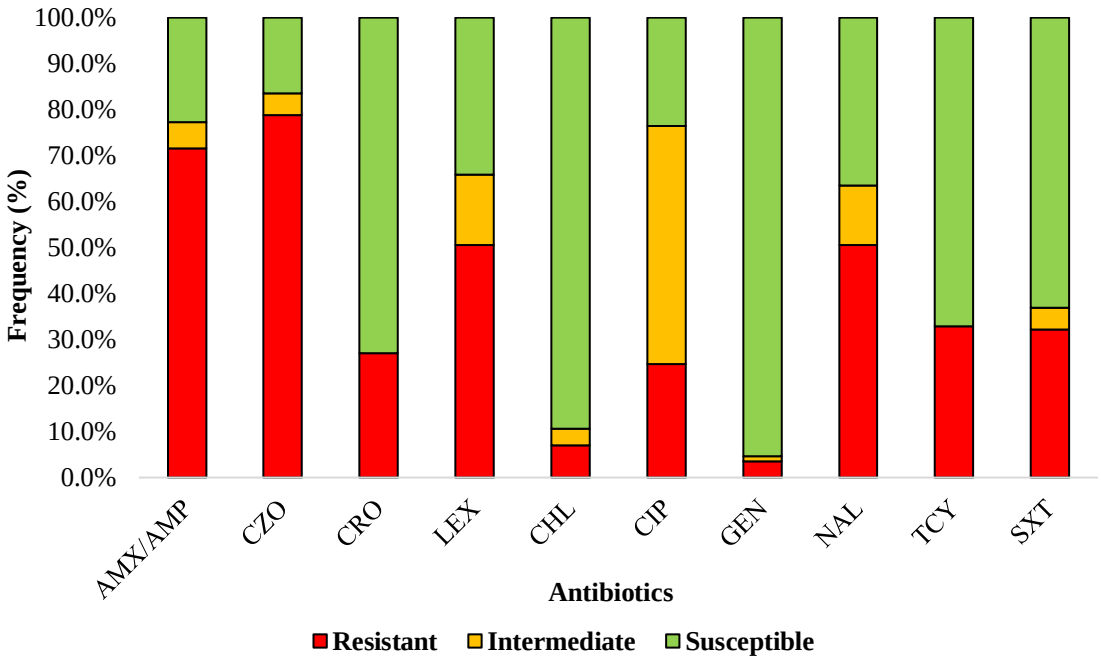

Figure 5: Antibiogram of enteric bacteria pathogens

AMX (Amoxicillin), AMP (Ampicillin), CZO (Cephazolin), CRO (Ceftriaxone), LEX (Cephalexin), CHL (Chloramphenicol), CIP (Ciprofloxacin), GEN (Gentamycin), NAL (Nalidixic Acid), TCY (Tetracycline), SXT (Trimethoprim and sulfamethoxazole)

Discussion

An adequate sample as expected (~ 100 samples/year/site) could not be collected or submitted for surveillance due to staff inadequacy at the surveillance centers since most were engaged in COVID-19 pandemic and other flagship programs. The distribution of enteropathogens showed that *Shigella, Salmonella, DEC,* and *Rotavirus* were the most potential enteric pathogen causing infected diarrhea than parasitic agents. Additionally, two cases of Giardia lamblia were also reported. From this surveillance, 57.0% (n=219) of the patients enrolled with acute diarrhea were under < 9 years of age. Diarrheal disorders in childhood account for a large proportion of childhood deaths, making it the second most common cause worldwide. Viral and bacterial enteropathogens are the causes of gastroenteritis in both developed and developing countries [6]. The seasonal prevalence pattern varied among the detected pathogens with viruses being more prevalent in winter and autumn months (dry – colder temperatures), while bacteria were more prevalent in summer months. The seasonal pattern is likely associated with environmental factors, such as ambient temperature, precipitation, and humidity, all of which may influence exposure frequency, agent vitality, host immunity, and pathogenicity. One case of dual (bacterial-viral) co-infection was reported from this finding. Suspecting possible multiple infectious etiologies and diagnosis of the right causative agent(s) can help in the better pharmacological management of acute diarrhea [7]. There were less or no reported cases in January until the first week of April due to the pandemic lockdown imposed during that time.

Among the tested antibiotics, Gentamycin, Chloramphenicol, and Ceftriaxone were found to be the most effective drugs to inhibit the invitro growth of these isolates. Thus, these drugs could be used for empirical treatment of these enteric pathogens in an area where a microbiology culture facility is not available. However, a substantial number of isolates were resistant to commonly prescribed antibiotics raising concerns for MDR. Strengthened antimicrobial surveillance is warranted to observe the potential emergence of enteric bacteria resistant to the antimicrobials that are presently important in the treatment of severe infections (8).

Limitations

1. The unavailability of the test kits resulted in incomplete testing for all enteric pathogens which probably resulted in an underestimate of positivity for enteric viruses.
2. Lack of consistency in data reporting from the sentinel sites.

3. In this surveillance, antibiotic susceptibility for *Campylobacterspecies*was not performed. Moreover, the resistance mechanisms of isolated organisms were not characterized due to limited testing facilities.
4. Seasonal incidence of enteric pathogens needs further elucidation.

Conclusion

This hospital-based sentinel surveillance highlighted the overall burden of major bacterial and viral pathogens in acute diarrhea. Nevertheless, the results suggested that DEC strains such as *EAEC, ETEC*, and *EHEC*, which are normally not screened routinely, should also be suspected in childhood diarrhea. However, more causative agents of acute diarrhea among children require further exploration. Seasonal variation was seen in the occurrence of the etiological agents. Laboratory monitoring of drug susceptibility of enteric pathogens appears necessary to formulate antibiotic policy for treating diarrheal illnesses. These findings are expected to help both clinicians in the treatment of diarrheal disease as well as public health officers in implementing public health measurements on the control and prevention of diarrheal disease.

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Surveillance of Measles and Rubella in fever with rash syndrome, 2022

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Infectious Disease Serology Laboratory

Introduction

Measles is a highly contagious disease caused by *measles virus,*a single-stranded, negative-sense, enveloped RNA virus of the genus *Morbillivirus* within the family *Paramyxoviridae* (1). Measles can easily spread to unvaccinated or under-vaccinated populations. Symptoms usually include a high fever with rash which begins about 10 to 12 days after exposure to the virus, and lasts 4 to 7 days. Other accompanying symptoms during the early phase are a runny nose, cough, red and watery eyes, and small white spots inside the cheeks. Approximately 14 after exposure to the virus, rashes erupt on the face and upper neck that gradually spreads to the extremities (2). Measles affects mostly children under the age of 5 years. Serious complications are more common in children under the age of five. The most serious complications include blindness, encephalitis (an infection that causes brain swelling), severe diarrhea and related dehydration, ear infections, or severe respiratory infections such as pneumonia. Severe measles is more likely among poorly nourished young children and immunocompromised individuals (3).

Global epidemics of measles were frequently reported before the introduction of the measles vaccine in 1963 and widespread vaccination. Each year, estimated deaths of 2.6 million were recorded. WHO reports a decrease in measles death by 73.0% from an estimated 5,36,000 in 2000 to 1,42,000 in 2018 (4). In 2021, nearly 40 million children were left at risk for measles by missing a life-saving vaccine dose. 25 million children missed their first dose of measles vaccine, and an additional 14.5 million children missed their second dose (4).

Bhutan's Expanded Programme on Immunization (EPI) was launched on 15 November 1979 with an objective of reducing the incidences of six vaccine-preventable diseases, of which measles is one. In 2006, rubella vaccine was introduced into the routine immunization schedule. With an aim to achieve 100% immunization coverage of all target populations, Bhutan today boasts of a sustained 95.0% coverage on routine immunization (6, 7). Bhutan was verified as having eliminated measles and controlled rubella in 2018 (6). In order to sustain elimination or detect any re-emergence of cases, a robust surveillance system is essential. Measles and rubella

are notifiable diseases and reporting of the incidences is captured by the National Early Warning and Alert Response Surveillance (NEWARS). Measles and Rubella surveillance is a nation-wide surveillance that encompasses epidemiological and clinical data collection along with laboratory sample testing.Measles and Rubella laboratory confirm case is consider as an outbreak and active case finding is done to establish the source of infection.

Methodology

Measles and Rubella surveillance is the nationwide case-based surveillance and among the immediately notifiable list of diseases and syndromes. Any cases visiting hospitals and Public Health Centers that meet case definition are enrolled for MR investigation and notified in the NEWARS information system. WHO case definition of measles and rubella is adopted, according to which, any person presenting with fever and rash (non-vesicular) in whom a clinician suspects measles infection irrespective of age, were enrolled as a suspected measles/ rubella case. As proactive surveillance, close contactof suspected cases was listed and samples were collected.

Contact tracing

Contact tracing was done for all sero-positive cases. To avoid delay in investigation, contact tracings were initiated as soon as any samples tested positive for measles or rubella IgM without waiting for the molecular test results.

Specimen Collection and shipment

Blood specimens are collected from a suspected case for serological testing of measles/ rubella virus. A throat or a nasopharyngeal specimen is also collected in viral transport media and shipped along with the blood specimen to the National Measles and Rubella Reference Laboratory in RCDC within five days from the date of collection.

Laboratory testing

Serological detection of measles/rubella IgM antibody is the confirmatory test for diagnosis of the infections. Anti IgM Enzyme Linked Immunosorbent Assay (ELISA) for measles and rubella are performed in parallel for all the samples at IDSL using Euroimmun® kits. Tests are performed as per manufacturer's instructions.

As per WHO's initial recommendation, molecular detection using quantitative real-time reverse transcriptase Polymerase Chain reaction was to be performed for only samples that tested positive by ELISA method. However, since Bhutan has achieved elimination of measles and targeting elimination of rubella, the country is required to perform qRT-PCR for every throat swab/ nasopharyngeal swab following the protocol by United States- Centers for Disease Control (US-CDC). Positive samples are subjected to end-point PCR testing for sequence analysis that will ultimately be used for phylogenetic analysis and determining molecular epidemiology.

Samples are referred to the WHO Regional Referral Laboratory (RRL) for measles and rubella for the purpose of sequencing and also serological result validation.

Results

***Laboratory results**:*

A total of 148 samples were collected in 2022, of which 120 samples were collected from the suspected cases notified and 28 samples collected through contact tracing. Seven samples (2 male and 5 female) tested positive for measles IgM antibody while four (3 male, 1 female) tested positive for rubella virus. The sero-positive cases were mostly children aged <10 years old (80%). Molecular detection by qRT-PCR for all throat swabs received in RCDC did not reveal any positive result for both MeV and RuV.

The cases were distributed across the country with some clustering in the western and central regions (**Figure 1&2**).

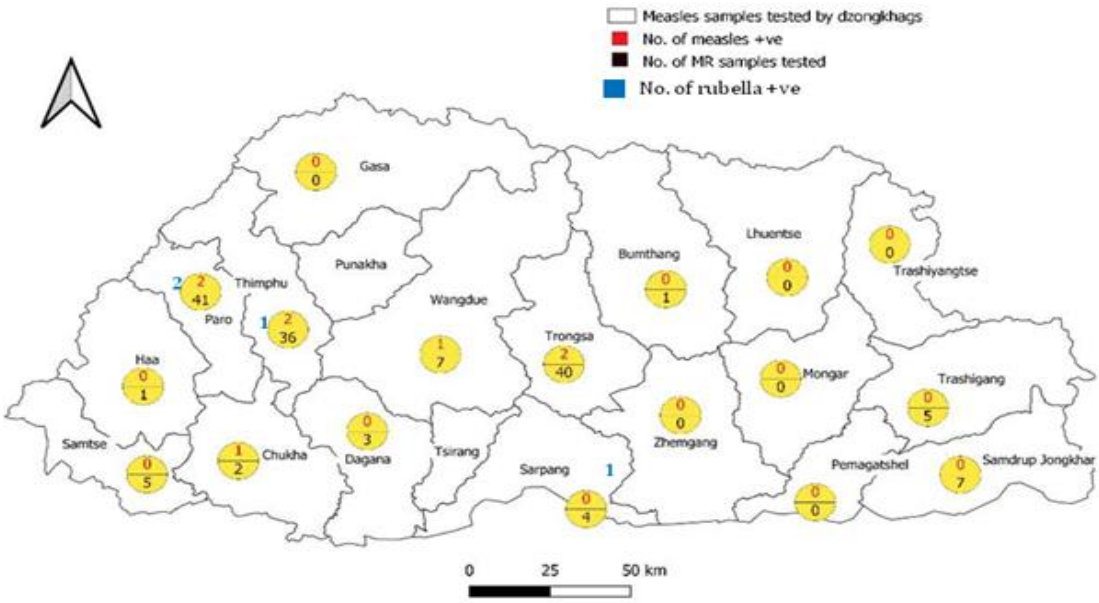

Figure 1: Map showing distribution of sample collection along with sero-positives for MR, 2022

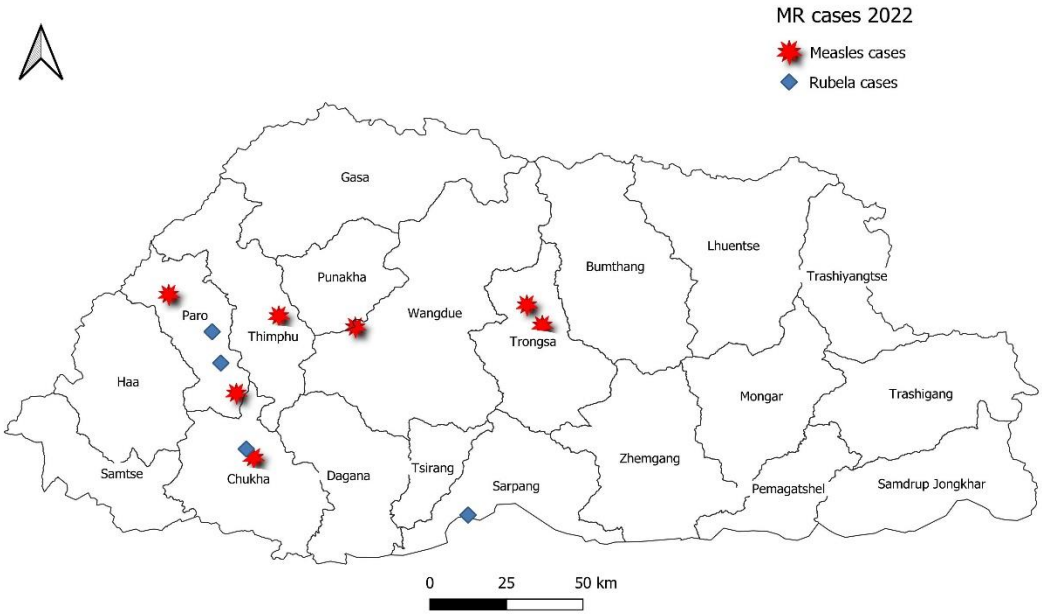

Figure 2: Map showing distribution of sero-positives for MR, 2022

Monthly notification:

Suspected cases were notified throughout the year from different parts of the country. Months of June, July and August, which corresponds to the summer and monsoon season in Bhutan, saw the maximum cases (40.0%)**(Figure 3).**

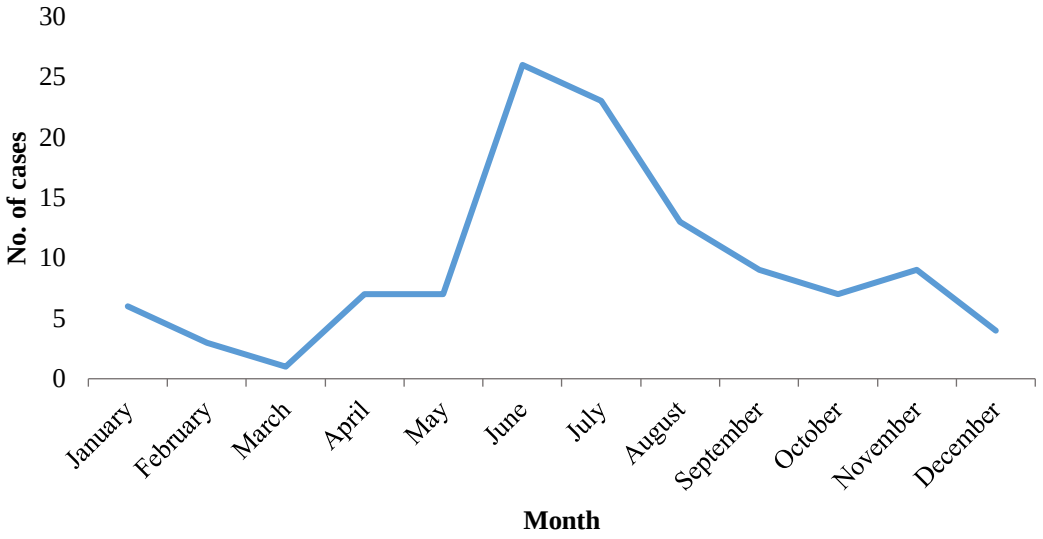

Figure 3: Monthly distribution of notified MR suspected cases

Age and Gender

In 2022, the most affected age group was <1 year of age (38.0%), followed by 1- 4 years (31.0%). Overall, fever with rash syndromes was more common in males (53.3%) than in females (47.0%) with a rate ratio of 1.1:1 **(Figure 4)**

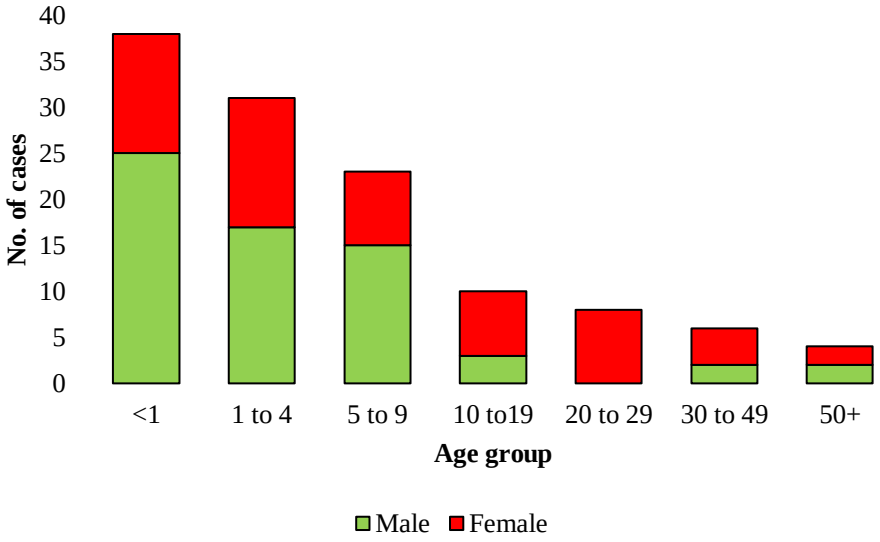

Figure 4: Age distribution of notified MR suspected cases

Vaccination status of positive cases

The serologically positive cases were assessed for their vaccination status. Except for the adults who did not have their vaccination records and the infant below nine months of age, the rest had all records of vaccination (**Figure5**). As for rubella sero-positives, a child of 11 months had missed the routine vaccination of measles, mumps and rubella (MMR).

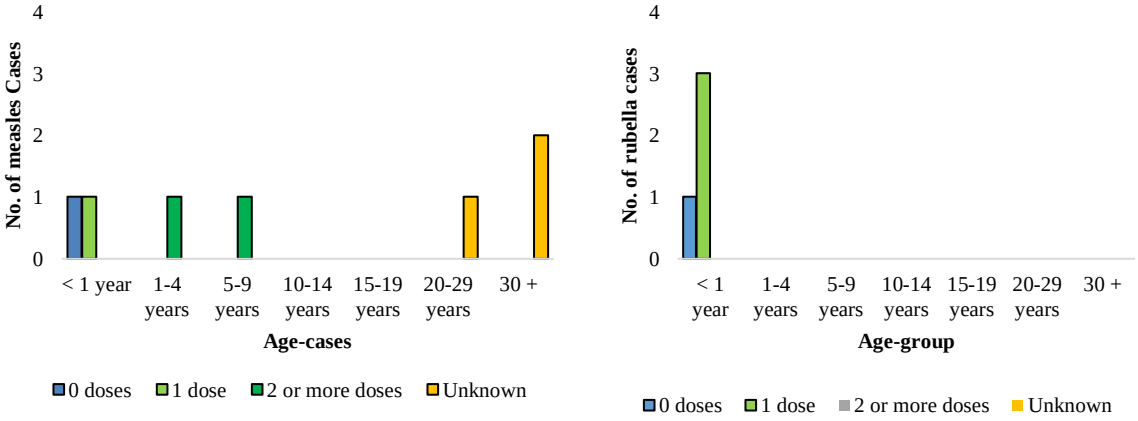

Figure 5: Vaccination details of serological positive cases for measles and rubella

Surveillance quality indicators

The laboratory surveillance quality indicators were assessed in 2 aspects, timeliness of specimen transport and timeliness of reporting lab results.

Number of days taken for samples to reach RCDC after collection range from 0 to 58 days, with an median of 7 days. Only 46.0% (68/148) of the samples fulfill the WHO criteria of reaching the laboratory within 5 days of collection **(Figure 6)**.

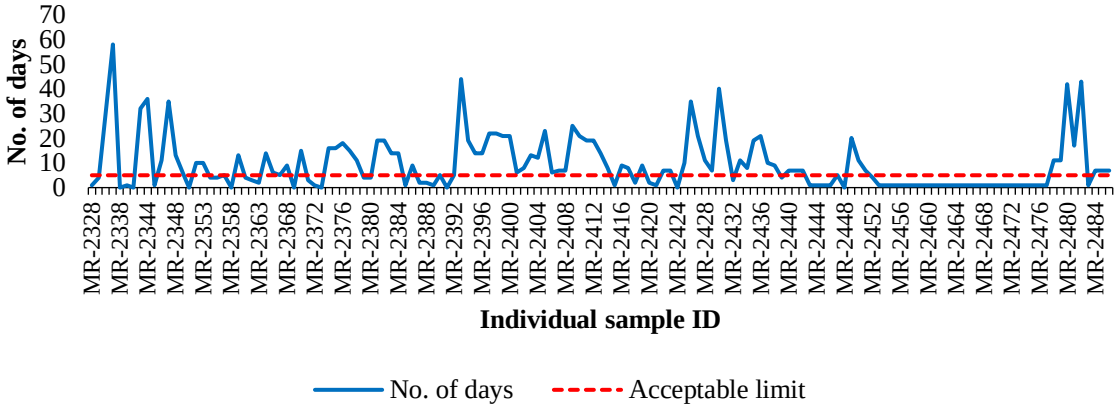

Figure 6: Timeliness of specimen transport

Similarly, the number of days taken for completion of the test after receiving samples range from 0 to 61 days. The mean and median for this indicator are 0 and 3.7 respectively.Percentage of samples tested within 4 days of receiving in the laboratory amounts to 86.5%**(Figure 7)**.

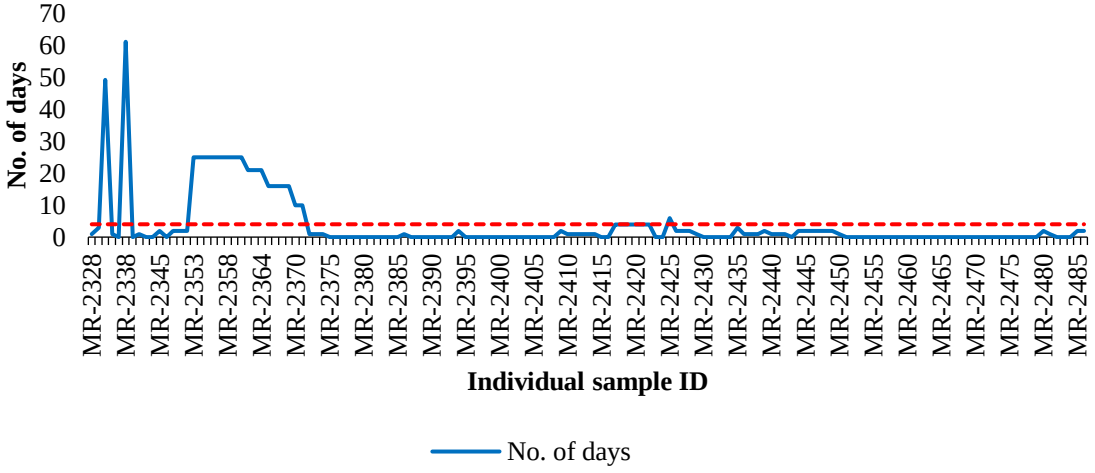

Figure 7: Timeliness of reporting laboratory results

Discussion

Our surveillance findings indicate that most of the fever with rash cases as well as sero-positive cases of measles and rubella occurred in pediatric age groups. The finding is in concordance with the age profile of measles and rubella cases in countries world-wide, whereby the high incidence in infants is attributed to inability of vaccines to protect these populations (8).

There were seven measles and four rubella cases detected in 2022 through serological testing. These cases were sporadic and isolated. Investigation of these cases did not reveal any epidemiological linkages. PCR test results of the throat swabs collected from all the cases were negative, probably indicating that the cases were passed their viremia phase. Except for an eleven month old child who missed his first dose of vaccine and the adults who did not have vaccination records, all others were adequately vaccinated. Infections in vaccinated cases are not unknown. However, they are said to have milder symptoms and less contagious as compared to the unvaccinated individuals (9)

Cross-reactivity of IgM assays with antibodies of other infections and interference from rheumatoid factors have been previously noted (10). On the other hand, it is well established that the predictive value of a test changes as the prevalence of a certain disease changes. False positive results are proportionately higher in low prevalence settings due to the lower predictive values of a test 10). Since Bhutan has a low or almost zero prevalence of measles and rubella as per the data of the past years, the laboratory was recommended by IVD-SEARO, WHO to perform PCR for MeV and RuV for all throat swabs collected irrespective of the serological test results. This recommendation was also made in order to sustain the elimination and controlled status of measles and rubella respectively. Anti-IgG avidity tests to measles and rubella viruses were carried out in the Regional Reference Laboratory, Thailand to determine whether the infections were of recent or past. Test results of 2 measles and 4 rubella samples revealed low-avidity, indicating that these IgG were recently formed either due to infection or vaccination (11). Further confirmation by capture ELISA will be performed by RRL and the results are awaited.

On the aspects of quality indicators, IDSL has achieved WHO recommended standard of testing at least 80.0% of samples within 4 days of receiving in the laboratory (12). There were some delays in testing noted towards the beginning of the year which occurred as a result of lock-down

imposed due COVID-19 outbreak in the community, and later due to shortage of reagents for a brief period of time.

There was a huge delay in samples reaching RCDC after collection. Although RCDC recommends hospital laboratories to make weekly shipments of samples, around 64.0% of the samples had reached RCDC after more than 5 days. While we cannot avoid the difficulties in transportation due to geographical conditions and uncertain shipment mechanisms, we are yet to ascertain the cause for those samples that have been delayed for too long. Timely shipment and proper cold-chain maintenance are crucial as these factors are known to affect the test results (12).

Besides the delay in shipment, there are several other limitations to our surveillance findings. Firstly, very few samples come with complete clinical and epidemiological information. Most of the cases would have attended out-patient clinic, or would have been discharged if admitted by the time samples reach RCDC, making it difficult for follow-up. IDSL has also observed different versions of case investigation forms being used in different health centres. Secondly, although shipments are made in cold-chain boxes with ice-packs, temperature maintenance during transportation is not regulated.In order to take the surveillance forward and sustain the status of elimination and control for measles and rubella respectively, it is imperative that we look into each of the limitations and resolve accordingly.

Conclusion

Considering the highly contagious nature of the diseases, and the feasibility to eliminate them with good control strategies, it is important that we have a good surveillance system for measles and rubella in the country. The current surveillance needs to be up-scaled with checks and controls in place in order to achieve all the quality indicators of the surveillance.

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Drug Resistance Surveillance of Tuberculosis in Bhutan, 2022

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Introduction

According to the global TB report, 6.4 million people were newly diagnosed with TB in 2021. This is an increase of 4.5% from the previous year. The year also saw an increase in drug resistant TB to a total of 450,000 cases of rifampicin resistant (RR-TB). The report states concern regarding the reversal of End TB Target due to COVID-19 pandemic. This is also indicated by the drop in reporting rate since 2019 (1).

In Bhutan, the national TB control program reported a total of 858 TB cases in 2021(2). This includes all pulmonary and extra-pulmonary cases. The younger age group was commonly affected with the average age being 35.8 years (National TB Control Program, Bhutan). As per the National Tuberculosis Reference Laboratory's (NTRL) drug resistance surveillance report of 2021, 511 bacteriologically confirmed samples were received at NTRL for culture and drug sensitivity test (DST). Overall, the prevalence of multidrug resistant tuberculosis (MDR-TB) in 2021 was 11.0% (n=55). The prevalence of MDR-TB in new cases & previously treated TB cases was 11.1% & 9.0% respectively (3).

The drug resistant surveillance was started in 2010 with the main aim to monitor the multi-drug resistant TB trend and burden in the country to facilitate the national program in policy decision and interventions.

Methodology

Case Enrolment

All bacteriologically confirmed cases confirmed by district hospitals & NTRL are enrolled for surveillance of MDR-TB. Clinical information including patient demographic and case history was used to classify new or previously treated cases while determining drug resistance. Patient details were extracted from the web-based online Tuberculosis Information and Surveillance

System (TbISS) and manual register at NTRL. WHO standard definition was used to categorize the case as follows:

Bacteriologically confirmed TB case: is one where a biological specimen is positive by smear microscopy, culture, or WHO-recommended Rapid Diagnostics such as Xpert MTB//RIF and Line probe assay.

Clinically Diagnosed TB case: A clinically diagnosed TB case is one who does not fulfill the criteria for bacteriological confirmation but has been diagnosed with active TB by a clinician or other medical practitioner who has decided to give the patient a full course of TB treatment.

New case: A patient who has never taken treatment for TB or a patient who has taken anti - tuberculosis drugs for less than one month OR, has no evidence of such history from adequate documentation if available with patients or hospitals. New patients may have positive or negative bacteriologically and may have a disease at any anatomical site.

Previously treated case: Patients who have received one month or more of anti-TB drugs in the past are classified as "previously treated" patients. They may have positive or negative bacteriologically and may have the disease at any anatomical site.

MDR-TB: Resistance of Mycobacterium tuberculosis strains to at least isoniazid and Rifampicin

RR-TB: Rifampicin Resistant TB detected with XPERT MTB/RIF

Laboratory Methods

Sampling strategy: Samples from all suspected TB cases are screened at district hospitals using microscopy and GeneXpert test. All(100.0%) smear-positive cases from microscopy centers across Bhutan and positive TB cases detected using Xpert MTB//RIF are shipped to NTRL, for culture and drug sensitivity testing (DST) of additional drugs (Isoniazid, Fluroquinolones& injectables). In addition, based on the clinician's judgement, samples from high risk groups including contact tracing, extra-pulmonary samples and sputum samples received for TB screening for VISA purposes are received at NTRL for culture and DST.

Sputum collection and transportation: Two sputum samples are collected from each patient and transported to NTRL using triple packaging and maintaining a cold chain. Sputum samples from each patient were accompanied by a sample shipment form.

Sputum culture and identification: Samples are decontaminated using NALC-NaOH method and inoculated on two slopes of egg-based Lowenstein-Jensen (L-J) and in liquid media using

the Mycobacteria Growth indicator tubes (MGIT). The solid cultures were then incubated at 37ºC and monitored weekly for growth up to 8 weeks and liquid culture in the MGIT 960 machine and monitored daily for growth & incubated for up to six weeks after which a culture was reported as negative if no growth was observed. For the positive cultures, identification of *Mycobacterium tuberculosis* complex (MTBC) was done based on the phenotypic appearance of colonies on the solid media and by rapid chromatographic identification test which detects mycobacterium protein MPT64. Microscopy was performed from liquid-positive culture to check for cord formation.

Drug Susceptibility Testing (DST)

DST was conducted at district hospitals using XPERT MTB/RIF and samples were referred to NTRL for testing additional drugs using Line Probe Assay & Liquid DST. For conducting DST, the following facilities are used in GeneXpert sites and NTRL (**Table 1**).

Table 1: Diagnostic tests used for drug sensitivity testing of TB samples

Facility	**Availability site**	**Drug Tested**	**Sample Type**
Xpert MTB/RIF	High burden hospitals*	Rifampicin	Sputum/extra-pulmonary samples
LineProbeAssay1st Line)	NTRL	Rifampicin & Isoniazid	Sputum/extra-pulmonary samples/Culture colonies
Liquid DST	NTRL	Isoniazid,Rifampicin, Ethambutol& streptomycin	Culture colonies
Line Probe Assay (2nd Line)	NTRL	Fluroquinolones& injectables	Sputum/extrapulmonary samples/Culture colonies

*Samtse, Phuntstholing, Gidakom, Nanglam, Mongar, Trashigang, Thimphu, Gelephu

DST for first line drugs were performed using GeneXpert test at district hospitals using sputum

Data collection, Management and Analysis

All data for samples shipped and patients treated for TB are extracted from TbISS for analysis. The variables in TbISS include the patient's demographic details, treatment history, clinical details and laboratory information. Validation of data for all TB treated patients is done by comparing with the TB registers at district hospitals. Descriptive data analysis was done for samples received at NTRL and MDR-TB patients detected using Liquid DST, LPA and GeneXpert tests.

Results

In 2022, NTRL received 1181 samples for culture and DST. Majority of the samples were received from hospitals in Thimphu dzongkhag (n=588) followed by Wangduephodrang(n=127) &Chukha dzongkhag(n=116) (**Figure 1**)

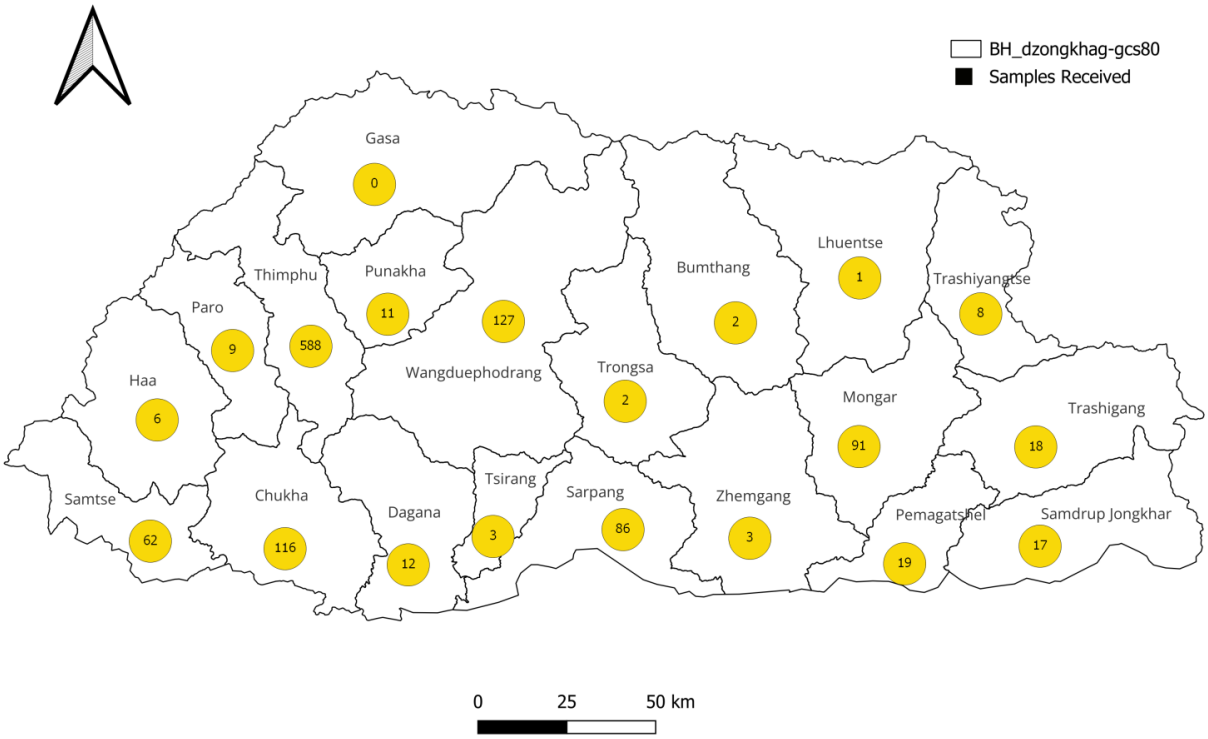
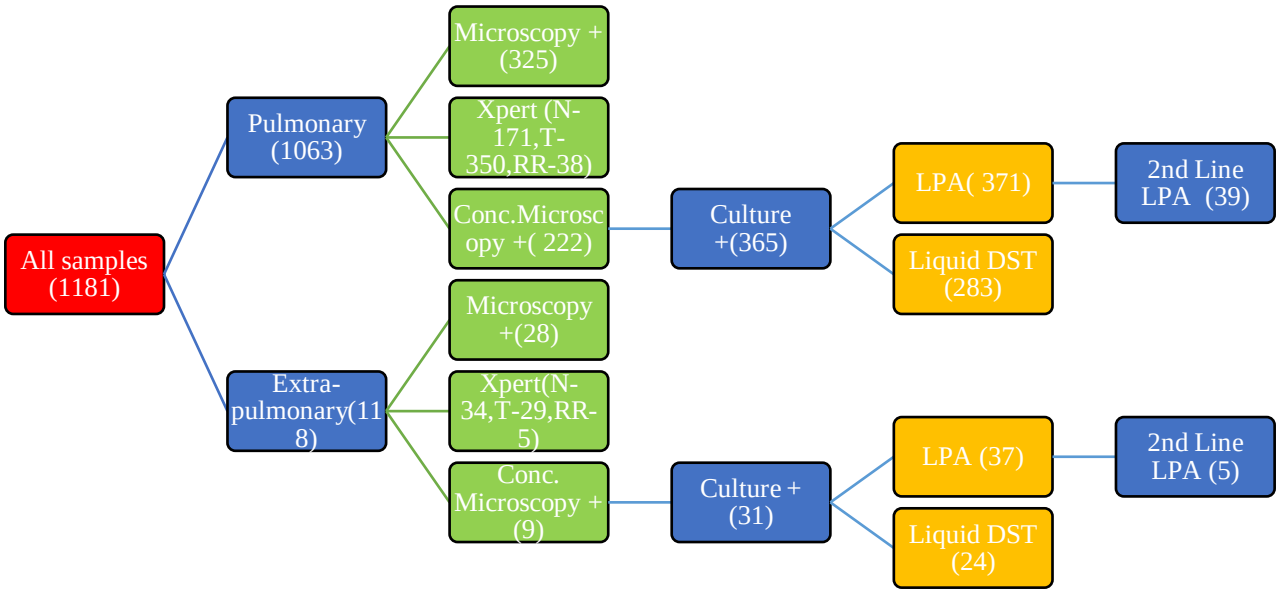


Figure 1: Number of samples received according to districts

Of the total 1181 samples received, 353 samples (325 pulmonary and 28 extra-pulmonary) were detected positive by microscopy and 422 samples positive for TB using Xpert MTB/RIF assay conducted atthe district hospitals. The remaining samples were negative at microscopy centers and GeneXpert centers but shipped to NTRL culture due to clinical suspicion or were contacts of positive cases.

At NTRL, samples were screened again by microscopy using concentrated method andsubjected to culture and DST. DST was done using liquid culture & LPA for first line drugs and LPA for second line drugs**(Figure 2).**



+: Positive; Conc. Microscopy: concentrated microscopy; N: Negative, T- TB detected,

RR- Rifampicin Resistant; LPA: Line Probe Assay; DST: Drug Sensitivity Test

Figure 2: Sample flow for pulmonary and extra-pulmonary samples for samples received at NTRL

Culture result

Among the 1181 samples received at NTRL, culture growth was seen in 322 (27.3%) samples in solid media and 388 (33.0%) samples in liquid media. The contamination rate for solid media and liquid media was 4.8 %(n=45) and 2.4% (n=29) respectively.

Drug Sensitivity Test Results

Liquid drug susceptibility testing*:* Of the 314 samples with liquid DST result, 84.1% (n=264) were new TB cases, 7.0% (n=22) were previously treated cases and 8.9% (n=28) cases with unknown treatment history. Resistance to all four first line drugswas seen in 3.5 % (n=11) cases. MDR-TB was detected in 8.3% new cases (n=22) and 27.3% (n=6) of the previously treated TB cases using liquid DST (**Table 2**).

Table 2: Drug resistant pattern for liquid DST

Resistance Pattern	New(n=264)		Previously Treated(n=22)		Unknown treatment history (n=28)		Total (n=314)	
	Cases	**%**	**Cases**	**%**	**Cases**	**%**	**Cases**	**%**
Streptomycin (S) Resistant	37	14	8	36.4	2	7.1	47	15
Isoniazid (I) Resistant	40	15.2	7	31.8	2	7.1	49	15.6
Rifampicin (R) Resistant	24	9.1	6	27.3	0	0	30	9.6
Ethambutol (E) Resistant	17	6.4	2	9.1	1	3.6	20	6.4
I+R Resistant	22	8.3	6	27.3	0	0	28	8.9
S+I+R+E Resistant	10	3.8	1	4.5	0	0	11	3.5
S+I+R+E sensitive	213	80.7	12	54.5	24	85.7	249	79.3

**Note: Resistance for individual drugs is irrespective of sensitivity for other drugs*

**S: Streptomycin; I: Isoniazid; R: Rifampicin ; E: Ethambutol*

L*ine Probe Assay drug susceptibilitytesting*:Of the 407 samples tested with LPA results**,** 80.8% (n=329) were new positive cases, 7.6 % (n=31) were positive among previously treated cases

and 11.5% (n=47) cases of unknown TB treatment history. MDR-TB was seen in 11.6% (n=38) new and 19.4 % (n=6) of the previously treated TB cases **(Table 3)**.

Table 3: Resistance pattern for samples tested using Line Probe Assay

Resistance Pattern	New(329)		Previously Treated(31)		Unknown treatment history(47)		Total(407)	
	Cases	%	Cases	%	Cases	%	Cases	%
Isoniazid & Rifampicin Sensitive	279	84.8	22	71	45	95.7	346	85
Isoniazid & Rifampicin Resistant	38	11.6	6	19.4	0	0	44	10.8
Rifampicin only Resistant	1	0.3	0	0	0	0	1	0.2
Isoniazidonly Resistant	11	3.3	3	9.7	2	4.3	16	3.9

Surveillance quality indicator: Compliance to MDR-TB surveillance protocol

In 2022, 809 patients (bacteriologically & clinically diagnosed) were put for TB treatment by TB units across the country. There were 571 (70.6%) bacteriologically confirmed TB and 238 (29.4%) clinically diagnosed TB. Of the 571 bacteriologically confirmed TB, 450 (78.8%) were pulmonary & 121 (21.2 %) were extra pulmonary TB.

Of 571 samples that needed to be shipped to NTRL for further testing, 475 (83.2 %) were received by NTRL. However, 96(16%) samples that were not shipped to NTRL had DST results based on GeneXpert MTB/RIF from district hospitals that had GeneXpert testing facilities. Of the 96 samples, MDR-TB was detected in 17.7 %(n=17) samples.

Multi-drug Resistant Tuberculosis/RR-TB

In total, MDR-TB/RR-TB was detected in 10.7% (n=61/571) of bacteriological confirmed TB cases using either liquid DST, LPA or GeneXpert tests. The median age was 27 years (IQR: 22-33 years). Majority of MDR-TB/RR-TB cases were in the age group of 15-29 years (n=40). The proportion of females that were affected was 65.6% (n=40) (**Figure 4**)

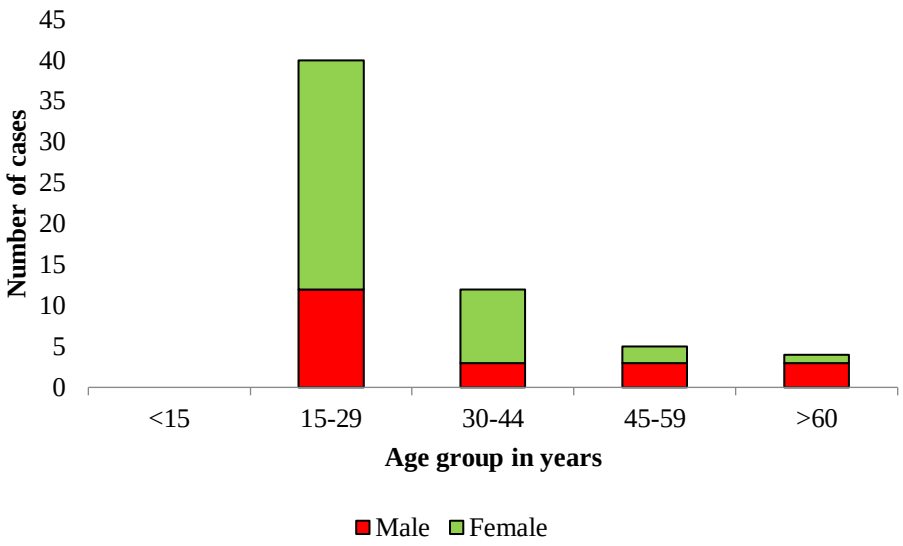


Figure 4: Distribution of MDR-TB by age group and gender

Of the total MDR-TB cases, 10.7% (n=55) MDR-TB cases were among new and 10.2% (n=6) were among previously treated cases (**Table 4).** Of the 61 MDR-TB cases, second line DST result was available for 44 cases. One case was detected resistant to fluoroquinolone. No cases were resistant to both fluoroquinolone & injectables.

Table 4: MDR-TB by method of detection among new and previously treated cases

Method of Detection	**New(512)**		**Previously Treated (n=59)**		**Total(571)**	
	Cases	**%**	**Cases**	**%**	**Cases**	**%**
Only LPA	6	1.2	0	0	6	1.1
Only Xpert	17	3.3	0	0	17	3
Only Liquid DST	0	0	0	0	0	0
LPA/XPERT/Liquid DST	55	10.7	6	10.2	61	10.7

Discussion

In 2022, 90.0% of samples screened at NTRL were pulmonary samples. Compared to the positivity rate for microscopy & Gene-Xpert test performed at district hospitals, the concentrated microscopy, solid & liquid culture tests performed at NTRL had lower positivity rate. Several

studies show that culture sensitivity & specificity is higher than that of microscopy or molecular assays (4). Therefore, sample storage, sample shipment protocol, sample collection procedures and culture methods need to be further investigated to understand the reason for lower culture yield at NTRL. For culture performed at NTRL, the culture contamination rate was within the acceptable range.

Due to the complexity of TB diagnosis, varieties of tools are used for the surveillance of drug resistant TB in Bhutan. With the availability of GeneXpert machines at district hospitals to screen MDR-TB, NTRL has been facing challenges in receiving all the samples for additional drug testing. NTRL did not receive samples for further testing from 96 bacteriologically confirmed samples. The reasons cited for not shipping extrapulmonary TB samples by district hospitals was the inadequate volume of sample for further testing whereas issues related to sample shipment facilities were mentioned for pulmonary samples.

NTRL received a large number of contact tracing samples that did not have microscopy or Xpertresults. Screening contact samples with Xpert MTB//RIF prior to sending samples for culture might help in reducing the volume of samples subjected to culture. Although culture has comparatively better sensitivity than Xpert, contact sample screening must be considered to reduce detection time as culture takes more than four weeks to declare the result.

Compared to the previous year, the proportion of MDR-TB among bacteriological confirmed TB is similar (10.0%). The proportion of MDR-TB among new cases (patients with no previous history of TB treatment) remains high in Bhutan compared to other countries. Thailand reported 0.8% and 13.0% MDR-TB among new and previously treated TB cases in 2017-2018 (5). Similar to the previous years, the most commonly affected age group is 15-29 years old and femaleshave a higher percentage of MDR-TB compared to men in contrast to findings from neighboring state of West Bengal in India where male predominance of MDR-TB was seen (6). The report of WHO also stated similar findings of higher MDR-TB among adult males (1). Among the MDR-TB cases, second line drugs DST report was unavailable for 27.9% (n=17) cases, of which the majority were extra-pulmonary TB samples.

DST performed at NTRL using liquid media, the drug testing results was available for streptomycin and ethambutol in addition to isoniazid and Rifampicin. The DST report need to be checked for usefulness as per the treatment guideline as some of the drugs has been discontinued

(7). Additionally, to keep up with the treatment protocol using delamanid&bedaquiline for drug resistant TB , diagnostic facilities need to be set up urgently.

Conclusion and recommendation

In order to make proper utilization of diagnostic facilities, the laboratory staff at district hospitals must be supported for shipment of samples to Xpert MTB//RIF facilities and NTRL for further testing. The TB unit in-charges also must ensure that all the diagnostic tests are performed for patients put on for TB treatment in close collaboration with the laboratory facilities. For contact tracing samples, implementation of proper diagnostic algorithm might be helpful in increasing the sample positivity rate while subjecting to culture. To understand the lower culture growth rate at NTRL compared to district microscopy and Xpert MTB//RIFresults, further investigation on the laboratory protocols for sample shipment and sample treatment procedures needs to be conducted. In order to understand the resistance pattern of drugs used for treatment, DST facilities for the newer drugs need to be set up.

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COVID-19 Integrated Influenza Surveillance, 2022

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Introduction

Influenza virus infections contribute considerably to morbidity and mortality both in the temperate and tropical regions, causing 3 – 5 million cases of severe illness annually and 250,000 – 650,000 deaths annually (1)(2). All age groups can be affected by influenza, however, pregnant women, children under 59 months, the elderly (>65 years), individuals with chronic medical conditions and individuals who are immunosuppressed), and health care workers are at higher risk(3).Seasonal influenza virus is a common cause of acute lower respiratory infection (ALRI) in young children. In 2018 alone, there were global episodes of an estimated 109.5 million influenza virus episodes, 10.1 million influenza-virus-associated ALRI cases, 870 000 influenza-virus-associated ALRI hospital admissions, and up to 34 800 overall influenza-virus-associated ALRI deaths among children under 5 years(4).

In Bhutan, acute respiratory infection (ARI) and the common cold is one of the top 10 diseases reported annually for the last ten years through the national early warning alert and response surveillance (NEWARS)(5). A study showed that the incidence of influenza-associated hospitalizations among all age groups was 50/100,000 persons in 2015 and 118/100 000 persons in 2016. The highest rates were among children <5 years: 182/100 000 in 2015 and 532/100 000 in 2016(6) Therefore, it is important to continuously monitor the trend of influenza on influenza-associated illnesses leading to hospitalizations and deaths, so that prevention and control interventions can be implemented more effectively.

Methodology

Surveillance Design and Setting

Surveillance design is sentinel-based surveillance for influenza and other viral respiratory pathogens including flu outbreak samples (7). Influenza sentinel hospitals are: Jigme DorjiWanghck National Referral Hospital, Thimphu, Punakha Hospital, Paro Hospital, Monggar

Regional Referral Hospital, Phuntsholing Hospital, Gelephu Regional Referral Hospital, Trongse Hospital, Tsirang Hospital and Trashigang Hospital. The sentinel sites were selected based on strategic geographical locations and demographic representation (Figure 1).

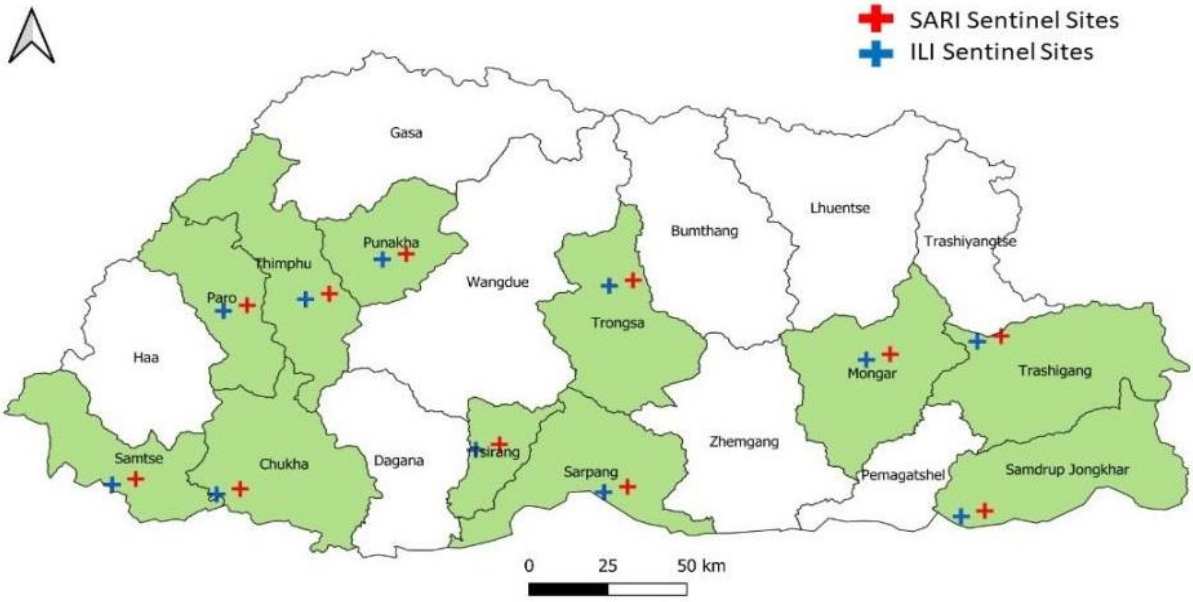


Figure 1: ILI Surveillance sites & SARI Surveillance Sites

Patients who meet case definition for influenza-like illness (ILI) and severe acute respiratory infection (SARI) were enrolled and collected their clinical and epidemiological information. As per the guideline, each identified sentinel site for ILI should enroll 5-10 cases per week (20-40 cases per month) for specimen collection representing all age groups while it is mandatory to enroll all SARI cases for specimen collection.

Case Definition

Influenza-like Illness (ILI): An acute respiratory infection with a measured fever of ≥ 38° C and cough with onset within the last 10 days (8).

Severe acute respiratory infections (SARI): An acute respiratory infection with a history of fever or measured fever of ≥ 38° C and cough with onset within the last 10 days and requires hospitalization (8).

Sample collection

Respiratory swab specimen was collected after getting the informed consent. Specimens were collected as part of routine patient care through a surveillance network of outpatient clinics, inpatient clinics, or from the Flu clinic.

Outbreaks sample collection and shipment

Influenza-like Illness (ILI) or acute respiratory infection (ARI) outbreaks are reported from Health Centers to RCDC through NEWARS information system. Representative samples of 5-10 throat swabs from clinically symptomatic suspected cases were collected and shipped to RCDC or to nearby COVID-19 Testing laboratories for confirmation by RT-PCR assay.

Laboratory Detection

RT-PCR

Viral RNA was extracted from 200µl of Throat/Nasal swabs collected in Viral Transport media using Spin-X Viral RNA Extraction Kit (SD Biosensor, South Korea) and KingFisherTM Flex Purification system (Thermo Fisher, India) following the manufacturer's instructions. The extracted viral RNA was subjected to RT-PCR using Influenza SARS-CoV-2 (Flu SC2) Multiplex Assay (US-CDC). 5µl of viral RNA was added in the tube containing the mixture of 7.75µl of nuclease-free water, 3µl of Flu SC2 combined primer mix, 3µl of Flu SC2 combined probe mix and 6.25µl TaqPathTM 1-Step Multiplex Master Mix (No ROX).

The cycling condition was set at 1 cycle of reverse-transcription step at 20ºC for 2 minutes and 50ºC for 15 minutes followed by 1 cycle of pre-denaturation at 95ºC for 3 minutes followed by 45 cycles of 95ºC at 15 seconds, 55 ºC for 33 seconds. Ct value <40 for InfA, InfB, SC2 and <35.00 Ct for RP was considered positive whereas, ct value ≥40.00 for InfA, InfB, SC2 and ≥35.00 Ct for RP were considered negative. For each protocol positive and negative controls were run in each test to validate the test result. Furthermore, samples that tested positive for Influenza A and B virus were subtyped and lineage determined respectively using Human Influenza Virus Real-Time RT-PCR Diagnostic Influenza A/B Typing Kit (US-CDC) based on manufacturer's instruction.

Data Collection

All the demographic details including clinical and laboratory information were collected through a structured surveillance questionnaire, which were verified by RCDC before entering into the COVID-19 Integrated Influenza surveillance system. All the laboratory results were entered into the system and the same were shared in PDF to relevant stakeholders and sentinel sites.

Data Analysis

Surveillance data were extracted from the COVID-19 Influenza Integrated surveillance system. Descriptive statistics were used to analyze the results including patient demographic to see the proportion of Influenza-associated ILI and SARI. All data were analyzed using Epi Info7. QGIS 3.16 was used to generate a mapping distribution of the Influenza positivity in the country.

Results

Weekly ILI and SARI cases were reported from 7 ILI sentinel sites and 11 SARI sentinel sites respectively to RCDC. A total of 4,287 ILI and 651 SARI cases were reported. For ILI, SamdrupJongkhar Hospital has reported the highest number of cases (1214) followed by Trongsa Hospital (1024). Gelephu CRRH has reported the highest cases of SARI (178) **(Figure 2)**

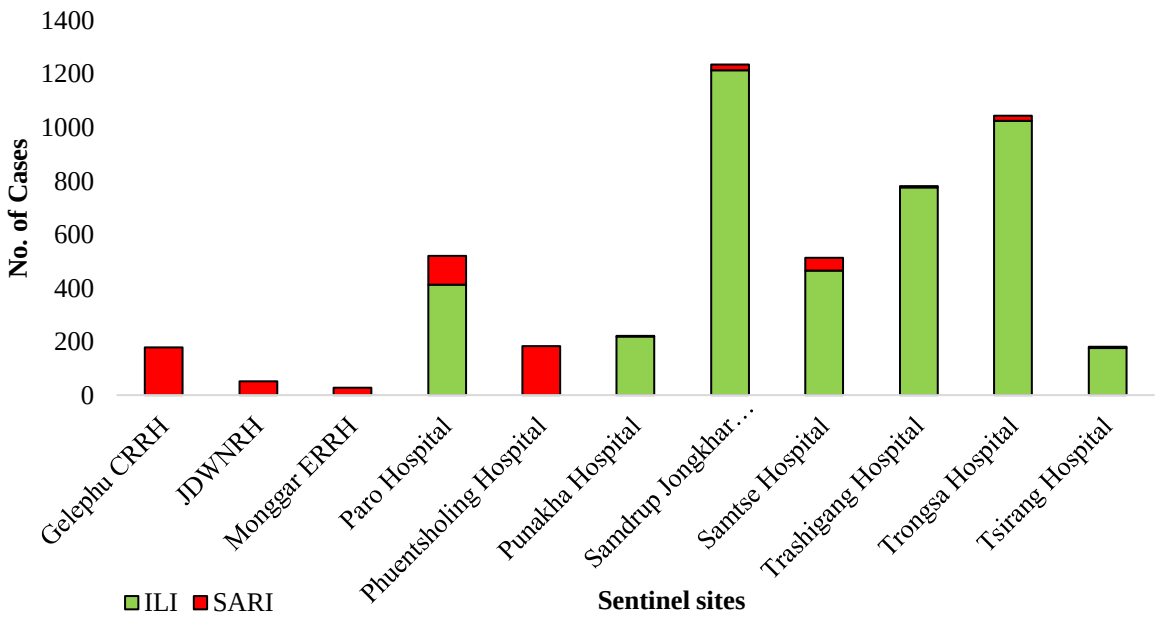


Figure 2: Number of ILI and SARI cases reported by Sentinel Hospitals in 2022

The most affected age groups for ILI were 5 – 14 years (25.4%) followed by 15 – 29 years (25.4%) and 30 – 64 years (24.1%). While for SARI the most affected age group was 0 – 1 years (30.4%) followed by 2 – 4 years (20.4%) **(Figure 3).**

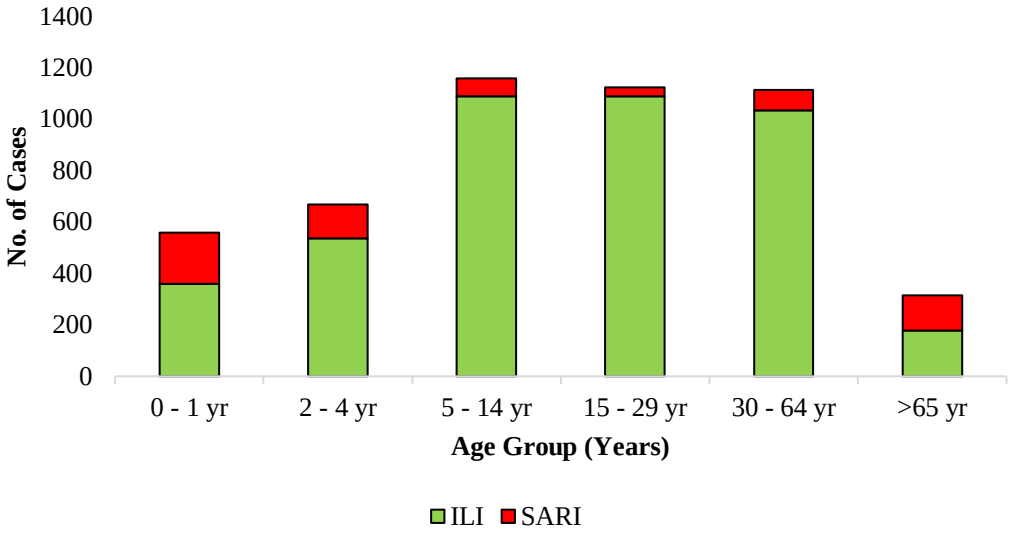
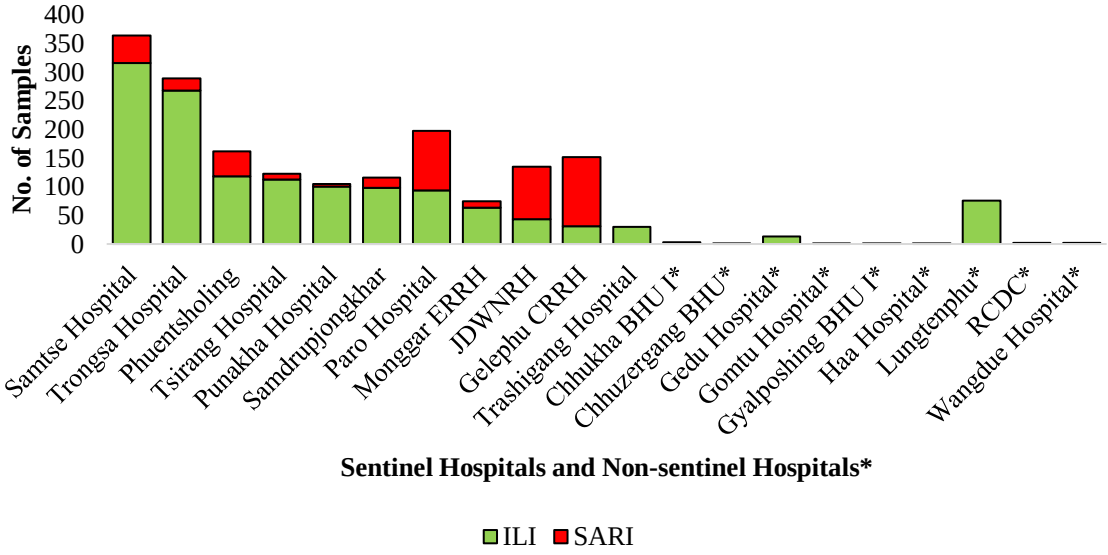


Figure 3: ILI and SARI cases by age group in 2022

A total of 2,015 respiratory swabs specimens were received in 2022, of which 1273 (63.2 %) were ILI, 473 (23.5 %) SARI, 169 (8.4 %) outbreaks samples and 100 (5.0%) samples from non-sentinel hospitals. Samtse Hospital (364) has enrolled the maximum number of cases followed by Trongsa Hospital (289) (**Figure 4).**



The weekly influenza positivity rate was monitored throughout the year. On average, weekly influenza positivity rate was 11.3% (0 – 64.5), there was a surge in cases and the positivity rate during Flu season (Oct – Dec) in epi week 41 – 48). Flu A/H3 (70.2%) subtype was found to be the most predominant circulating strain, followed by Flu A (H1)/pdm09 (4.7%) and Flu B/Victoria (2.5%) (**Table 1**). Five of ILI cases were detected with coinfection (1.4%) for both Flu A/H3 and SARS-CoV-2. SARS-CoV-2 positivity among ILI and SARI cases was 20.6 %. The influenza positivity rate was high in Paro, Thimphu and Punakha (12 – 15%) compared to other sentinel hospitals **(Figure 4).**

Table 1: Number of Influenza subtypes and SARS-CoV-2 received and tested in 2022

Variables	**n (%)**
Time taken to receive sample (Days)	
Mean (range)	7.2 (0 - 137)
Median (IQR)	6 (2 - 10)
TAT for Testing (Days)	
Mean (range)	3.2 (0 - 26)
Median (IQR)	2 (1 - 3)
Dewathang Hospital	4 (2 - 7)
Gelephu CRRH	2 (0 - 5)
Monggar ERRH	6 (3 - 18)
Phuentsholing Hospital	3 (1 - 4)
Royal Centre for Disease Control	2 (1 - 3)
Influenza Subtypes	
Flu A/H3	252 (70.2)
Flu A/H3 and SARS-CoV-2	5 (1.4)
Flu A/Not Subtyped	2 (0.6)
Flu A/Pdm09	17 (4.7)
Flu B/Victoria	9 (2.5)
SARS-CoV-2	74 (20.6)

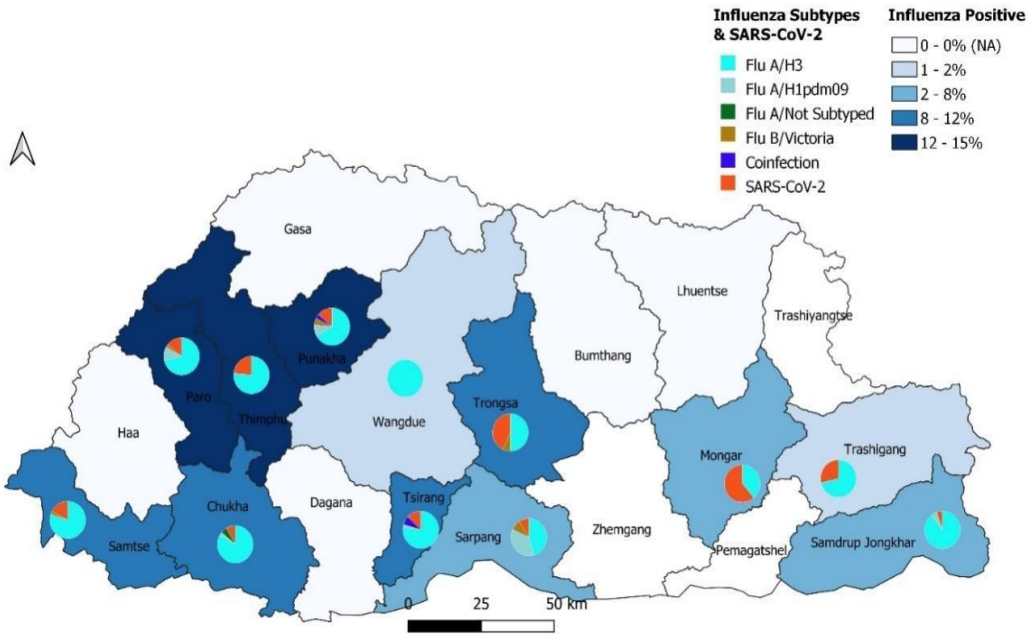


Figure 4: Proportion of Influenza subtypes and SARS-CoV-2 by district

All specimens were tested for influenza and SARS-CoV-2 viruses by using influenza SARS-CoV-2 (Flu SC2) multiplex assay and detected 15.2% (280) influenza positive, 4.0% (74) SARS-CoV-2 and 0.3% (5) coinfection. The median age was 12 (5 – 28)years,while median age among SARI was 6.2 (2.5 – 15) years. The most affected age group by influenza was 15 – 29 years (35.9%) followed by 5 – 14 years (28.5%). Males (20.2%) were affected slightly more than females (19.1%).Clinically the most common symptoms presented were cough (96.7%), fever (77.8%) and sore throat (64.8%) and the least were diarrhoea (3.3%) and nausea/vomiting (6.7%) **(Table 2)**.

Table 2: Patient demographic and clinical characteristics of ILI and SARI

Variables	AllILI n	Influenza n(%)	SARS-CoV-2 n(%)	Coinfection n(%)	All SARI n(%)	Influenza n(%)	SARS-CoV-2 n(%)
Total	1373	270 (19.7)	65 (4.7)	5 (0.4)	473	10 (2.1)	9 (1.9)
Median age (IQR), years	13 (6 – 29)	12 (5 – 28)	12 (5 – 26)	14 (8 – 54.5)	2.6 (1 – 29)	6.2 (2.5 – 15)	4 (1 – 63)
Age group							
0 – 1 years	133	11 (4.1)	4 (6.2)	0	199	2 (20.0)	3 (33.3)
2 – 4 years	147	12 (4.4)	3 (4.6)	0	87	1 (10.0)	2 (22.2)
5 – 14 years	438	77 (28.5)	8 (12.3)	3 (60.0)	45	5 (50.0)	0
15 – 29 years	330	97 (35.9)	21 (32.3)	0	24	1 (10.0)	0
30 – 64 years	293	69 (25.6)	24 (36.9)	2 (40.0)	50	0	2 (22.2)
>65 years	32	4 (1.5)	5 (7.7)	0	68	1 (10.0)	2 (22.2)
Sex							
Female	666	127 (19.1)	30 (4.5)	1 (0.2)	214	6 (2.8)	3 (1.4)
Male	707	143 (20.2)	35 (5.0)	4 (0.6)	259	4 (1.5)	6 (2.3)
Clinical symptoms							
Fever	1125 (81.9)	210 (77.8)	53 (81.5)	3 (60.0)	425 (89.8)	9 (90.0)	7 (77.8)
Cough	1309 (95.3)	261 (96.7)	62 (95.4)	5 (100)	452 (95.6)	10 (100)	9 (100)
Sore throat	889 (64.8)	198 (73.3)	51 (78.5)	3 (60.0)	311 (65.8)	7 (70.0)	4 (44.4)
Breathing problem	192 (13.9)	55 (20.4)	9 (13.8)	1 (20.0)	271 (57.3)	5 (50.0)	5 (55.6)
Headache	719 (52.5)	169 (62.6)	30 (46.2)	3 (60.0)	110 (23.3)	1 (10.0)	9 (100)
Nausea/vomiting	98 (7.1)	18 (6.7)	1 (1.5)	0	51 (10.8)	1 (10.0)	0
Smell/taste	238 (17.3)	79 (29.3)	11 (16.9)	1 (20.0)	39 (8.3)	1 (10.0)	0
Chills	259 (18.9)	44 (16.3)	11 (16.9)	1 (20.0)	82 (17.3)	1 (10.0)	1 (11.1)
Muscle pain	370 (26.9)	125 (46.3)	16 (24.6)	3 (60.0)	45 (9.5)	3 (30.0)	0
Diarrhoea	60 (4.4)	9 (3.3)	1 (1.5)	0	38 (8.0)	2 (20.0)	0
Abdominal pain	114 (8.3)	24 (8.9)	5 (7.7)	0	32 (6.8)	1 (10.0)	0

Of these 169 respiratory specimens received from outbreak places for confirmation by RT-PCR, Almost all samples were positive for Flu A/H3 (61.5%). Two cases from Lhamoizingkha were detected as co-infection for both Flu A/H3 and SARS-CoV-2 (1.2%) **(Figure 9).** All samples were tested for Influenza and SARS-CoV-2 and results were disseminated to respective health centers within 24 hours. The median age of patients in outbreak samples was 16 years (range 13-17). Students (88.2%) were the commonly affected occupation group **(Table 3)**.

Table 3: Patient demography and Influenza subtypes of samples obtained from ARI outbreaks and non-ILI sentinel sites

Variables	n (%)	Flu A/H3	Negative	SARS-CoV-2	SARS-CoV-2 and FLUA/H3
Age					
Mean (Range), years	15.5 (6 - 38)				
Median (IQR), years	16 (13 - 17)				
Gender					
Female	105 (62.1)				
Male	64 (37.9)				
Outbreak Location					
Lhamiozingkha School	92 (54.4)	56	34		2
Paro School	23 (13.6)	19	4		
Phuentsholing Hospital	5 (3.0)		5		
Punakha School	28 (16.6)	11	16	1	
Trashiyangtse Hospital	4 (2.4)	3	1		
Trashigang school	6 (3.6)	4	2		
Trashiyangtse Hospital	11 (6.5)	11	0		
Occupation					
Health Staff	1 (0.6)				
Others	15 (8.9)				
Student	149 (88.2)				
Teacher	4 (2.4)				
Flu subtypes & COVID-19					
Flu A/H3	104 (61.5)				
Negative	62 (36.7)				
SARS-CoV-2	1 (0.6)				
SARS-CoV-2 and FLU A/H3	2 (1.2)				

Discussion

In 2022, Bhutan experienced more influenza activity due to influenza A(H3N2) which became the predominate strain during the year. Globally, influenza activity remained elevated due to higher activity in the northern hemisphere for the same reason. Influenza activity was seen increased towards the end of the year from October – December 2022 which is flu season in the country. In the Northern Hemisphere, influenza outbreaks and epidemics typically occur between October -December, whereas in the Southern Hemisphere, influenza activity occurs between June and September

During the year, ARI outbreaks were reported in many schools compare to previous year due to change is influenza A serotype. In tropical regions, influenza can occur throughout the year (3)(5).

SARS-CoV-2 (20.6%) positivity among ILI and SARI cases was found proportionately high and same trend was also observed globally from sentinel surveillance in the WHO Regions of the Americas and Western Pacific (10).

ILI are commonly observed in young adults while SARI among children less than 5 years and among elderly similar to the previous year's findings and reported elsewhere (11)

Conclusion

Influenza and SARS-CoV-2 positivity rates were detected substantially high particularly during Flu seasons in 2022. Influenza A(H3N2) subtype was found to be the most predominant circulating strain in the country and globally as well. Continuous monitoring and laboratory surveillance is recommended monitor influenza subtypes and SARS-CoV-2 variants.

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COVID-19 surveillance in Bhutan, 2022

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Introduction

The COVID-19 pandemic has not only resulted in a massive loss of human life worldwide, but it has also posed an unprecedented threat to public health, food, the economy, and education systems. As of April 7, 2023, more than 762 million COVID-19 confirmed cases and 6.8 million deaths were reported globally. Bhutan detected the first COVID-19 case in March 2020 through active surveillance and experienced four outbreaks until January 2023.

COVID-19's global impact has underlined the importance of comprehensive surveillance systems, data-driven decision-making and evidence-based interventions.Continuing the COVID-19 surveillance was crucial for understanding the epidemiological trends, such as the incidence and mortality rates among different age groups, and the virus's transmission. It is also essential to monitor the frequency of mutations/variations in all the significant proteins of SARS-CoV-2. The national COVID-19 surveillance continued in 2022 through periodic review and implementation of the national COVID-19 surveillance strategy based on regional and global situations.

Methodology

Sampling strategy and population

Enhanced Surveillance was implemented in both high and low-risk districts of Bhutan to detect any person(s) with COVID-19 promptly and to prevent further transmissions and outbreaks. Apart from symptomatic testing in flu clinics established in all 20 districts, active surveillance of frontline workers identified by the respective dzongkhag/task force was initiated by testing every two weeks. During the Omicron wave, mass testing strategy and contact tracing was conducted

as per the national testing protocol. Both nasopharyngeal and oropharyngeal swabs were collected and transported in viral transport media for testing in COVID-19 testing laboratories.

Epidemiological data collection

A web-based electronic data collection system namely Health Facility System (HFS) was used to extract demographic and epidemiological data from quarantined individuals, positive contacts, and frontline workers while an active case investigation form was used in symptomatic cases. The citizenship identification (CID) number was used as the case identification number for nationals and parent's CID was tagged for children who do not have a CID. Passport and Immigration permit numbers were used as case identification numbers for other nationals and migrant workers residing in the country.

Laboratory tests

Fluorescent-based immunoassay test STANDARD F Ag FIA (SD Biosensor, South Korea) and rapid immunochromatography-based assay STANDARD Q COVID-19 Ag were used to test in hospitals and Public Health Centers as per the manufacturer's instructions.

The viral RNA was extracted from 200µl of pooled samples using Spin-X Viral RNA Extraction Kit (SD Biosensor, South Korea) and KingFisher^{TM} Flex Purification system (Thermo Fisher, India) following the standard manufacturer's instructions. Extracted Viral RNA was subsequently subjected to RT-PCR using Standard M nCOV Real-Time Detection Kit (SD Biosensor, South Korea) that detects envelope gene and ORF1ab gene. A Cycle threshold (Ct) value of less than 36 was considered positive. For each test, positive and negative controls were run to validate the test result.

Data analysis

The data for cases confirmed by RT-PCR was obtained from RCDC online COVID-19 integrated influenza surveillance system database and from the Health Facility System (HFS) database for the cases confirmed by FIA antigen testing. The extracted data was cleaned and analysed by Microsoft Excel and Graphpad Prism v9. Electronic shapefile of the geographical map of Bhutan was processed using QGIS for the geographical representation of cases.

Phylogenetic analysis

Phylogenetic analysis using Nextstrain, an open-source bioinformatics visualization platform. We used Nextclade v2.13.0, a web-basedNextstrain tool, to align our sequences pairwise, assign them to clades, and construct a Maximum-likelihood phylogenetic tree (1)(2). The phylogenetic tree generated by Nextclade was exported as a JavaScript Object Notation (JSON) and visualized by auspice.us 0.120 and then downloaded as a Newick format. The final annotation was generated using iTOL v6.7.4 (1).

Results

Epidemiological characteristics

In 2022, a total of 59,790 COVID-19 (SARS-CoV-2) cases were detected in the country with a cumulative incidence rate of 0.082 cases per person-year. A surge in cases was observed during Omicron outbreak in the early epidemiological weeks of 4 – 17, which falls during the months of February (7,811 cases), March (15,398 cases), and April (31,062 cases) in 2022 **(Figure 1).** The weekly mean positivity rate was 6.3%, with a range of 0.1 – 36.2%. This was higher compared to the previous two years, with a positivity rate of 0.4% (734 cases) in 2020 and 0.2% (1926 cases) in 2021.

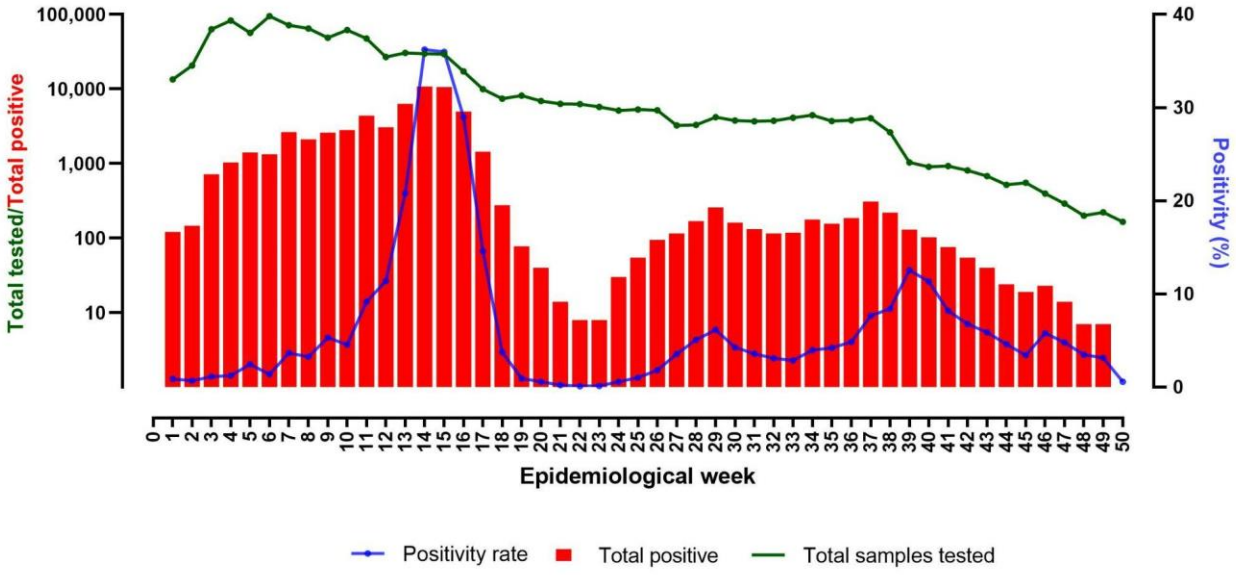

Figure 1: COVID-19 cases detected by epidemiological week along with the total number of samples tested in 2022 (Source: HFS & RCDC system).

Age and sex

The mean age of COVID-19 cases in 2022 was 28.9 years. The most affected age group was those aged between 25 – 49 years (50.2%), followed by those aged between 20 – 24 years (13.4%). The least affected age group was those aged between 1 – 4 years (0.9%) **(Figure 3).** Males (54.1%) were more affected than females (45.9%).

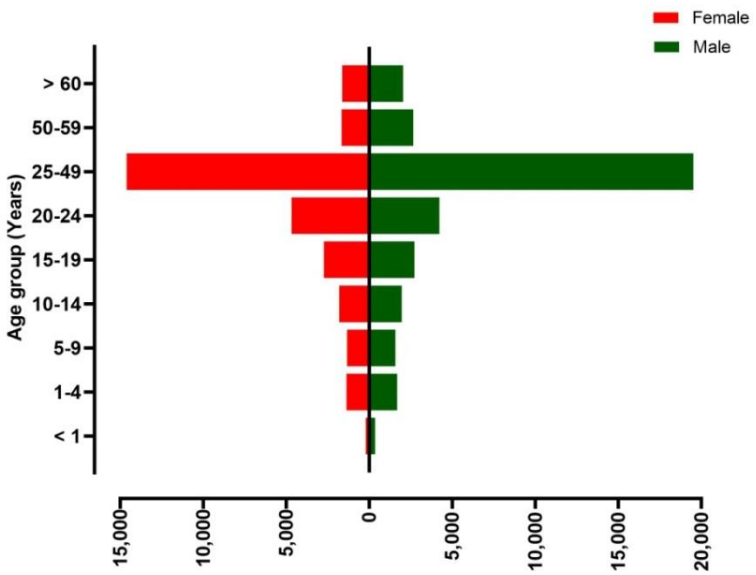

Figure 3: Population pyramid showing the cumulative number of COVID-19cases by age and sex 2022.

Distribution of cases

COVID-19 cases were detected in all 20 districts of the country. Thimphu reported the highest number of cases with a total of 17,850 cases, followed by Chhukha with a total of 8,333 cases, and Sarpang with a total of 5,914 cases. Gasa reported the least number of cases with a total of only 109 cases **(Figure 4).**

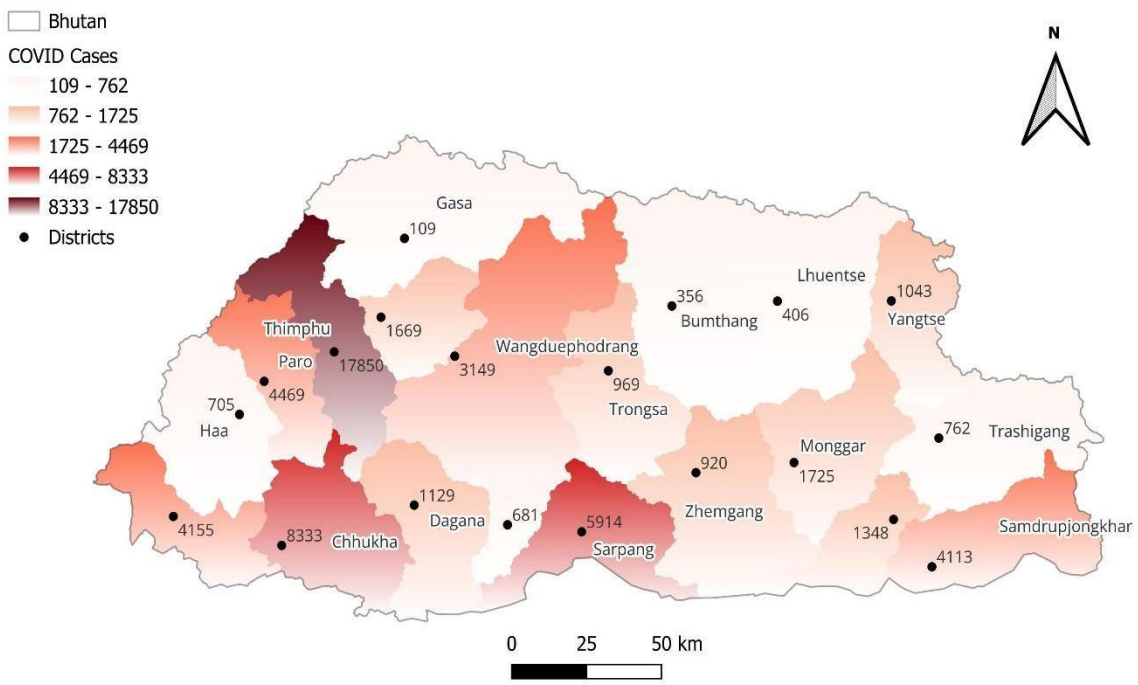

Figure 4. Geographical distribution of COVID-19 cases in 2022

SARS-CoV-2 genome sequence analysis

We analyzed 46 complete genomes of SARS-CoV-2 from Bhutan in 2022 for their variation and phylogeny. The phylogenetic analysis showed that the SARS-CoV-2 strains in 2022 were distributed among five Nextstrain clades and 13 Pangolin lineages. The most prevalent lineage was BA.2.10 (48%), followed by BL.1 (15%), XBB (9%), BM.1.1.4 (4%), BA.2.10.1 (4%), B.A.2(4%), XJ (1%), XBF (2%), XBB.2.1 (2%), BJ.1 (2%), BA.5.2.1 (2%), CA.3 (2%), and BM.1.1.3 (2%). As shown in Figure 6, the PANGO variants of the samples changed over time. BA2.10 was the most prevalent variant in April 2022, but it was replaced by BL.1 from September to November 2022. By December 2022, BM.1.1.4 became the dominant variant. There were five VOIs (Omicron B.A.2, XBB, XBB.2.1, XBF, and BJ.1) and one VOC (Omicron BA.2) detected from the representative samples sequenced and submitted to GISAID**(Figure 5**).

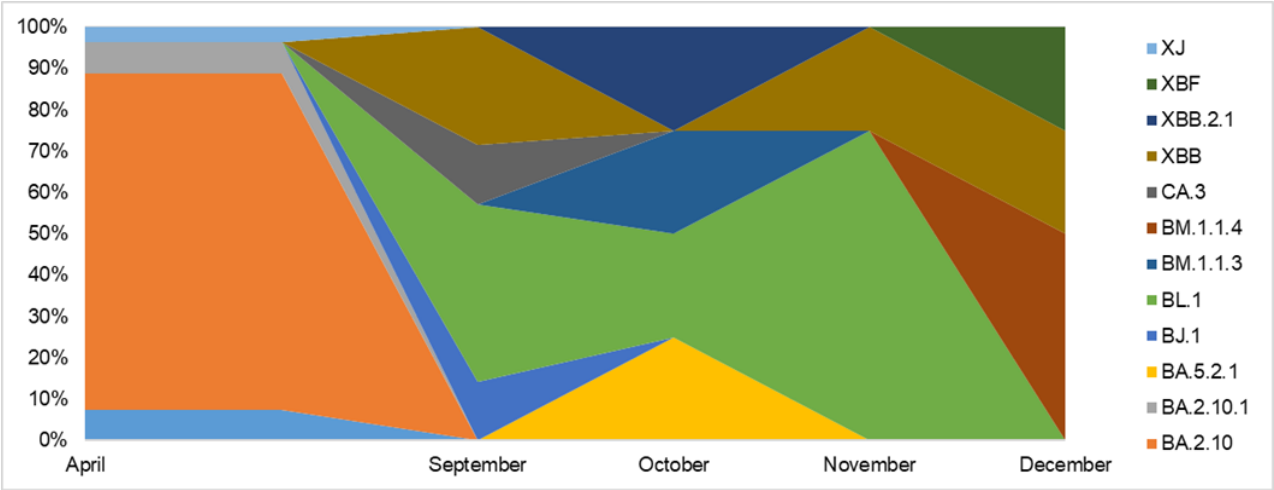

Figure 5: Lineage relative frequencies of SARS-CoV-2 in Bhutan in 2022

Discussion

In 2022, Bhutan experienced a surge in COVID-19 cases that was temporally associated with the introduction of the Omicron variant in the country during the third epidemiological week. Omicron is a variant of concern (VOC) of SARS-CoV-2 that has acquired mutations that may affect its transmissibility, severity, and immune escape potential. This variant was shown to have a two- to three-fold increased risk of reinfecting individuals who have previously acquired immunity from natural infection or vaccination, due to its ability to escape the immune response elicited by prior exposure (3). Among the subvariants of Omicron, BA.2.10 was the most prevalent in Bhutan in early 2022. This subvariant has been reported to have higher transmissibility than BA.1, the original subvariant of Omicron that emerged in late 2021 (4).This would explain the substantial increase in the burden of COVID-19 cases in 2022 compared to the previous years of the pandemic. To mitigate the impact of this variant, the national taskforce for COVID-19 implemented several public health interventions, such as a fourth nationwide lockdown on January 19, 2022, enhanced testing and tracing, increased isolation, and treatment capacity, and enforced strict public health measures (5)(6). These actions helped to prevent community transmission and break the chains of infection. The high positivity observed also reflects the high level of testing conducted during the year 2022. Bhutan had one of the highest testing rates per capita in the world, reaching over 100 tests per 1000 population by December 2021(7).

The age distribution of cases observed in 2022 is consistent with studies conducted in different countries that have shown that younger adults are more likely to be infected and symptomatic than older adults (8)(9). This may be due to factors, such as higher exposure risk due to occupational or social activities, or lower adherence to preventive measures. The gender difference observed with males being more affected than females, is also consistent with previous studies that have reported higher infection rates among males than females (10). This may be due to biological or behavioural factors, such as differences in immune response, comorbidities, or hormone levels. This disparity in the positivity rate may be also contributed by higher exposure risk in men than females due their predominant roles in sectors such as security, transportation, or construction.

The geographic distribution of cases observed reflects the population density and mobility patterns of Bhutan. Thimphu, which reported the highest number of cases, is the capital and largest city of Bhutan, with a population of over 100,000 people (11). It is also a hub for economic, political, social, and cultural activities. Chhukha and Sarpang, which reported the second and third highest number of cases respectively, are districts that border India, which was severely affected by the twin combination of omicron and delta variants in 2022 (12). These districts also have high population movement due to trade and commercial activities. Gasa, which reported the lowest number of cases, is the least populated district of Bhutan, with a population of less than 4000 people (27). It is also located in a remote and mountainous area with limited access.

The analysis of 46 complete SARS-CoV-2 genomes offered valuable insights into the virus's genomic variation and evolutionary dynamics. We found that the virus in Bhutan was distributed among five Nextstrain clades and 13 Pangolin lineages. The most prevalent lineage was BA.2.10 (48%), followed by BL.1 (15%) and XBB (9%). This finding was consistent with the diversity and lineages reported from neighbouring countries like India. Zaman et. al reported the predominance of Omicron BA.2 lineage in SARS-CoV-2 positive cases during the third wave in North India in early 2022 (13).

The phylogenetic analysis revealed that SARS-CoV-2 strains in Bhutan were evolving rapidly, with changes in dominant lineages over time. The most prevalent variant shifted from BA2.10 in April 2022 to BL.1 from September to November 2022 and finally to BM.1.1.4 by December

2022. This highlights the need for ongoing genomic surveillance to track the emergence and spread of new variants. Six variants of concern or interest (VOCs/VOIs) were identified, including Omicron B.A.2, XBB, XBB.2.1, XBF, BJ.1, and Omicron BA.2. These variants have been linked to increased transmissibility, severity, or immune evasion. Their detection underscores the importance of continued monitoring and response efforts to mitigate COVID-19's impact.

Conclusion

The year 2022 saw the maximum cases of confirmed COVID-19 till date which was mostly after the emergence of the Omicron variant. Although the number of cases was high, severity and death were minimal, indicating the success of vaccination and strategic lifting of lockdowns and other restrictions.

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National Drinking Water Quality Surveillance, 2022

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Introduction

According to the United Nations, every individual must have access to safe, sufficient, and affordable water, sanitation, and hygiene facilities as it is recognized as a human right, reflecting the fundamental nature of these basics in every person's life (1). As per the World Health Organization, in 2017, 71.0% of the people used improved quality, easily accessible, and affordable drinking water services and about 90.0% of the population got at least access to safe drinking water (3) while 785 million people still lack basic drinking–water service. Sustainable Development Goal 6.1 aims to achieve safe and affordable water for all universally by 2030. The goal also targets improving the quality of drinking water by reducing pollution by eliminating and minimizing the dumping of waste such as human excreta and hazardous chemical waste into the water (2).

In Bhutan, 97.7% of the population has access to safe drinking water from which, 58.8% of the water was piped into a compound, 24.0% piped into a dwelling, 7.8% public tap, and 5.9% to the neighbor (4). The Ministry of Health (MoH) is responsible for looking after the quality of drinking water as per the Water Act of Bhutan, 2011 (5). The National Water Reference Laboratory (NWRL) under Royal Centre for Disease Control (RCDC) conducts Drinking Water Quality Surveillance and continues to strengthen the capacity for monitoring drinking water quality across the country. NWRL serves as a national reference laboratory for drinking water quality testing and monitoring, assesses and certifies the technical competency of drinking water quality testing laboratories. The centre also reports to the National Environment Commission (NEC) in the event of non-compliance with the standard which may pose a potential threat to consumer health and undertake water quality testing in Thimphu thromde.

The drinking water surveillance was established in 2015 with the objective to monitor the drinking water quality both in urban and rural areas of the country and free residual chlorine where water treatment plants are to ensure that safe drinking water is portable.

Methodology

There are two types of water surveillance systems, the urban drinking water and the rural drinking water quality surveillance system. A total of 257 health centers are in the rural water supply system and 44 health centers are in urban water supply systems as of 2022. The frequency of testing the drinking water sample is monthly for the urban health centers and bi-annually for the rural health centres. Sampling points in urban areas are selected based on housing clusters, in schools and institutions.In rural areas, sampling points are decided based on community water supply system.The water quality monitoring reporting is done through a web-based system hosted by RCDC **(Figure 1).**

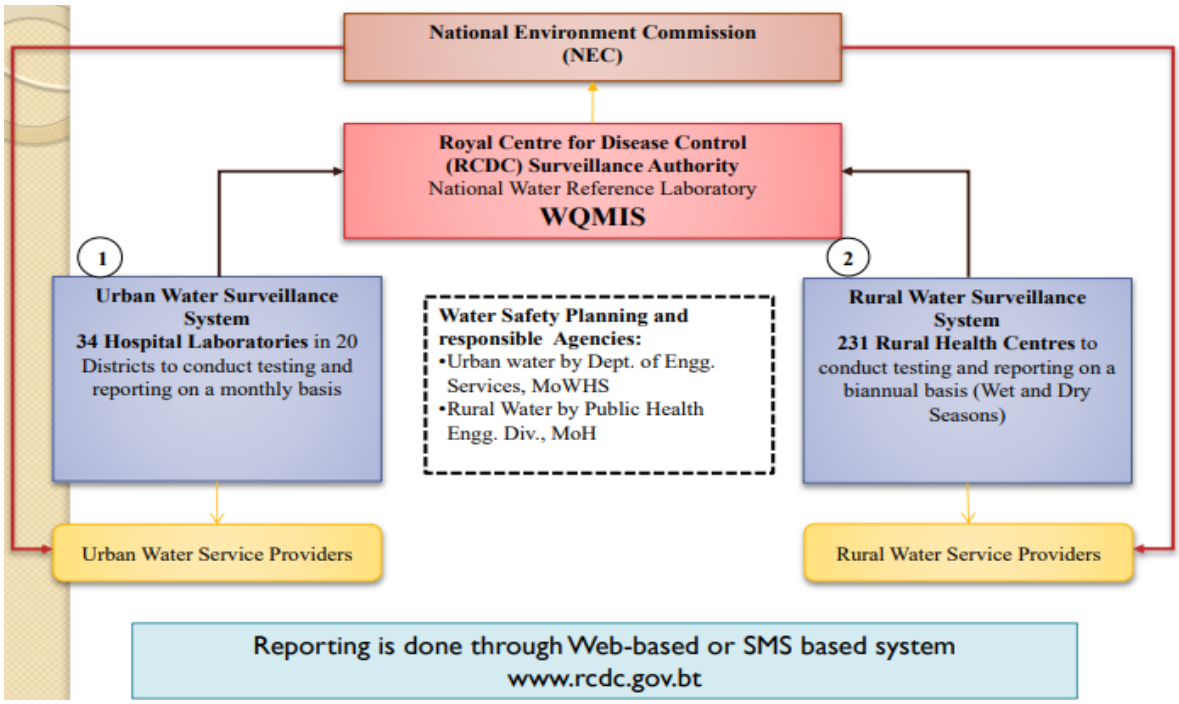


Figure 1: Drinking water quality surveillance framework

Water sample collection

Water samples are collected as per the standard operating procedure in the sterile bottle from different collection points in urban areas which were identified based on risk assessment. Water is then shipped to the lab for bacteriological testing. Free residual chlorine is tested on site using the test kit.

Laboratory Testing

The laboratory analyses of the samples are conducted following the standard operating procedure for the test parameters listed below for urban and rural health facilities **(Table 1).**

Table 1: List of test parameters in Urban Health centers for monitoring drinking water quality

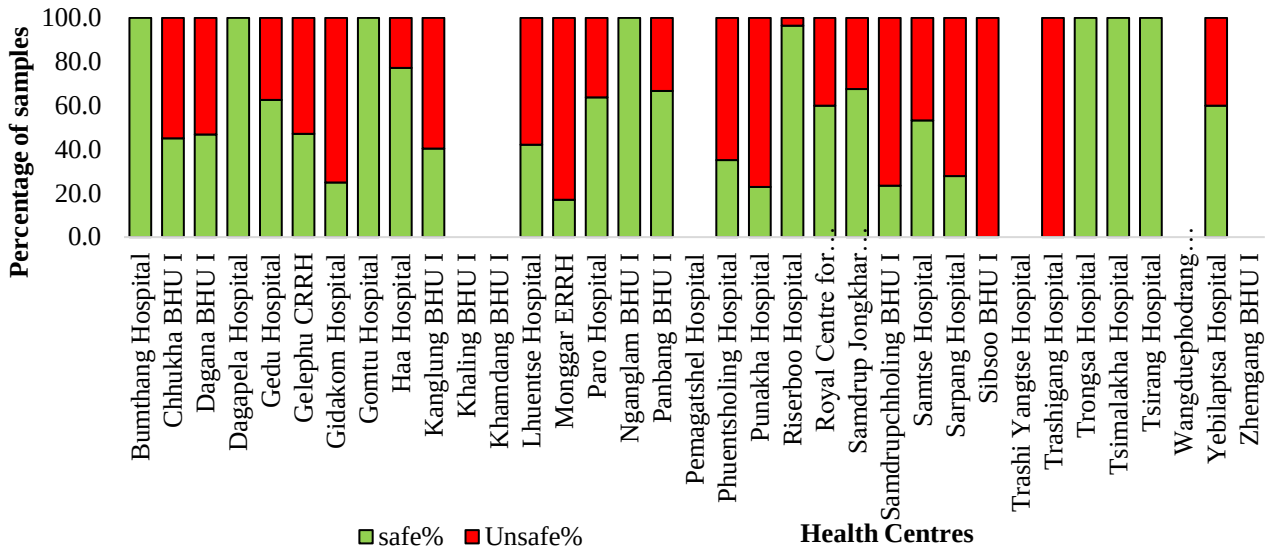
Currently Tested Parameters(Urban)	**Currently tested parameters (Rural)**
Thermo tolerant Coliform	*E.coli*
Turbidity	Conductivity
pH	Turbidity
Free Residual Chlorine	pH
Conductivity/TDS	Odor
E. Coli	Colour

Results

Urban Drinking Water Quality surveillance

Thermo tolerant coliform

A total of 2940 samples were collected and tested for bacteriological testing of thermo tolerant coliform from 29 urban health centers in the country for routine water quality surveillance in 2022. Of the total, 55.8% of samples were found safe (0 CFU/100mL) and the rest (44.2%) were found unsafe (>0 CFU/100mL Thermo tolerant Coliform) for consumption **(Figure 2).**



***Note**: Samples not received from Khaling BHU I, Khamdang BHU I, Pemagatshel Hospital, Trashiyangtse Hospital , Wangudephodrang hospital & Zhemgang BHU I*

Figure 2: Urban Thermo-tolerant coliform report

Turbidity

A total of 770 samples were monitored for turbidity from 35 urban health centers for routine water quality surveillance. Of the total, 83.9% of the samples were found within the acceptable limit (<5NTU). It was noted that a maximum number of turbid samples were observed in June, July & August **(Figure 3)**

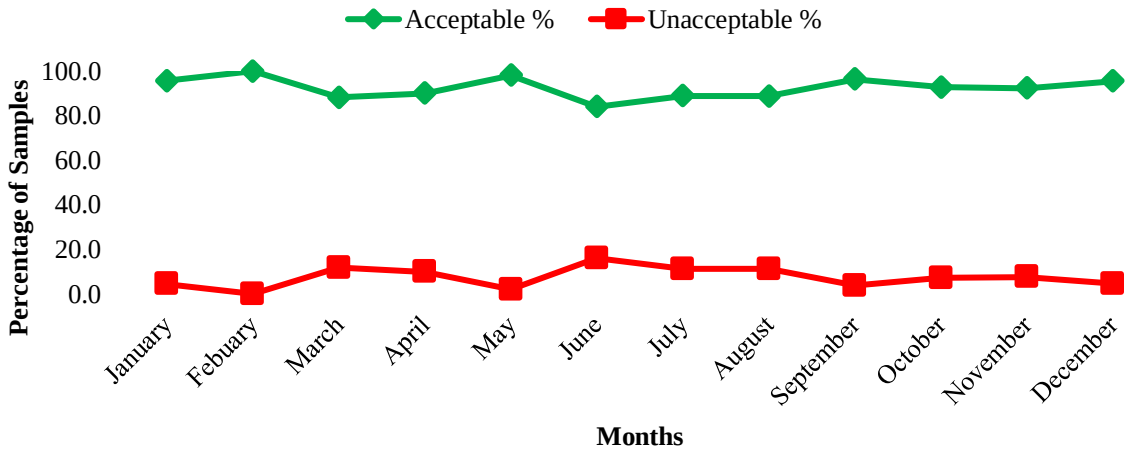


Figure 3: Monthly variation of turbidity from 35 health centers

pH

The allowable range for pH is 6.5 to 8.5 as per BDWQS. Out of 1691 samples tested, 11.5%of the samples were found non-compliant. The highest pH tested was found to be 9.6 and the lowest value was tested at 6. There was no noticeable difference during wet and dry seasons.

Free Residual Chlorine

Out of 20 dzongkhags, only eight Thromde (Bajo, Bumthang, Gelephu, Phuntsholing, Thimphu, Gomtu, Samtse and Tsirang) monitor free residual chlorine level in drinking water. Wangdue did not submit the test records. Other districts do not have treatment facilities or are non-functional. A total of 478 treated water samples were tested for Residual Chlorine from which 83.3%were observed inadequately chlorinated. The acceptable range of free residual chlorine is 0.2-0.5 ppm which is recommended concentration to kill bacteria in the water **(Figure 4).**

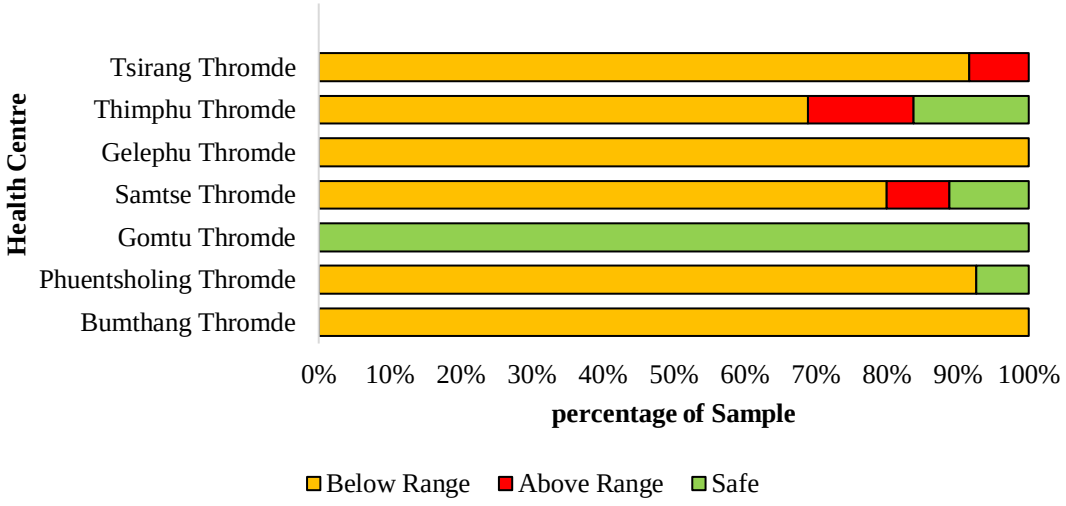


Figure 4: Free Residual Chlorine maintained at different urban drinking water treatment facilities

Rural Drinking Water Quality Monitoring (RDWQM)

A total of 2023 samples were tested for *E.Coli* from rural health centers, out of which 58.2% were found fit for consumption and the rest (41.8%) were found to be potential health risk if consumed without boiling **(Figure 5 & 6).**

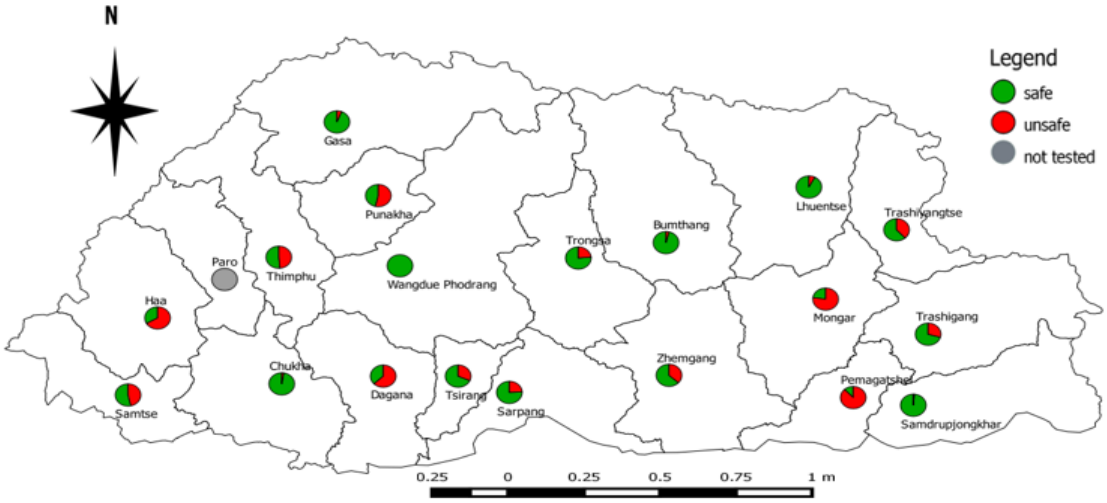


Figure 5: Rural *E.Coli* report for drinking water quality monitoring

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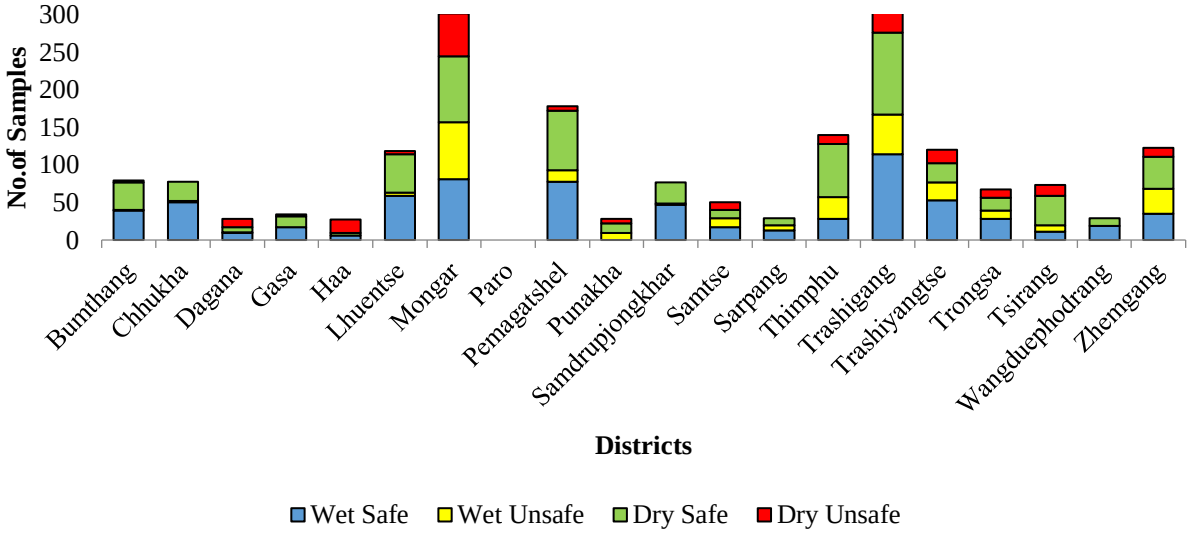

Figure 6: Variation in dry and wet season of rural drinking water quality

Discussion

Thermotolerant Coliforms are bacteria that can be introduced into drinking water supplies through human or animal feces. For water to be considered safe, Thermo tolerant Coliforms should not be detected in a 100mL sample of drinking water. If detected, it is an indication of the contamination of drinking water by feces and also suggests that other potentially-harmful microbes may be present. Comparing the microbiological results of urban and rural health

centers, results from the urban health centers showed more contamination which could be because of the large number of settlements in urban areas, problems with the water supply system and inadequate or improper chlorination where water treatment plants are present. Drinking water sources were found contaminated more during the wet season as expected because of the rain water washing away the contaminants into the water sources and contaminating the drinking water supply system. Therefore, it is important to sensitize the public to drink boiled water during the wet season.

The presence of chlorine residual in drinking water indicates that a sufficient amount of chlorine is added into the water to inactivate the bacteria and some viruses that cause diarrheal disease; and the water is protected from recontamination during storage and distribution. We observed inadequate free residual chlorine in more than 50% (83.3%) of water samples tested. This problem is likely due to the addition of inadequate chlorine in the water or the sodium hypochlorite used for water treatment do not have adequate chlorine content in it. It is important that the chemical procured for water treatment has adequate chlorine content which can be validated by testing in the lab. Further, where chlorination is done, the concerned district thromde must monitor the free chlorine at the treatment plant on a regular basis.

Turbidity is a measure of the cloudiness of the water caused by suspended particles or colloidal matter. Although turbidity may not have a direct health effect it may have a negative impact on consumer acceptability.

Conclusion

Drinking water quality status will change continuously due to many factors.Therefore, regular water quality monitoring is crucial to understand the quality of drinking in the country and provide the evidence necessary to make decisions on managing water quality.

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Drug Quality Monitoring Report, 2022

KelzangWangdi, Amin NgawangTashi, Sonam Gyeltshen, Sonam Choden

National Drug Testing Laboratory

Introduction

The World Health Organization (WHO) estimates that 1 in 10 medical products circulating in low-and middle-income countries is either substandard or falsified, which indicates that people are taking medicines that fail to treat or prevent disease (1). Apart from increased morbidity and mortality from substandard and falsified medicines, it is also a financial loss to individuals and health systems that purchase these products (1,2). As the COVID-19 pandemic strained health systems worldwide, there is a growing concern about its potential impact on the supply chain for medical products in low- and middle-income countries (LMICs) (3). As country borders closed and countries dependent on imports for essential medicines were left empty-handed, substandard and falsified (SF) products began proliferating in LMIC markets, further threatening public health and imperiling ongoing health programs (3). Quality medicines are essential for effective management and treatment of illnesses (4).

National Drug Testing Laboratory (NDTL) was established under Royal Centre for Disease Control (RCDC) in 2013 as the National Quality Control Laboratory for medicinal products. The laboratory has been officially recognized as the reference laboratory for Drug Regulatory Authority (DRA) by the Bhutan Medicines Board. To enhance and ensure the quality of medicines, the quality monitoring and surveillance of medicines was strengthened by development of Medicinal Product Quality Monitoring System (MPQMS) and Substandard and Falsified Medical Product Reporting System (SFPRS) in collaboration with Drug Regulatory Authority. The SFPRS is a web-based database system for reporting of any defective medicines by health centers. In addition, sentinel surveillance is also carried out by NDTL.

Methodology

There are three categories of samples that NDTL receives;

Surveillance – Samples are collected quarterly by RCDC from surveillance sites for drug quality monitoringin the country. Presently, NDTL has four surveillance sites; Jigme DorjiWangchuck

National Referral Hospital, Thimphu, Mongar Eastern Regional Referral Hospital, Mongar, Gelephu Central Regional Referral Hospital, Sarpang, Medical Supply & Distribution Division (MSDD), Phuentsholing, Chukha. From the rest of the health centres, DRA collects the samples for quality monitoring

Complaint – Samples referred by other agencies based on complaints received on the quality of the samples/ adverse drug reactions

Registration – Samples referred by DRA for registration of medicinal products

Testing

The samples are analyzed as per their pharmacopeia claim and availability of reference standards. If the prescribed pharmacopeia method is not achievable, the selection of the method follows the following order; International Pharmacopeia, British Pharmacopeia, United States Pharmacopeia and International Pharmacopeia.Primary reference standards from Indian Pharmacopeia Commission, United States Pharmacopeia, French Reference Standard, European Pharmacopoeia (Ph. Eur.) were used for the analysis of samples. Drugs are tested for the following parameters **(Table 1):**

Table 1: List of Physical parameters and chemical parameters

Physical Parameters	Chemical Parameters
Identification Test	Assay
Average Weight	Dissolution Test
Average volume	Microbiological Assay
Uniformity of weight	Uniformity of Content
Friability Test	
Disintegration Test	
pH	
Sterility Test	

Data Analysis

Data was generated from Medicinal Product Quality Monitoring System (MPQMS) and was imported into the MS excel 2019. All data was analysed using Epi Info v.7 (CDC, USA) and descriptive analysis is presented as frequency and percentage.

Results

Collection of samples

In 2022, a total of 399 samples were collected from 21 sites of which 151 (37.8%) were collected by DRA and 244(61.2%) were collected by RCDC.The non-compliance rate for samples collected by DRA was 7.9% (n=12), RCDC was 3.3% (8) and the overall rate was 5.3 % (n=21) (**Table 2**).

Table 2: Distribution of samples collected along with non-compliance

Collected By	Complies	Does not comply	Total	% Non-compliance
Drug Regulatory Authority	139	12	151	7.9
Medical Supply & Distribution Division	1	1	2	50
Medical Supply & Procurement Division	1	0	1	0
Paro Hospital	1	0	1	0
Royal Centre for Disease Control	236	8	244	3.3
Total	**378**	**21**	**399**	**5.3**

Maximum number of samples were collected from MSDD comprising 19.3 % (n=77), followed by samples from JDWNRH and Mongar ERR Hospital with 11.5 %(n=46) and 7.8 % (n=31) respectively. Samples from 13 health centres were found to be substandard with the four samples failing from MSDD, followed by three samples from LhamoyzingkhaBHU**(Figure 1).**

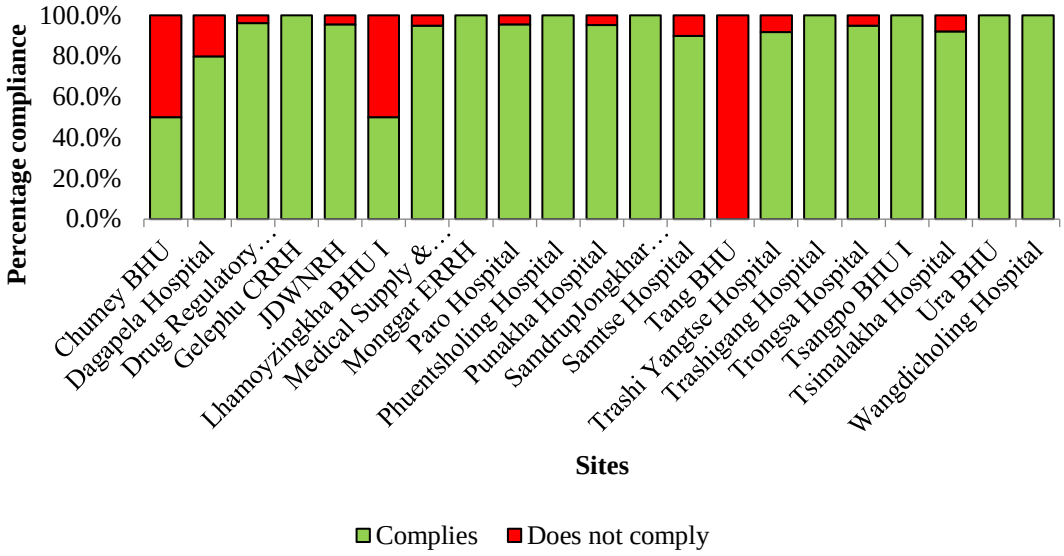

Figure 1: Compliance of samples according to collection sites

The highest number of samples were tested from April to June constituting 61.4 % (n=245), followed by October to December, July to September and January to March with 16.3 % (n=65), 15.3 % (n=61) and 7.0 % (n=28) respectively.

Category of samples

From the total of 399 samples analyzed, seven comprised of complain samples, 25 registration samples and 367 surveillance samples. Of those one (14.3%) samples from complain, one (4.0%) from Registration and 19 (5.2%) from surveillance does not comply.

From a total of 21 samples (5.3%) found substandard; Cloxacillin 250 mg capsulesconstituted 23.8% (n=5) followed by Chloroquine 150mg tablet at 19.0 % (n=4)**(Figure 2).**

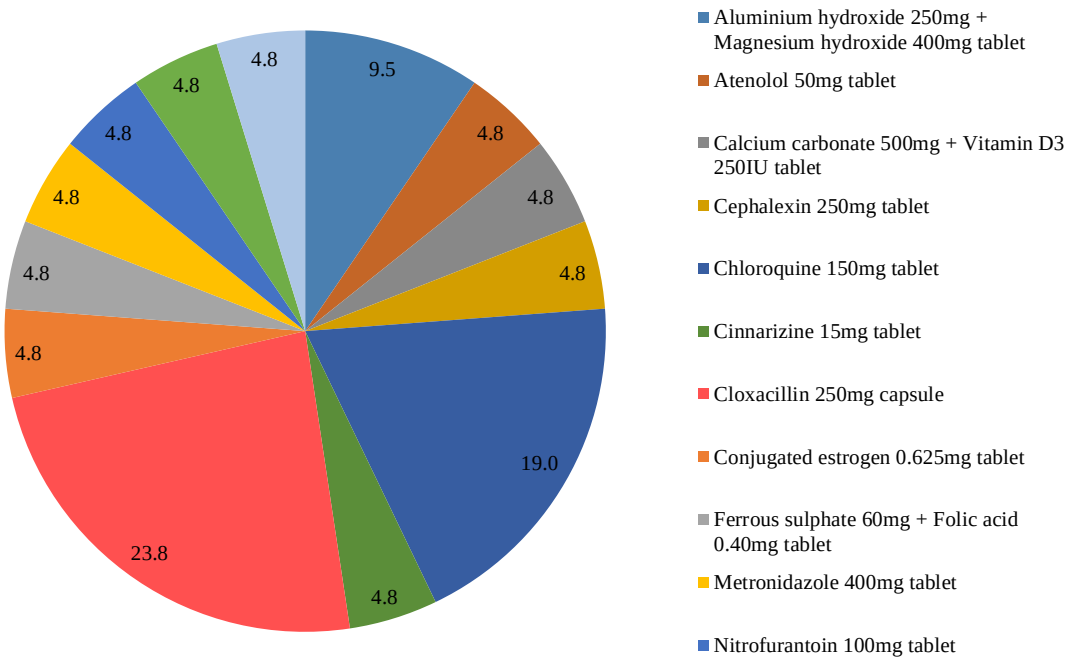

Figure 2: Distribution of non-compliance drugs

Category of formulation

The types of samples are categorized into 25 formulations. Presently tablets are the most frequently tested samples with a proportion of 85.2 % (n= 340), followed by capsules with a proportion of 11.8 %(n=47). From these, 15 tablets and 6 capsules did not comply with the requirements of the standard monograph with the percent of non-compliance at 4.4 % (n=15) and 12.7 % (n=6) respectively**(Table 4).**

Table 4: List of formulation tested

Formulation	Complies	Does not comply	Total	% Non-compliance
Capsules	41	6	47	12.7
Effervescent	1	0	1	0.0
Injections	9	0	9	0.0
Insert	1	0	1	0.0
Others	1	0	1	0.0
Tablets	325	15	340	4.4

Test Parameters

A total of 7.5 % (n=30) samples were analysed for both physical as well as chemical parameters and92.5 % (n=369) samples were analysed for only physical parameters. The non-compliance rate for assay was 16.7 % (n=5) from the total of 30 samples tested for assay. The non-compliance rate for the uniformity of weight and friability test was 2.8% (n=11) and 2.3 %(n=5) respectively**(Figure 3).**

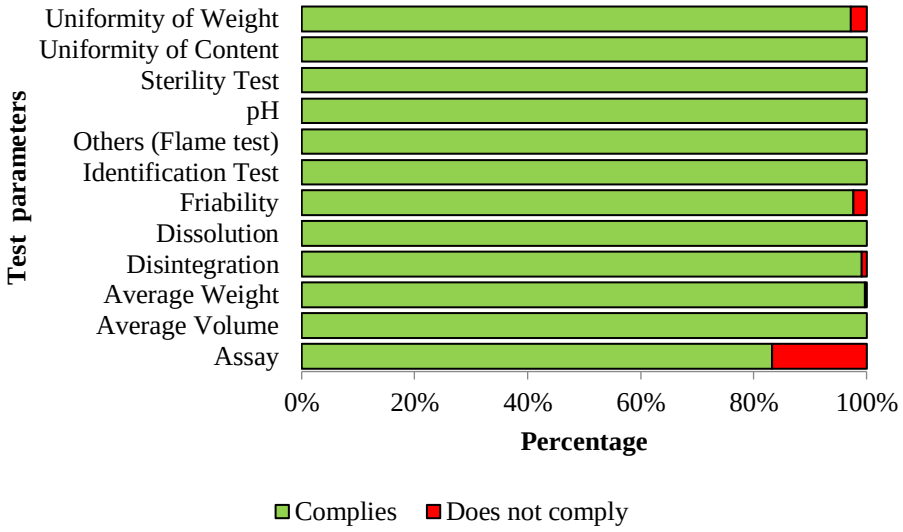

Figure 3: Compliance rate according to test parameters

Pharmacological category

The samples are broadly categorized into 30 pharmacological categories as per the National Essential Medicines List 2018. Samples belonging to 21 various categories were analysed. The most frequently tested samples were Cardiovascular medicines constituting 20.0 % (n=111), followed by anti-infective medicines at 15.5% (n = 86). Medicines used in haemodialysis had the maximum percent non-compliance at 100% (n=2). Anti-infective medicines and Cardiovascular medicines had 12.8 % (n=11) and 3.6% (n=4) of non-compliance rate respectively which is rather high and alarming**(Table 5).**

Table 5: Distribution of samples as per their pharmacological category

Category	Complies	Does not comply	Total	NC %	Total %
Muscle Relaxants	1	0	1	0.00	0.18
Oxytocics And Tocolytics	1	0	1	0.00	0.18
Anaesthetics	2	0	2	0.00	0.36
Medicines For Use In ICU	2	0	2	0.00	0.36
Solutions For Water, Electrolyte, Acid-Base Disturbances	5	0	5	0.00	0.90
Immunologicals	9	0	9	0.00	1.62
Vitamins	15	0	15	0.00	2.70
Anti-Allergic& Medicines Used in Anaphylaxis	35	0	35	0.00	6.31
Hormones, Endocrine Medicines & Contraceptives	66	1	67	1.49	12.07
Medicines Used in Neurological Disorders	51	1	52	1.92	9.37
Genitourinary Medicines	59	2	61	3.28	10.99
Cardiovascular Medicines	107	4	111	3.60	20.00
Others	25	1	26	3.85	4.68
Medicines Affecting the Blood	17	1	18	5.56	3.24
Analgesics, Antipyretics& Non-Steroidal Anti-Inflammatory Medicines	34	4	38	10.53	6.85
Anti-Infective Medicines	75	11	86	12.79	15.50
Ophthalmological And ENT Preparations	6	1	7	14.29	1.26
Minerals	7	2	9	22.22	1.62
Antineoplastic Agents & Immunosuppressive Medicines	3	1	4	25.00	0.72
Respiratory Tract Medicines	3	1	4	25.00	0.72
Medicines For Use inHemodialysis	0	2	2	100.00	0.36

Discussion

In 2022, 399 samples were analysed at NDTL comprising 367 surveillance samples, 25 registration samples and 7 complain samples with an overall non-compliance rate at 5.3% (n=21). The non-compliance rate for 2022 is lower than the estimated WHO rate (10%). The average non-compliance rate for the past five years is 6.9 % with the year 2018, 2019, 2020 & 2021 with 9.1 %, 4.6%, 10.6%, 4.7% & 5.3% rate of non-compliance respectively.

From the total of 21 substandard samples, 4 were detected at MSDD before distribution and 17 were detected at the hospital level after distribution and use by the hospitals. This could contribute to adverse events related to these medicines and also may contribute to the increasing AMR. Though no major adverse events related to medicines has been noted, if the prevalence of substandard medicines remains constant, there could be adverse events.

The most and frequent substandard medicines belonged to anti-infective medicines and cardiovascular medicines since 2018. This may have contributed to anti-microbial resistance and also adverse events which are not known.

On aggregate, 30 samples have failed in assay, 29 in friability, 17 in disintegration and 13 in uniformity of weight from 2018- 2022. This suggest that testing the samples for only physical parameters such as Uniformity of weight, friability and disintegration is equally as good as testing the samples for chemical parameters for identification of substandard medicines. Therefore, testing of surveillance samples for physical parameters can be practiced to improve the NDTL services and minimize the waste of resources.

Conclusion and Way forward

With consistent number of substandard medicines found from 2018-2022,there is a huge likelihood of prevalence of more substandard medicines in the market. The need to strengthen Drug Quality Monitoring and Surveillance in the country is paramount. Further there is an urgent need to institute a mechanism to test the medicinal products at the point of entry and distribution centre prior to distribution of the products to health centres to avoid any adverse events and also minimise the waste of resources.

Way Forward:

- Increase the number of surveillance sites.
- Timely field monitoring and evaluation to provide recommendations and suggestions.
- Encourage active participation from health centres in reporting defective medicines through SFPRS

Collaboration with National Medical Services to ensure timely testing of medicines to guarantee distribution of only quality medicines.

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Food Safety surveillance in Bhutan, 2022

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Introduction

Globally, the World Health Organization (WHO) estimates that foodborne diseases (FBD) cause 600 million illnesses with 420,000 deaths and 33 million disability adjusted life-years (DALYs) annually. The highest burden was recorded in the Africa region (1300 DALYs per 100000 population) followed by South East Asia (1200 DALYs per 100000 population) with the second highest burden of food borne diseases (FBD) incidences per population among WHO regions (Organization, 2015). Foodborne diseases (FBDs) represent a threat to public health and hinder socioeconomic development (Botelho et al.). In the South East Asian region approximately 50% of FBD's are caused by diarrheal agents (*E. coli, Salmonella, Campylobactera, Norovirus*).

In Bhutan diarrhea causes significant morbidity and FBD is mostly associated with diarrheal symptoms. More than 60 events of food borne disease have been recorded with the Royal Centre for Disease Control (RCDC) from 2012 to 2020 (www.rcdc.gov.bt). Our review paper published in 2022 states that laboratory confirmation rate of FBD investigation outbreaks increased from 25.5% (2012–2018) to 91.7% in 2020, and at least 0.2% of the total population had suffered FBD from 2015 to 2020.The baseline report shows that most of the FBD events occurred during hot and wet seasons (June and August). The common pathogen causing FBD was *Salmonella* species accounting for 42.9% of cases, followed by *Shigella* species (21.4%). Most of the FBD events were reported in schools (46.5%) (1). The FBD linked to bakery products was also studied and found that *Salmonella* being the common pathogenic organism isolated (Chhetri et al., 2022). Also the earlier food safety surveillance report findings shows that the RTE foods can likely be hazardous as 12.3% of samples were contaminated with indicator organisms and 8.7% of the samples were unacceptable due to pathogenic organism contamination (2).

Foodborne diseases (FBDs) mostly present as gastrointestinal dysfunction. They are caused by consuming foods that are contaminated due to the use of polluted water for food preparation,

pollution in soil where food is grown, as well as unsafe practices at any stage of the food preparation, delivery, and consumption chain, involving the entire process from farm to fork. Therefore, food safety surveillance is important to identify risk and mitigate necessary action to prevent FBD.

Methodology

Food safety surveillance is a sentinel based surveillance consisting of the five sites (Paro, Thimphu, Phuentsholing, Gelephu and Monggar) **(Figure 1)**. These sites were chosen because these are the towns with higher population and functional hotels/restaurants.

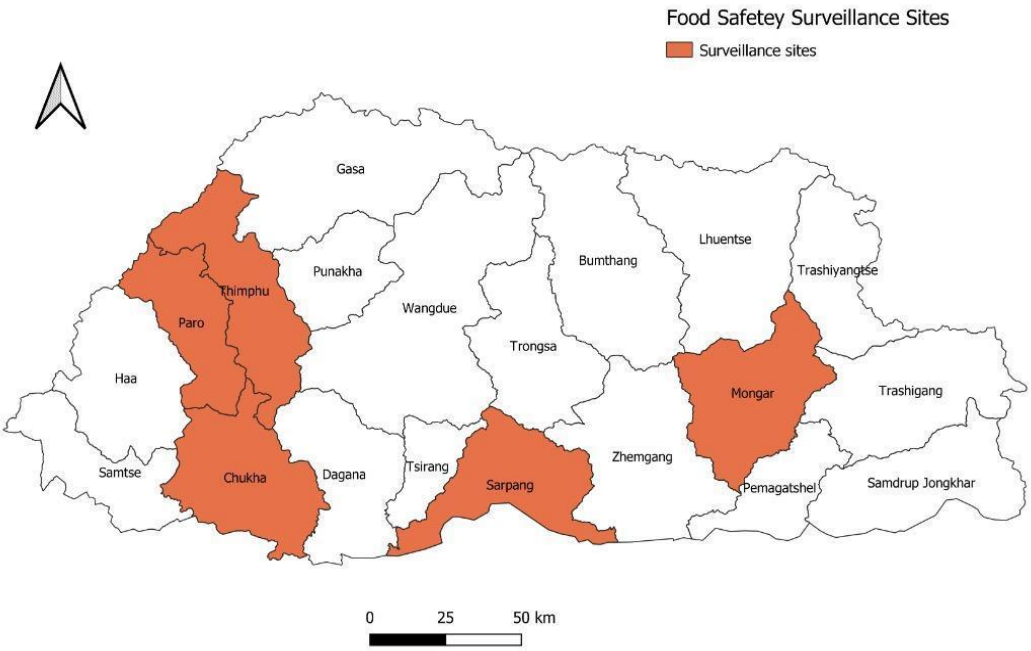

Figure 1: Food safety surveillance sites (highlighted) in Bhutan (2022)

Samples collection

The food samples were aseptically collected (five samples per month) from randomly selected restaurants/hotels/bakery by the Food Inspectors (BAFRA). Any ready to eat food samples; including both vegetables and meats 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 coldchain to the Food and Nutrition Laboratory, Royal Centre for Disease Control, Thimphu for laboratory analysis.

Sample preparation and Laboratory Analysis

Samples were prepared according to the Association of Official Analytical Chemist (AOAC) method (Feldsine et al., 2002). Twenty-five grams of each sample was weighed and homogenized for 30 seconds at 230 rotation per minute (RPM) by stomacher in 225 mL of sterile buffered peptone water (BD, USA). Furthermore, the serially 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/gram). 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 35ºC ±2ºC for 48 hours with 5 % CO_2. After which a loopful of enrichment broth was cultured on *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 35ºC ±2ºC for 48 hours with 5 % CO_2.

Isolate identification

Colonies were presumptively identified by colony pigmentation and Gram staining characteristics. Pure cultures were obtained by streaking a portion of an isolated colony on nutrient agar and incubated aerobically at 37 °C for 24 h (Gilbert et al., 2000). Biochemical identification test and furthermore API 20E was performed on isolates with discordant results.

Polymerase chain reaction (PCR)

The PCR test was performed onto the *E. coli* isolates including *Enterotoxigenic E. coli* (ETTC), *Enterohemorrhagic E. coli* (EHEC), *Enteroinvasive E. coli* (EIEC), *Enteroinvasive E. coli*

(EIEC), and*Enteroaggragative E. coli* (EAEC) to study the presence of any pathogenic strains. The primers used were manufactured by Macrogen.

Statistical Analysis

Statistical analysis was performed using MicroSoft excel and descriptive analysis is presented as percentages and ratios.

Results

A total of 136 ready to eat food samples were collected from five dzongkhagsthrome (Figure 2). Of the total ready to eat food samples collected, 100% samples were solid food with pH ranging between 5.8-7.3. Overall, 22.1% of food samples were unacceptable based on indicator test contamination and/or pathogenic organisms detected that are capable of causing foodborne disease. The highest contamination due to indicator organisms was isolated from Sarpang food samples (47.2%) and highest pathogenic organisms isolated from Paro food samples (30.0%) **(Table 1).**

Table 1: Percentage of samples with un-acceptable level of indicator and pathogenic organism, Food safety surveillance 2022

	Indicators test organism				**Pathogenic organism**			
Place	TPC	*E.coli*	*Enterobacteriaceae*	Y/M count	*B. cereus*	*S. aureus*	*Aeromonas*	*Shigella*
Chhukha (n=25)	8.00% (n=2)	0	0	4.0% (n=1)	12.0% (n=3)	4.0% (n=1)	0	0
Paro (n=20)	40.0% (n=8)	5.0% (n=1)	15.0% (n=3)	10.0% (n=2)	20.0% (n=4)	20.0% (n=4)	0	0
Thimphu (n=30)	0	0	0	0	3.33% (n=1)	0	0	0
Mongar (n=25)	4.0% (n=1)	0	4.0% (n=1)	0	4.0% (n=1)	12.0% (n=3)	0	0
Sarpang (n=36)	33.3% (n=12)	19.44 % (n=7)	30.5% (n=11)	11.1% (n=4)	16.6% (n=6)	13.8% (n=5)	0	0

Of the total samples tested, 22.0% was contaminated with indicator organisms which includes total plate count, total *E.coli* count, *Enterobacteriaceae* and total yeast mold count. The common type of food contaminated with the indicator organisms were chowmein, channa, aluchop followed by other foods items**(Figure 2).**

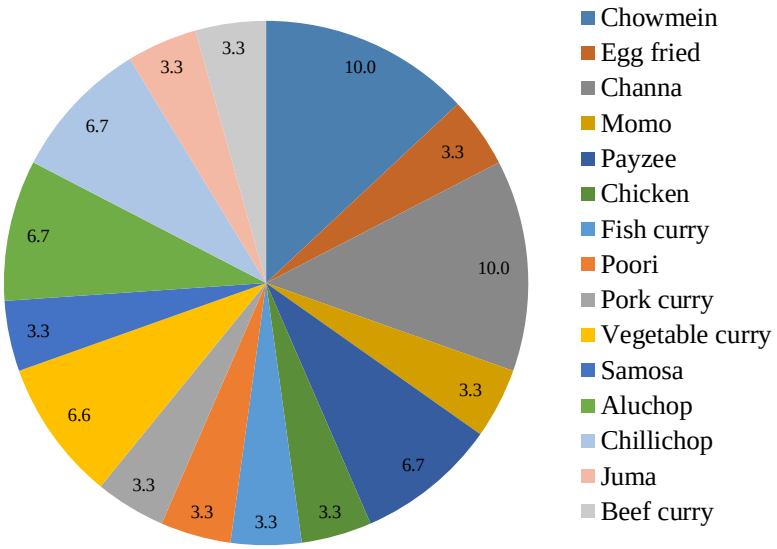

Figure 2: Common types of RTE foods contaminated with indicator test organism (Food safety surveillance 2022)

Of the total samples, 19.1% of foods were being contaminated by pathogenic organisms. The common types of pathogenic organisms isolated from food samples include *Staphylococcus aureus* and *Bacillus cereus*. The common type of food with pathogenic organisms were sausage/jumma (19.2%) followed by other food items **(Figure 3).**

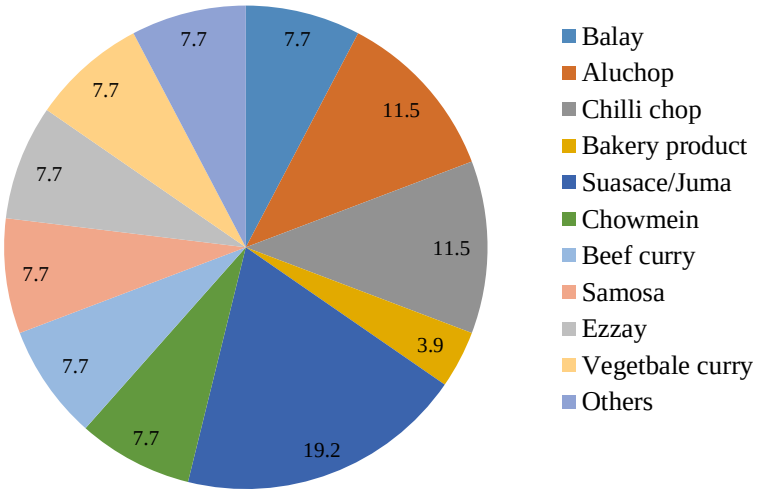

Figure 3: Common types of RTE foods contaminated with common pathogenic organism (Food safety surveillance 2022)

Polymerase chain reaction

Multiplex PCR was performed onto the isolated *E.coli*. An isolate of the *E.coli* was found to be positive for pathogenic *E.coli* (EAEC)**(Figure 4).**

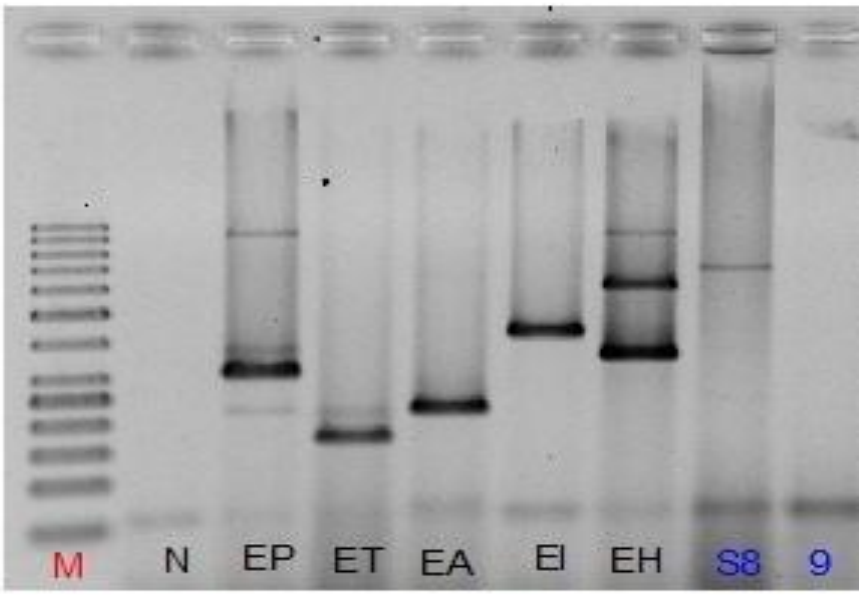

Figure 4: Gel electrophoresis (Sample S8 EAECfrom food sample)

Discussion

Due to the pandemic, surveillance was reactivated only after June, 2022. Therefore, the current report contains data for June to December 2022. Since the initiation of food safety surveillance from June 2019, 767 RTE food samples have been laboratory investigated. In total, 136 food samples were sampled during the current study period, this contributed by 21.5% to the overall sample size analyzed (2019 to 2022).

The common food parameter that attracts customer preference as well as actsas a quality indicator of food or storage information is pH value. The other advantage of low pH food or acidic foods is most of the pathogenic organisms cannot survive low pH. Therefore, the pH of RTE food was measured but there were no high acidic or high alkaline foods being sampled during the current study period. A few food samples were low acid, and the overall pH ranged between 5.8 &7.3.

Of the total 136 food samples tested between June to December 2022, 22.0% (n=30) was contaminated with indicator organisms as compared to 12.3% (n=78) between 2019-2021 food safety surveillance (2). Foods were classified as acceptable or hygienic based on the inference from indicator teste limits. The common indicator test limit for total bacterial count or total plate count is $<10^5$ CFU/g, Yeast mould count$<10^2$ CFU/g, Total *Enterobacteriaceae* count $<10^2$ CFU/g and *E.coli* count $<10^5$ CFU/g . Total Plate count of aerobic microorganisms and yeast mould count found in food is one of the microbiological indicators used as surrogacy for hygiene indicators (3).

During the current food safety surveillance period it was found that indicator test (total plate count and total *Enterobacteriaceae* count) un-acceptability was slightly higher. The majority of the indicator test violation was present in the samples from Paro and Sarpang. The highest number of indicator test unacceptable was due to $>\log^5$ growth of total plate count/total bacterial count 40.0% (Paro samples) followed by total *Enterobacteriaceae* count 30.5% (Sarpang samples). This indicates that there are possibilities of unhygienic cooking process, poor standards of cleaning of cooking utensils and lack of proper hand-washing hygiene or temperature abuse. The earlier report (2019-2021) presented that the samples collected from Phuentsholinghad highest indicator test organism contamination [TPC (13.8%), *E. coli* (12.3%) and *Enterobacteriaceae* (12.3%), respectively] (2). The most common type of food group contaminated with indicator organisms were the fast foods; such as chowmein and Channa (10.0% each), these findings remain similar to the earlier report (2019-2021) (2). This could be due to the fact that these foods require frequent direct hand contact, and long storage after preparation.

Most of the samples from Sarpang and Paro were contaminated with indicator organisms and later corresponds to the pathogenic organism. Thus, indicates that a food contaminated with indicator organisms has higher chances of contamination with pathogenic organisms and potential for causing FBD outbreaks. Moreover, during the current study period it was observed that three food samples had the growth of both *B. cereus* and *S. aureus*. This also indicates that the proper adherence to a good manufacturing process is inevitable as the pathogenic organism can contaminate food extensively that would cause devastative public health concern and loss to business.

The common type of pathogenic organism isolated remains similar to the previous report of 2019-2021 and of the total 19.1% of samples in 2022 was contaminated with pathogenic organisms. Both *Bacillus cereus* and *Staphylococcus aureus* were isolated from several foods with maximum from the samples collected from Paro. The contamination from these two organisms is common as they are abundantly present in the environment. The risk of FBD outbreak from these organisms cannot be undermined as they are capable of causing intoxication due to toxins.

The most common type of food in which pathogenic organisms are isolated were from sausage/jumma (19.2%) this shows slight change from previous report where chowmein was the top fast food with pathogenic organism contamination. The fact that the jumma/sausage is prepared from the animal intestinal lining, the chances of contamination is high. Therefore, if prepared with inadequate washing several pathogenic organisms can be isolated that can cause FBDs.

Pathogenic *Escherichia coli* was identified through PCR which are a diverse group of organisms with at-least six groups based on the possession of one or more virulence characteristics. Such characteristics contribute to the ability of *E. coli* strains to cause disease in humans and have been used to define the groups.There are several associations drawn between EAEC and persistent diarrhea in children, predominantly in low-income countries. More recently, EAEC have been identified as an important cause of acute diarrhea in children and adults in a wider range of countries. There are evidences of foodborne transmission of EAEC (EAEC serotype O104:H4, first identified in northern Germany and epidemiologically associated with the consumption of fenugreek sprouts- large FBD outbreak) (4).

Conclusion

Bhutanese food is diverse and to ensure it safe for consumption requires a systematic, proactive method to reduce contamination. Moreover, access to safe running water plays a paramount role in food safety. The findings demonstrate that ready-to-eat food is a likely hazard to human health. The isolation of both the indicator (19.1%) and pathogenic micro-organism 22.0%) and pathogenic *E.coli* from ready-to-eat foods is a good indicator of post-processing contamination or inadequate cooking. Therefore, health education to the vendors on personal hygiene and good

manufacturing practices, food safety and proper disposal of waste would improve food quality thereby reducing food borne incidents.

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Prevalence of Vitamin D deficiency in two districts in Bhutan

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Background

Vitamin D (ergocalciferol-D2, cholecalciferol-D3, alfacalcidol), naturally soluble in fat is an important steroid hormone required for proper growth, development, and healthy skeletal structure that regulates calcium and phosphorus metabolism in the body (Kader et al., 2019). Vitamin D consists mainly of two forms; ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) caused by exposure to ultraviolet rays in the skin. In the human body vitamin D is synthesized in the skin by the reaction of 7-dehydroxy cholesterol with UVB radiationof sunlight. Dietary source alone is usually not adequate to meet the body requirements (Taseen and Beaulieu, 2017).

The marker, plasma 25(OH)D or calcidiol (a summation of D3 and D2 forms) is the most reliable way to study vitamin D status. Since vitamin D undernutrition is largely silent and subclinical, the indication for testing remains controversial (Gupta, 2014). In children, severe deficiency leads to bone-malformation, known as rickets. Milder degree of insufficiency causes reduced utilization of dietary calcium. Vitamin D deficiency causes muscle and bone weakness. Insufficiency has been associated with chronic diseases such as diabetes, cancer, cardiovascular disease and autoimmune diseases (Wacker and Holick, 2013). Vitamin D deficiency in pregnancy has been associated with an increased risk of pre-eclampsia, gestational diabetes mellitus, preterm birth, impaired fetal skeletal formation causing infant rickets and reduced bone mass (Organization, 2012).The status of deficiency is estimated reliably by measuring the level of 25-hydroxycholecalciferol (25 (OH)D) in the serum (Regmi et al., 2017).

Vitamin D deficiency is a global health issue and estimated 50% of the global population have vitamin D insufficiency (Siddiqee et al., 2021). In south East Asia, Thailand has 41.5% insufficiency and 34% deficiency (Pratumvinit et al., 2015); Bangladesh has 12- 83% deficiency; India has 27-42% deficiency (Arabi et al., 2010). Till date there is no data representation of

Bhutan although the risk factors such as low exposure to sun, skin tone and increasing indoor activities amongst the Bhutanese population increases the probability of Vitamin D deficiency. The other factors include the season of the year, environmental pollution, use of sunscreen cream, aging and geographical location (Cannell et al., 2008).Therefore,the objective of this study is to determine the prevalence of vitamin D level in the Bhutanese population and provide evidence for public health interventions.

Methodology

The study was conducted in the third quarter of 2021 on serum samples from Infectious Disease Serology Laboratory (IDSL), Royal Centre for Disease Control (RCDC). The blood samples were collected from young adults from Thimphu and Paro, who were free of any chronic illness and mostly working in offices and few students. The samples were collected for COVID-19 vaccine efficacy study and stored at -40 °C. The level of vitamin D level in serum samples was tested using SD Bio-sensor (F200 analyzer) immune chromatography technique by measuring the level of 25-hydroxycholecalciferol (25 (OH) D) in the serum (Gupta, 2014). The laboratory testing was performed following the manufacturer instruction (SD Biosensor F-VTD-01).

Assay calibration and quantification

Test method was validated by internal calibration of equipment and running known value samples and plotting a regression graph. The calibration curve was obtained by weighted linear regression of the known value sample and serial dilution.

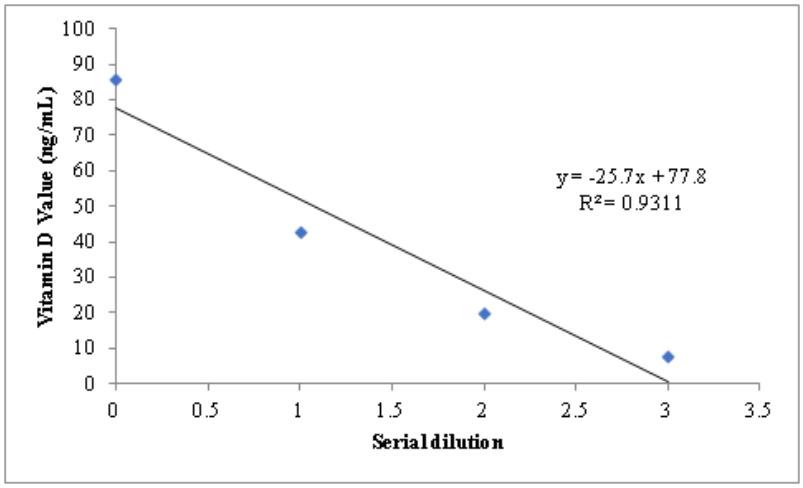

Figure 1: Method validation linear regression graph

Data analysis and report interpretation

All the data were analyzed using ANOVA and descriptive analysis presented as means and percentages. The blood report was interpreted following the criteria mentioned as below:

Table 1: Criteria for diagnosis of serum vitamin D level (Kader et al., 2019)

Serum Vitamin D level	Interpretation
<10ng/mL	Deficiency
10-30 ng/mL	Insufficiency
30-100 ng/mL	Sufficiency
>100 ng/mL	Toxicity

Results

A total of 181 serum samples were analyzedfor25-hydroxycholecalciferol, of which it was found that 77.0% had vitamin D level <30ng/mL (deficiency). Of the total samples analyzed 61.0% were male and 39.0% female. The mean age was 29 years (Range: 10 to 62 years) with the maximum group belonging to 21 - 30 years. The average vitamin D was found to be lowest (18.22±8.0ng/mL) within the age group of 41-50 years. However, amongst the different age groups there was no significant difference at (p-value:0.418) observed in serum vitamin D level, except that the age group 41-50 years had slightly lower average vitamin D level (18.22±8.0ng/mL)**(Table 2).**

Table 2: Percentage of different age group and average serum vitamin D level

Age group	**Percentage of different age groups**	**Averageserum Vitamin D level (ng/mL)**	**p-value (between groups)**	**F-ratio value (between groups)**
10 - 20 years	21% (n=38)	21.97±10.7	0.4731	0.518
21 - 30 years	40% (n=73)	21.93±12.4	0.5137	0.4292
31 - 40 years	21% (n=38)	21.51±12.1	0.3794	0.7973
41 - 50 years	14% (n=25)	18.22±8.0	0.4005	0.7596
51 - 60 years	3% (n=6)	24.68±7.2	0.6494	0.2180
≥60 years	1% (n=1)			

It was found that only 21.0% (n=23) of male and 27.0% (n=19) of females had sufficient serum vitamin D level (>30ng/mL). The average serum vitamin D (25 (OH) D) level was 21.7±11.0 ng/mL and there was no significant difference between male (21.8±10.0 ng/mL) and female (21.9±12.4 ng/m\L)**(Figure 2)**.

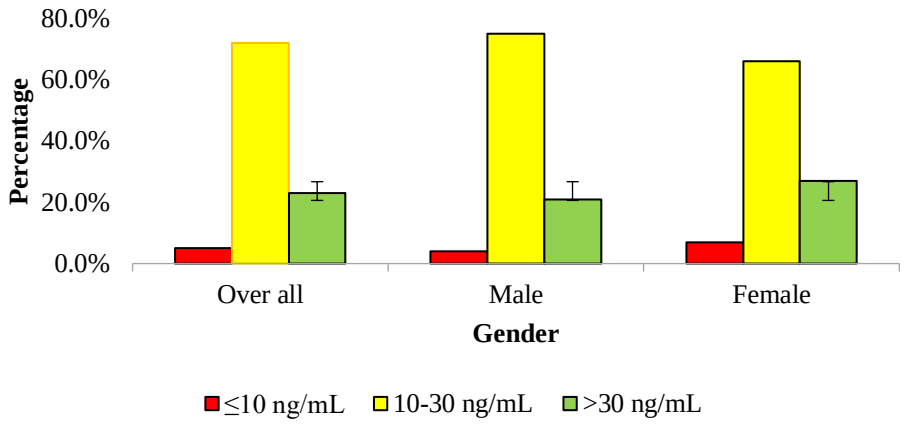

Figure 2: Percentage of different level of serum vitamin D

The study found that only 23.0% (n=42) had vitamin D levels above 30 ng/mL and the distribution was similar for both the sexes. The maximum samples had vitamin D levels ranging between 10-30 ng/mL (insufficient) and 5.0% (severe deficiency) had below 10ng/mL. None of the samples had vitamin D toxicity levels.

Discussion

Study on vitamin D in Bhutanese population is limited. This study analyzed the serum vitamin D level of various age groups and by sex. The overall deficiency/insufficiency of vitamin D was 77.0%. A recent study in Bhutan (Nishizawa et al., 2017) stated the population could be at risk of severe vitamin D deficiency. The finding of the study is comparable to other neighboring countries; 74.1% in Nepal (Shrestha et al., 2019), 83.0% in Bangladesh (Acherjya et al., 2019) and >90.0% in Tibet (Gelsor et al., 2017) had vitamin D deficiency.

The comparison was done between the different age group and sex. It was found that there was no significant (p-value:0.95) difference observed between different sex groups. This finding is in

contrast to findings from other studies (Siddiqee et al., 2021, Gelsor et al., 2017), whereby they mentioned females could be affected with vitamin D deficiency more than the males.

The mean average difference of vitamin D level was studied among different age groups and found that the three age groups (10-20 years, 21-30 years, and 30-40 years) had the same range of vitamin D level and SD. On the other hand, the 41 - 50 years age group had the lowest average vitamin D value of 18.2±8.0ng/mL.Similarly the high prevalence of vitamin D deficiency (92.0%) was found in the 41 - 50 years age group followed by 31 - 40 years age group (79.0%). A study by (Siddiqee et al., 2021) found that 68.0% [95% CI: 64% to 72%] of South Asian adults (mid age) are affected with vitamin D deficiency. These age groups of people are usually office goers who attend indoor jobs in urban areas and have very minimal exposure to external sunlight.

Though high altitude receives adequate sunshine with low air pollution,limited exposure to sunlight is a common factor for low vitamin D (Kapil et al., 2017). Additionally due to short summer and cold winter (Lips, 2007) and clothing that covers the whole body. South East Asians typically have higher concentrations of skin melatonin resulting in a longer time period required to synthesize vitamin D at higher latitude (Laird et al., 2020). The human serum 25(OH)D level is influenced by their occupation and work environment (Gelsor et al., 2017) and it is anecdotal that farmers in Bhutan have strong bones as compared to urban dwellers.

The WHO Bhutan fact sheet presents, 40.0% of Bhutanese women are either overweight or obese, whereas 27.0% of men were reported to be either overweight or obese (Chhetri et al.) and the higher BMI is associated with a lower 25(OH)D measurement possibly due to either adipose sequestering 25(OH)D, or due to a higher fluid volume in those with a larger body size (Darling, 2020). Rickets is a condition of bony deformity related to poor mineralization of growing bones and Vitamin D deficiency was established as the primary cause of rickets (Erbele et al., 2016). Rickets occur in Bhutan, but the causes and prevalence are unknown. Several correctable risk factors have been identified.

Conclusion

From the study it can be concluded that the Bhutanese population (77.0%) is deficient in vitamin D. The small sample size and unavailability of other information such as type of work they do,

personal dietary habits are few limitations of the study. Further studies are recommended for conducting the comprehensive study to represent the whole country.

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Estimation of formaldehyde in dry fish and health risk assessment

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Introduction

Fish is an important source of protein (15.0-20.0%) with long chain omega-3 fatty acid (1). However, fish is a very perishable food commodity and risk of long storage and deterioration during transportation is a major challenge (2). Fish fermentation and drying fish is a common technique for increasing the shelf-life of fish and icing of fresh fish can maintain its freshness up-to one week (3). Therefore, adding chemical preservatives, such as formalin to increase the shelf-life of fish is a common illegal practice (4).

Formalin or 37.0% formaldehyde is a reactive, strong odored, colorless and a poisonous well known carcinogen (WHO, IARC carcinogenic to humans- Group 1) and has a determinable effect to the human health (LD50 30 gram for human) (5). Although formaldehyde is an antimicrobial agent, its use in food has been prohibited due to its adverse effect to human health. Formaldehyde exposure can cause several complications, such as bronchial asthma, nasopharyngeal cancer (NPC), leukemia, dermatitis and mutations (6, 7, 8). However, a small amount of formaldehyde is produced naturally by fish when it undergoes postmortem changes, the trimethylamine oxide (TMAO) breaks down to give trimethylamine, dimethylamine and formaldehyde (9).

Domestic production of fish in Bhutan has not been able to meet the consumer demand. Thus, most of the fish (both wet and dry fish) are imported. According to the National Research and Development Centre for Aquaculture, Bhutan imports 2049.32 tons (annual mean for 2007 to 2017) from India and other countries. Consumption of both wet and dry fish among the Bhutanese population has recently been cumulative and fish accounts for 3.18% of all protein consumed. The per capita consumption of fish was 6.3 kg/person/year in Bhutan based on the Food and Agriculture Organization report 2017 (Fishery10).

In recent years several public media from India have reported on the addition of formaldehyde in fish causing public concern and need for action (10). Hence, the Bhutan Agriculture and Food Regulatory Authority has started screening for formaldehyde during the import of wet wish. However, the need for testing of formaldehyde in dry fish is also deemed necessary due to its large consumption in population. Therefore, in this study we determine the amount of formaldehyde content in dry fish imported in Bhutan and perform the exposure assessment and characterize the risk of formaldehyde in the Bhutanese population.

Methodology

Sample preparation and Laboratory analysis

Dry fish were randomly collected from 20 Dzongkhag towns. Twenty five grams of dry fish samples were collected in a ziplock plastic bag and assigned a unique sample code. The samples were stored at room temperature until tested.

Five grams of samples weighed and shredded/crushed and put into 100mL flask with 50mL distilled water. The flask was allowed for 30 minutes at room temperature and then filtered using Whatman No. 1 filter paper. Briefly, 5mL of filtrate was transferred to a clean test tube with 5 mL of 0.5% solution of Chromotropic acid (FINAR, AR grade) in 60.0% sulfuric acid (60°, Fisher Scientific). The solution was heated for 15 minutes at 100°C in the Fume hood. The test was cooled and measured using UV-Vis spectrophotometer (Genesys 10S, Thermo Scientific) at 520 nm wavelength. This is a modified method from Utama C etal., (2021) (11).

Standard graph was prepared using formaldehyde (formalin 37%, Sigma). Different concentrations of 0.156, 0.312, 0.625, 1.25 and 2.5 parts per million (PPM) were prepared. A known concentration of formaldehyde was used as internal test control.

Human Health risk assessment

Estimated daily intake of formaldehyde

The estimated average daily intake (EDI) depends upon two factors; the amount of formaldehyde content in fish samples and the average quantity of fish consumed daily. The formula for EDI is calculated using (Bhowmik et al., 2020);

$$EDI = (\frac{FC}{DI})/ABW$$

Where, FC is the concentration of formaldehyde content (mg kg^{-1} or PPM) in fish samples, DI is average quantity of fish consumed daily per person (0.017 kg $person^{-1}$) and ABW is the average body weight (kg $person^{-1}$).

Risk assessment

USEPA has described the target hazard quotient (THQ) for health risk assessment through consumption of fish, thus THQ as estimated using the formula (Environmental Protection Agency, 2000);

$$THQ = 10-^{3}(EFxEDxDIxFC)/(RODxABWxTA)$$

EF = exposure frequency (365 days $year^{-1}$)

ED = exposure duration (71.5 years)

DI = average daily intake (kg $person^{-1}day^{-1}$)

FC = formaldehyde concentration in fish samples (mg kg^{-1} or PPM)

ROD = oral reference dose (0.2 mg $day^{-1)}$

ABW = average body weight (kg $person^{-1}$) (Adult 60kg, children 15kg)

TA = average exposure to non-carcinogens (365 days $year^{-1}$)

Health Risk index

The health risk index is obtained using the formula (Huang et al., 2008, Bhowmik et al., 2020);

$$HRI = EDI/ROD$$

EDI = estimated average daily intake (kg $person^{-1}$)

ROD = oral reference dose (0.2 mg day^{-1})

Cancer Risk

$$CR = (EDIxCSF)$$

EDI = estimated average daily intake (kg $person^{-1}$)

CSF = Cancer slope factor (0.0455mg $^{-1}$ kg $^{-1}$ day $^{-1}$)

Data analysis

Data analysis was performed using SPSS and descriptive analysis is presented as frequency and percentage. Ap-value of <0.05 was considered significant and confidence interval (CI) of 95.0 % with 5.0 % margin of error.

Results

The recovery rate was established at 87.5% following the spiking technique. A total of 45 dry fish samples were collected and tested for formaldehyde content using Chromotropic acid method. Standard calibration graph was plotted to determine the formaldehyde in the samples (**Figure 1A**).

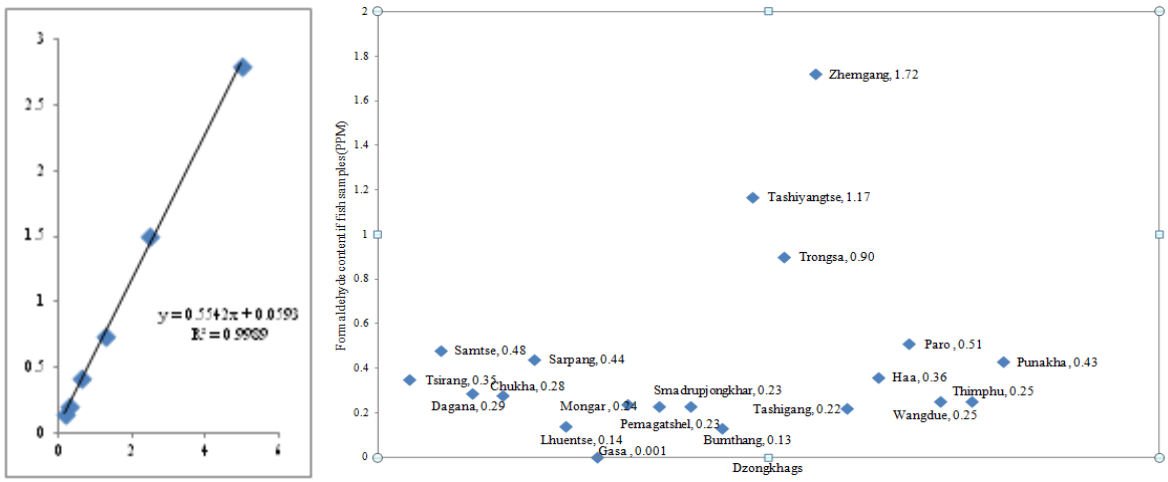

Figure 1A: Standard curve of formaldehyde standard solution using chromotropicacid, **B** Formaldehyde content (in PPM) in dry fish collected from 20 Dzongkhags

The overall average formaldehyde content was 0.38±0.33PPM (range 0.0003 – 1.72 PPM). The lowest amount of formaldehyde from dry fish samples was detected from samples collected from Gasa (0.001PPM) and highest from Zhemgang (1.17 PPM), statistically significant at p<0.05 between these two dzongkhag. **(Figure 1B).**

Table 1: Estimated daily intake in two groups by different concentration of formaldehyde

Estimated daily intake			
Groups	Formaldehyde concentration		
	Average (0.38PPM)	Minimum (0.0003PPM)	Maximum (1.72PPM)
Adult	0.0001	0.0000	0.0005
Children	0.0004	0.0000	0.0019

The estimated daily intake of formaldehyde (EDI) through the fish ingestion for adults and children is given in Table 1. It was assumed that oral exposure to formaldehyde in humans from dietary sources to be approximately 0.017 kg per person body weight per day for 60 kg and 15 kg for adults and children, respectively. The calculated EDI is used to find health risk index and found to be <1 in both the groups **(Table 2).**

Table 2: Health Risk Index

Formaldehyde concentration	HRI		THQ	
	Adult	**Children**	**Adult**	**Children**
Average (0.38PPM)	0.0005	0.0022	0.0000	0.0001
Minimum (0.0003PPM)	0.0000	0.0000	0.0000	0.0000
Maximum (1.72PPM)	0.0022	0.0097	0.0001	0.0006

Discussion

Although standard for dried fish and dried salted fish was drafted in pursuant to the Food Rules and Regulations of Bhutan 2007, the allowable limit of formaldehyde is not mentioned in the standard. The formaldehyde limitation set by FSSAI in India is 4 PPM for most sea and freshwater fish (12). Thus, of the total samples tested 100.0% were within the allowable limit. However, this does not guarantee that the imported dry fish is always safe with formaldehyde contamination differing with each lot/batch of production. Of the 45 samples collected from 20 Dzongkhags, only three samples (Zhemgang 1.42PPM), Tashiyangtse (1.17PPM) and Trongsa (0.90PPM)) had the higher range of formaldehyde detected. The type of dry fish samples

collected were a mixture of few species (Sermeling, Pakpa, Banmatsha, Snake fish, Salsa, Puttimacha, Sitara, Jingemasa) thus giving slight differences in the amount of formaldehyde content detected; either added artificially or naturally formed. The several factors that influence the quantity of formaldehyde produced are the fish species, source of catch, duration and temperature storage (13).

The World Health Organization has set a maximum total daily intake of formaldehyde to 0.15 mg kg^{-1} day^{-1} (14)and United States Environmental Protection Agency (USEPA) set it to 0.2 mg kg^{-1} day^{-1} (15). The THQ was calculated for adults and children (**Table 2**) and found to be <1 in both the groups. These values are interpreted as <1 or >1; if THQ is <1 it is assumed to be safe of carcinogenic effect and if >1 it is considered with the increasing probability of carcinogenic effects as the THQ value increases (16). Since all the results had THQ <1, it signifies that the analyzed samples were less likely to have potential adverse health effects. It was also found that the calculated health risk index to be within the safe interval (<1).

Cancer risk was assessed to calculate the probability of an individual developing cancer due to formaldehyde ingestion over a lifetime. The cancer risk factor (CSF) is defined by Integrated Risk Information System (IRIS) as 0.0455$(mg/kg/day)^{-1}$(17). The US Environmental Protection Agency (US EPA) prescribes an acceptable risk level value of the order 10^{-6} (1 in 1,000,000) (17). The analysis found the cancer risk with average formaldehyde concentration of 0.38PPM to be 4.89883E-06 for adults and 1.95953E-05 for child (adult < child). If cancer risk is greater than 1.0E-6 in-depth toxicological effects analysis needs to be conducted.

The other contaminants commonly reported in dry fish, such as heavy metal contaminations, pesticides and microbiology food safety (18) needs to be studied. Since there is no distinguished difference between naturally occurring and added formaldehyde in fish, it remains as the major limitation. However, more samples of different batches and lots from the point of entry would be of high value.

Conclusion

The laboratory analysis established that the amount of formaldehyde in the dry fish samples is lower than the maximum allowance as per FSSAI and Malaysian Food Act and Regulation. The EDI value was also found below both the WHO and USEPA range. However, it is important to

continuously monitor the level of formaldehyde contamination in dry fish as exposure to cancer risk will depend on the level of contamination which may vary from time to time depending upon supply chain and source.

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Preliminary findings on levels and health risk assessment of mercury contamination in dried fish consumed in Bhutan

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Introduction

Dried fish is the most affordable and abundantly available source of proteins to the Bhutanese population. It is considered a delicacy and are consumed by all ages including children in Bhutan. Annually, 372 metric ton of dry fish are imported from the neighboring country India and there has been a steep increase in the consumption of dry fish (1).

However, the concentration of heavy metals above the permitted level is a global public health concern (2). Lead, cadmium and mercury is the most commonly found contaminants in the dry fish (3). These heavy metals, also referred as toxic elements occur naturally in the environment from various sources and are often at higher levels from past industrial uses and pollution (4). These metals do not have any physiological role in the human system and even a trace amount is detrimental to the human health (5).

Of note, methylmercury is one of the toxic contaminants that is of greatest concern due to its acute and chronic toxicity to the CNS, CVD, respiratory system, hepatic, renal and musculoskeletal system (6). Several species of environmental mercury are commonly found, including elemental mercury (Hg), inorganic mercurial salts (Hg + and Hg 2+), and methylated mercury (MeHg) (7).

More specifically, excessive levels of methylated mercury are associated with various negative effects on human health, mostly the neurological damage, reproductive and developmental abnormalities (8). Methylated mercury, the most common inorganic form of mercury, exists in the water and quickly enters the aquatic food chain (9). In most adult fish, 90-100% of the mercury is methylated mercury (10). Globally, heavy metals contamination in aquatic organisms is an emerging phenomenon (11).

Currently, there is no available data in the country in regard to the maximum residue level (MRLs) of toxic element mercury in dried fish. This is further aggravated with the Bhutanese dietary habit of high consumption of dried fish, as it is a delicacy and served in almost all-important occasions due to its easy availability and a cheap high source of protein. In contrary, there is no regular monitoring programs for food safety in terms of regulating MRL of mercury in dried fish samples. Therefore, this study aims to determine the toxic metals (mercury) content in different dried fish available in the country and to assess the potential health risk in the Bhutanese population.

Methods

Sample collection

Bhutan imports all the dry fish from neighboring country India. Different species of dried fish that are widely available and consumed in the country were included in the study. Samples were randomly collected from grocery and supermarket shelves. The collected samples were stored at room temperature until analysis. Only edible portions were used for the analysis

Sample preparation

Samples were prepared for analysis according to the AOAC standard official methods (12). Briefly, the edible portion of the samples were grinded and homogenized in a clean metal free sample processing container. A 0.5 grams of samples were weighed in a mercury free sample boat and inserted directly for analysis. For further confirmation with other techniques for method validation, samples were digested in ultra-pure nitric acid. For this, 5 grams of dry fish tissue samples was weighed and transferred to the digestion vessel and 5 ml of ultra-pure nitric acid and 2 ml of hydrogen peroxide was added. Blank digest was also carried out to validate the digestion procedure accordingly.

The samples were initially pre-digested for 30 minutes followed by micro-wave assisted digestion using the following temperature-controlled program:

i. PH-30 bar
ii. TH-190^0 C
iii. Holding time-600 seconds

Toxic Metal analysis

Determination of mercury was conducted using direct mercury analyzer MA-3000 cold vapor atomic absorption spectroscopy as per the standard US EPA method 7473 (SW-846) (12). Briefly, 5 level of multi-standards of certified reference materials was used in ppb level including the blank (5, 10, 25, 50 ppb) to constructed a calibration curve. Samples will be diluted if the concentration of metals in the samples are beyond the limit of quantification. Calibration curve with the correlation coefficient (R^2) of 0.9999 will be used to quantify the level of each heavy metals in the samples. The following figure demonstrates the calibration curve for mercury.

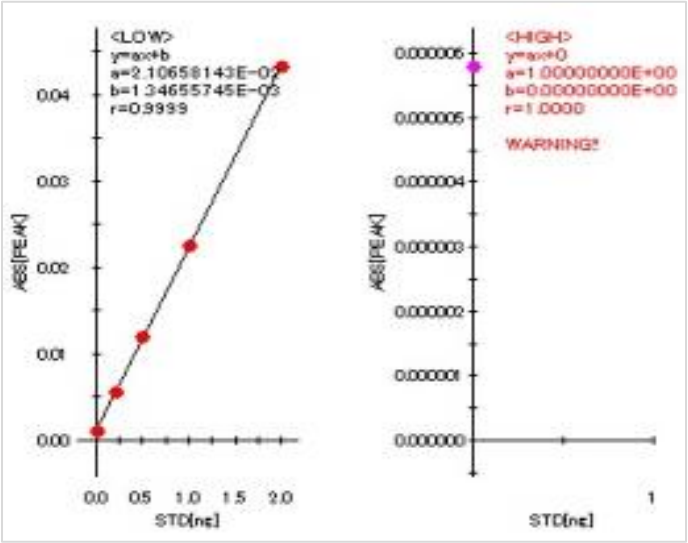

Figure 1. Calibration curve for mercury

Health risk estimation

The estimated daily intake (EDI), Target hazard quotient (THQ), hazard index (HI) and target cancer risk (TCR) was calculated as per the US EPA guidance for assessing mercury contaminant data and WHO chemical risk assessment guidelines (13).

Data analysis

The levels of toxic element mercury are expressed in mean ± standard deviation. One-way analysis of variance (ANOVA) and turkey's method was used to compare the differences in

contents of heavy metals among the groups of sample collection area of dried fish. A p-value of less than 0.05 was considered significant wherever applicable.

Results

There was a total of 35 samples collected for determination of mercury in dried fish samples. Of the total of 35 samples, 100.0 % of the samples were detected with mercury in the range of 8.33-122.79 µg kg^{-1} with the mean concentration of 49.7±32.9 µg kg^{-1}. The concentration at 95th percentile was 116.8 µg kg^{-1}**(Table 1).**

Table 1: Concentration of mercury in dry fish samples

Sl. No	Dzongkhags	Average Hg (µg/kg)
1	Tashiyantse	53.8
2	Tashigang	76.1
3	Pema Gashel	22
4	SamdrupJongkhar	115.7
5	Lhuntse	22.5
6	Trongsa	70.1
7	Paro	26.5
8	Haa	19.3
9	Chukkha	31
10	Gasa	122.7
11	Punakha	42.8
12	Thimphu	24.9
13	Sarpang	80.3
14	Samtse	21.1
15	Bumthang	46.7
16	Tsirang	49
17	Monggar	8.3
18	WangduePhodrang	61.5
	Average	49.7
	Maximum	122.7
	Minimum	8.3
	75th percentile	68
	95th percentile	116.8

From the mercury concentration in dried fish, it is correlated with the frequency of fish consumption by Bhutanese, the estimated daily intake of dry fish for an adult consuming four pieces of dry fish (28.0 grams x 4 pieces = 112.2 grams) for four times a week (52.1 x 4 = 208.5 days) was ~0.2 mg/day/kg body weight **(Table 2).** An average body weight of 65 kg with a lifetime exposure of 67 years was considered for the current assessment. The results showed that the Hazard quotient is 1 which poses a risk for the consumers.

Table 2: Health risk assessment of Mercury in sampled dried fish species

	Estimated Daily Intake	**Hazard Quotient**
Mercury (Hg)	~0.2 mg/day/kg	1

Discussion

Mercury is a well-established cumulative neurotoxic agent that can have serious adverse effects on the development and functioning of the human central nervous system, especially when exposure occurs prenatally. In the study, mercury was found to vary in concentrations in the studied fish samples. Based on the risk assessment of fish mercury concentrations, we reckonfish diets are high-risk to health.

The source of dried fish samples collected were either imported from India or Bangladesh. Contamination of heavy metals in dried fish was studied in Bangladesh with Mercury at the third highest concentration among heavy metals. The probable source of contamination has been suggested to arise from leaching from the drying pans into fish, atmospheric deposition and the river/marine waters in which fish were harvested (14).

In Mumbai, mercury levels in fish were 0.03-0.82mg total Hg/Kg dry weight compared to the permissible limit of 0.5mg/kg. The North Koel river showed mercury concentrations almost 600-700 times above the limits (15). Currently, in Bhutan, there is no restriction on the import of dried fish from any country. However, in a study from India, the highest sediment concentration

of Methyl mercury (202.0ng/g) was obtained during monsoon season (10). In this regard, import of dried fish during different seasons of the year should also be considered and monitored.

Along with mercury, many other heavy metals such as lead, cadmium, Arsenic, Copper and Zinc can also be found in the dried fish. Additionally, residues of organochlorine pesticides were found in a study conducted at Bangladesh and Kerela, India (15,16). Further studies on the contamination of other heavy metals such as lead, cadmium and arsenic are also required for assessment of human health risk. Our preliminary study has the limitation with a smaller number of samples collected, and the unknown types of fish. Further, an increased sample size and categorization of fish species is essential.

Conclusion

The results from this preliminary finding suggest that the dried fish sampled were contaminated with mercury posing significant health risk to the consumers. Moreover, consumption of various delicacies prepared with dried fish in a long run may lead to bioaccumulation of mercury in tissues that may pose greater impacts. Therefore, continuous monitoring of heavy metals through surveillance programs is pertinent in the dried fish imported from India and other neighbor countries.

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Retrospective review of suspected poisoning cases from hospitals, 2013-2019

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Poison information and toxicology laboratory

Introduction

Poisoning is a significant public health concern worldwide causing an estimated 1,93,460 deaths from unintentional poisoning (1). Of which 84.0 % occurred in low- and middle-income countries including south Asia region (2). In addition, one of the main reasons for emergency hospitalization, utilization of healthcare resources and high mortality is attributed due to poisoning.

Many types of poisoning occur in the community caused by different agents responsible. Poisoning by pesticides, acetaminophen and benzodiazepines are reported as a common agent of exposure in urban areas of India, Singapore and other developing countries (3-5). Some are fatal resulting in death and some although recovered also causes significant disabilities.

The epidemiological surveillance specific for each country is highly necessary to determine the characteristics and extent of the problem, in order to develop the most effective measures. At present very little is known and documented on poisoning in the country. There are no published reports available on the patterns of poisoning. The most prevalent poisoning reported and managed in the respective hospitals and the most affected age groups still remains indefinite. It is very pertinent to have knowledge on the types of poisoning in the particular geographic locations. This can help in formulation of important public health measures to reduce mortalities and morbidities. Therefore, we conducted a retrospective analysis of the profile of poisoning cases admitted between 2013-2019 in the hospitals.

Materials and Methods

Data were reviewed from the medical records of the respective hospitals and retrospective analysis of all the poisoning cases in the country were conducted **(Figure 1).** All the names were removed before the analysis and the confidentiality was maintained.

Demographic data, type of exposure was extracted and recorded from the medical records. Diagnosis of poisoning was made based on history of exposure or contact and characteristics clinical features. All data were tabulated in Microsoft excel and data analysis was conducted using Epi info 7.2.

Figure 1: Sample collection sites

Results

There were 549 cases of poisoning admitted to hospitals during the period of 7 years from 2013 to 2019 respectively. Of the 549 cases, 286 (52.0%) were males and 263 (49.7%) were females. The maximum cases were in the age group 25-49 years followed by 15-24 years **(Figure 2)**.

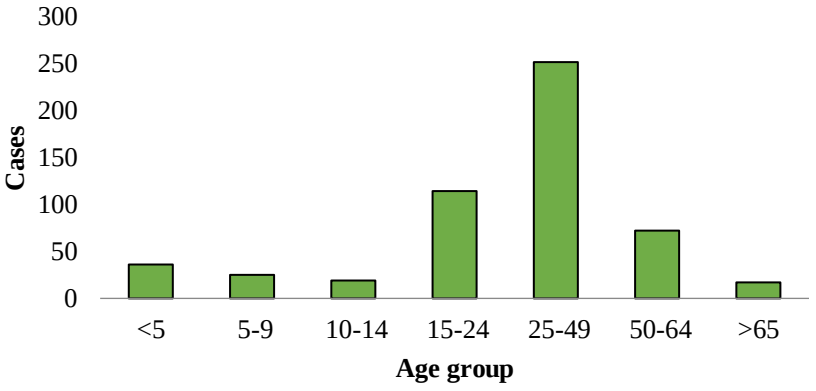

Figure 2: Distribution of poisoned patients based on age

Among the total poisoning incidents, majority of the cases were owing to Snake bites (43.5 %) and mushroom poisoning (16.2 %) respectively. Other predominant exposure was via toxic agents like medicine/drugs (11.7%) and pesticides (6.4%) that were responsible for poisoning**(Table 1).**

Table 1: Common agents of exposure

Types of poisoning	Number	Percentage
Snake bite	239	43.5
Mushroom	89	16.2
Medicines/drugs	64	11.7
Pesticides	35	6.4
Food	33	6.0
Household cleaning substances	26	4.7
Wild plants	20	3.6
Hydrocarbons	17	3.1
Unspecified	11	2.0
Toxic Alcohols	8	1.5
Cannabinoid, Bear bile & cloth dye	7	1.3

Discussion

The findings from the current study showed snake bite to be the predominant cause of poisoning in the country followed by mushroom poisoning. Snakebite causes huge mortality and morbidity in many tropical and subtropical areas (6, 7). Mushroom poisoning is another public health concern that most of the middle-income countries face. Similar studies have reported mushroom poisoning to be the most common agents of exposure (8, 9).

Most of the cases in our study belonged to the age group of 25-49 years old in all 7 years of data 2013-2019. This is similar to studies elsewhere (10-12). Notably, there were more males than the female cases as reported in other studies (10, 13). This can be speculated due to the easy accessibility of poisons and increased risk-taking behavior of men as compared to women.

Apart from snakebite envenomation, mushroom poisoning was the major reason for acute poisoning in the country. Bhutan being at the tropical regions with the country's rich fauna

diversity, wild mushrooms are easily available and are routine for food among the Bhutanese population. This might be the cause for poisoning in these patients (14)

A detailed analysis of poisoning epidemiology that includes the pre-disposing factors such as whether it was intentional due to suicidal, social conflicts, abuse and psychiatric problems were not conducted. The route of exposure that includes ingestion, inhalation and dermal exposure were also not included. In addition, the clinical presentation of each case, case management with different antidotes and supportive measures and treatment outcome were also left out. This information was very pertinent in determining the targeted public health interventions. For instance, these data can help the public health authorities to advocate the community via targeted education, mass media education, changes to environment, behavioral strategies and WHO's safe community approaches (15,16)

However, in the present analysis these were not included due to limited data available in the records of the poisoning cases maintained in respective hospitals. Therefore, a robust web-based surveillance system is crucial in collecting all the information related to poisoning in the country.

Conclusion

The present review of the data provides the insights of poisoning in the country. The findings demonstrate that snake bite and poisoning via exposure to mushrooms were the main cause of poisoning in the community. However, a robust web-based surveillance system is crucial in collecting all the information related to poisoning in the country. This may aid in generating evidence for targeted public health interventions in the country.

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Report on toxicological analysis of unknown samples, 2022

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Introduction

Toxicology is an interdisciplinary field dealing with adverse (toxic) effects of exogenous chemical substances on living organisms (1). Toxicology covers two aspects: medical, which focuses on the effects of poisons and optimal therapy of intoxications, and analytical, which deals with demonstration and determination of the amount of usually unknown substance or mixture of substances and their metabolites in biological or non-biological material (2).

Analyses of unknown compounds are integral to toxicological sciences and can be extremely useful in certain scenarios. For example, using toxicological analyses to identify substances (e.g., tablets, powders, liquids) found on a patient may aid clinicians in cases of poisoning, particularly if the patient is unconscious. This is also used mainly to identify compounds and toxins from various environmental, food and biological matrix samples. Mainly, in the case of poisoning, substance abuse and from seized narcotic and psychotropic drugs (2).

Notably, the sample preparation step is very pertinent in any toxicological analysis that constitutes either solid phase extraction or liquid-liquid extraction (3). However, with the technological advancements and the availability of mass spectrometers with increasing sensitivities, the need to remove potential interferences, such as matrix components or non-relevant analytes to the analysis is essential to optimizing an analytical method (4). On the other hand, an appropriate sample preparation technique should be considered. This is because the toxicological analysis involves diverse analytes involving both biological and non-biological with a variety of chemical properties.

Therefore, the development, validation and application of a gas chromatography mass spectrometry (GC-MS) for the identification of toxicants necessities the importance in delivering fast, accurate and reliable results. This report describes the method we have developed and validated for the analysis of unknown narcotic drugs, pesticides that are commonly used in the country and toxic alcohols.

Methodology

Workflow of analytical toxicology

The workflow includes several stages

- Screening
- Identification of a chemical entity
- Quantitative estimation of exogenous substances in biological material
- Evaluation of findings

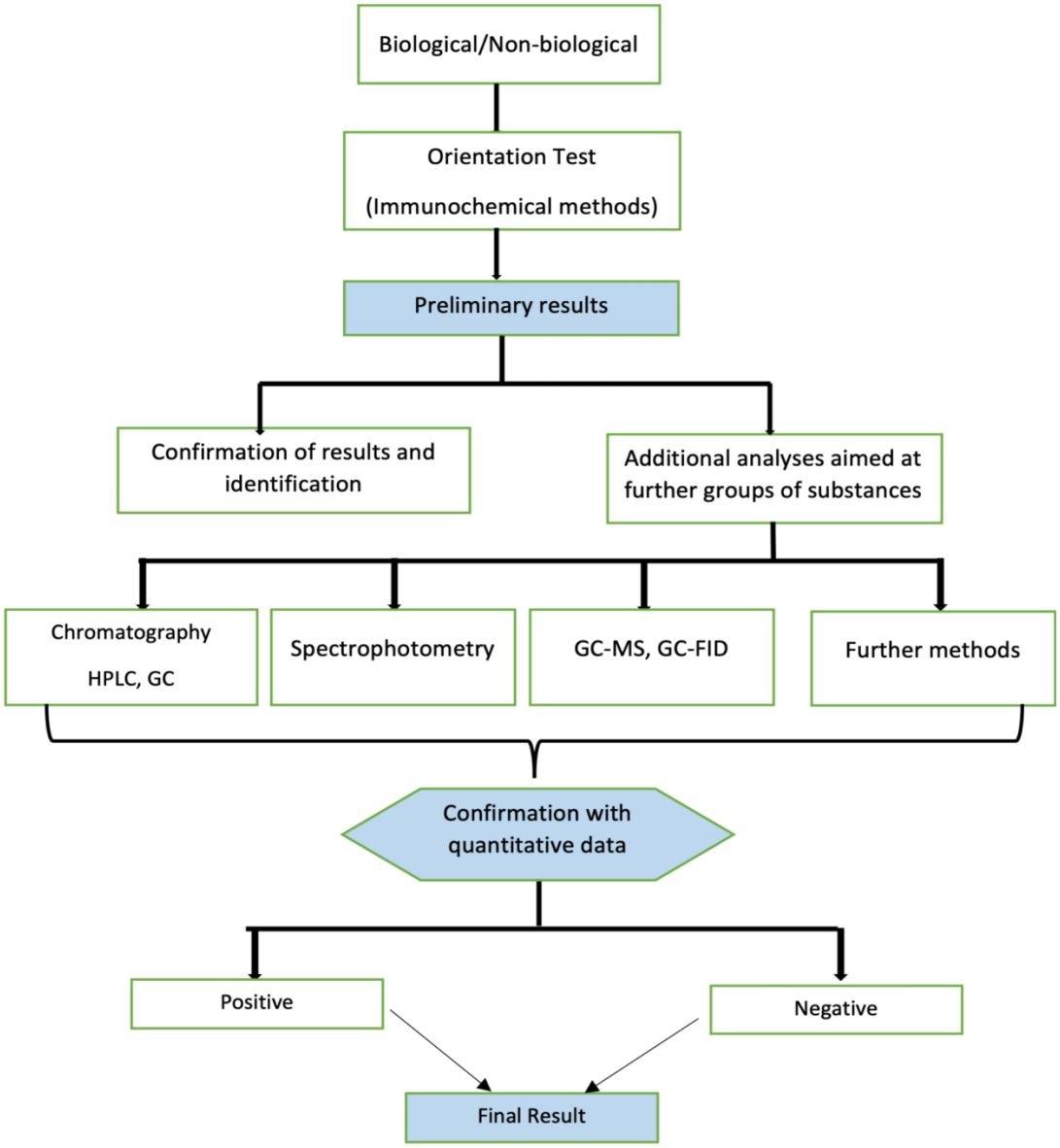


Figure 1. Scheme of the workflow in toxicological analysis of unknown compounds

Types of Samples analyzed

- Urine for analysis of narcotic substances
- Ara (locally brewed alcohol)
- Pesticides

Sample preparation

There are numerous techniques that range from simple to rigorous to prepare samples for analysis. Common techniques we adopt are dilution, liquid-liquid extraction, and solid-phase extraction.

1. **Liquid-Liquid extraction**

These are based on the concept of immiscible solvents, typically one aqueous and another organic, which are added to an aliquot of a biological sample. A common LLE extraction is protein precipitation which uses inorganic acids or solvents such as acetonitrile, chloroform, methanol, ethyl acetate, etc which are selected based on the type of compounds that are to be analyzed. Then, followed by centrifugation and separation of two organic and in organic layers. Final step is drying the compounds present in the organic layer under gentle nitrogen gas flow. Depending on the method of analysis, the dried sample can be reconstituted with recommended solvent and injected into the instrument directly.

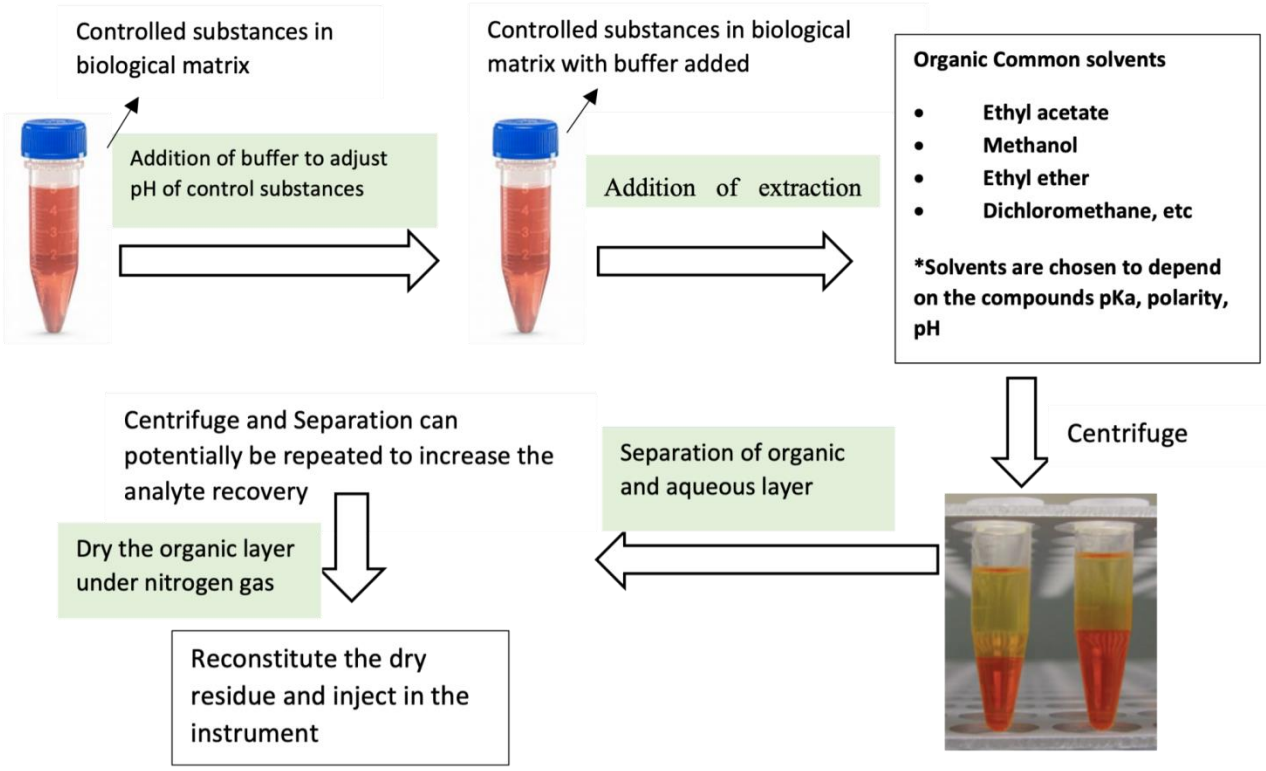


Figure 2. Liquid-Liquid extraction of samples

2. Solid-phase extraction

When analytes of interest have similar polarities, or acid/base properties, LLE may not be suitable for their recovery, so SPE is selected. These extractions aid in removing interferences such as phospholipids found in blood, other interfering drugs, and other components found in the matrix and results in clean extracts.

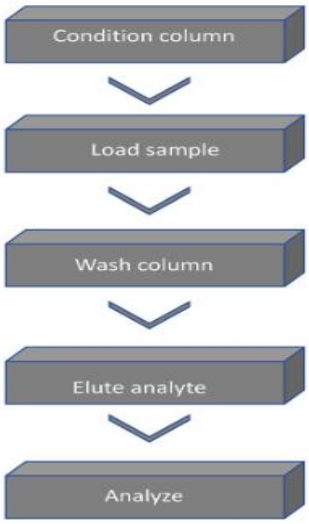


Figure 3: General flow of SPE

Analytical measurement

Identification of unknown toxicants were then analyzed using a gas chromatography-mass spectrometry (GC-MS) and gas chromatography flame ionization detector (GC-FID). Drug identification in various biological sample matrices like urine and blood is accomplished by comparison of chromatographic retention times and mass spectra to certified reference standards analyzed under the same condition. Moreover, it is also compared with the mass spectral from commercialized and pre-established validated in-house library of drug standards using the NIST 11 library (National Institute of Standards and Technology, USA) software. Different analytical conditions were set for an individual drug, compound, or pesticides. Another analytical method used is Ultraviolet spectrophotometry which is employed for drug analysis. It is used for the quantitative purpose of toxicants in various samples.

In GC-MS, conditions used for analysis of toxicants featured an Agilent 7890B GC including a split/splitless inlet, together with a 5977B GC/MSD and a 7693A automatic liquid sampler (ALS) system. Agilent MassHunter GC/MS software was used to acquire and process data. All the analysis was done using column HP-5MS 5% phenyl Methyl Silox,30m * 250μm* 0.25μm; Agilent 19091S-433, with syringe size of 10μl and injection volume 1μl. The carrier gas used was helium. All methods are as follows **(Table 1)**:

Table 1: Method conditions for drug of abuse

Parameters	Value
Column temperature	150 ˚C
Injection mode	*Split*
Oven temperature	150˚C (1 min), 18.7 ˚C/min, 300 ˚C (7min)
Split flow	*16.4ml/min*
Split ratio	*10:01*
Mode	*Scan*
Range (m/z)	*50-450*
Ion Source	230
Run time	*18 mins*

The 250ng/ml standard used in testing drugs of abuse was an Agilent GC/MS toxicology checkout mixture containing 16 compounds where amphetamines, benzodiazepines, opiates and other commonly screened drugs **(Table 2).**

Table 2: GC method conditions for alcohol

Parameters	Value
Column temperature	*325˚C*
Injection temperature	*280˚C*
Inlet type	*Split*
oven temperature	*220˚C*
Split flow	*25ml/min*
Split ratio	*50:01:00*
Mode	*Scan*
Range (m/z)	*35-550*
Run time	*16 mins*

The 500ng/ml standards used in testing of alcohol was an Agilent GC/MS toxicology checkout mixture containing 13 compounds (**Table 3)**.

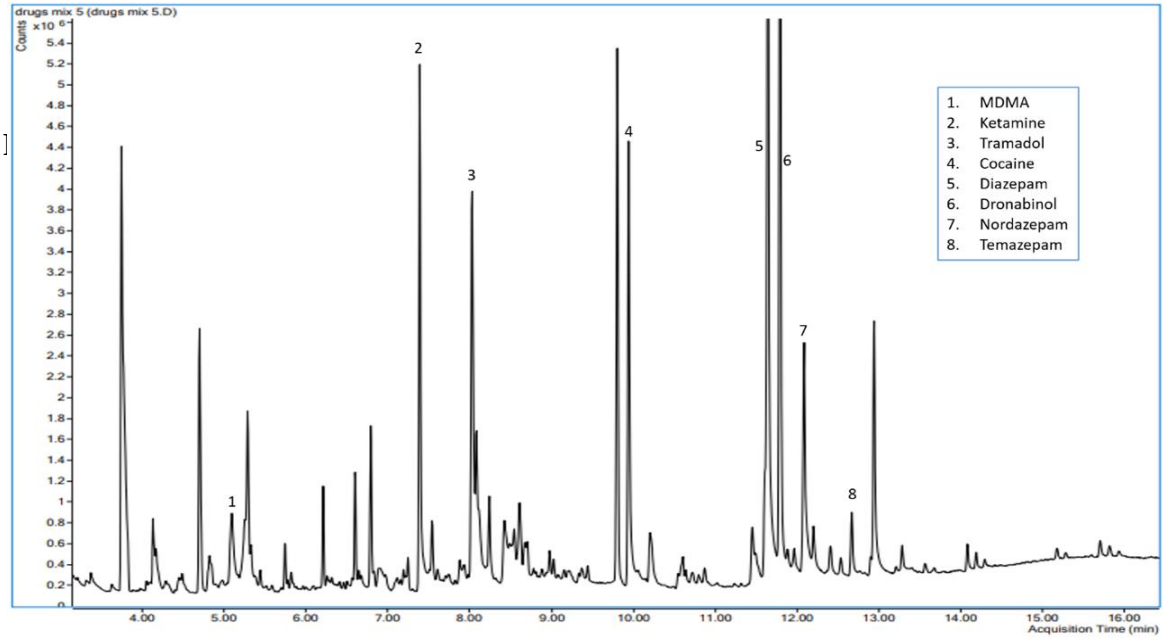
Table 3. GC method conditions for pesticides

Parameters	Value
Column oven temperature	75°C (1 min), 10°C, 300°C (4.5 min)
Injection temperature	250°C
Inlet type	*Split*
oven temperature	320°C
Split flow	*17.4 ml/min*
Split ratio	*10:01*
Mode	*Scan*
Ion source	230°C
Range (m/z)	*50-450*
Run time	*28 ins*

Results

Confirmation of drugs of abuse

The analysis method for confirmation of drug of abuse was developed for eighttype of drugs, validated using the certified reference standards, compared with the NIST 17.0 library and use of internal standards/spiking methods **(Figure 4).** A total of 11 samples were received from Bhutan Narcotics Control Authority (BNCA) for confirmation of Tramadol, Ketamine, benzo-diazepam and its derivatives and delta 9 tetrahydrocannabinol in urine samples (**Figure 5).**



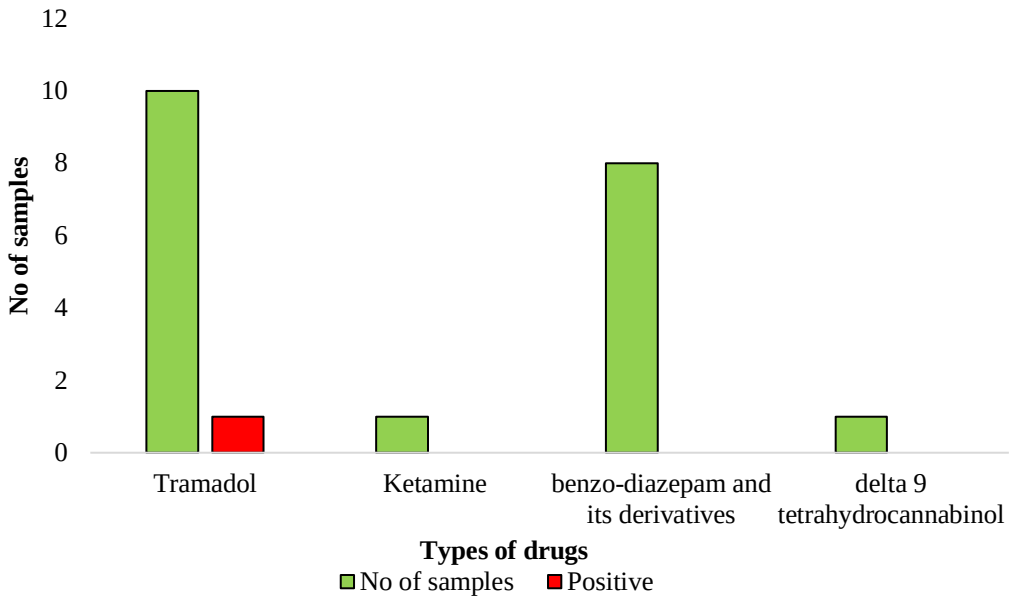


Figure 5: Identified drugs of abuse in samples tested in 2022

During the method optimization, enough response was generated with a 10:1 Split to integrate peaks and generate a high-quality match for the mentioned drugs of abuse. Excellent resolution was attained with the high-efficiency column. All the 16 peaks were easily identified, and when the resulting spectra were processed using a NIST library, every peak was properly identified as the top match.

Analysis of toxic alcohols

A total of 85 home brewed unrecorded samples (Ara) were collected from local producers from 12 different identified sites in Thimphu and analyzed for the toxic contaminants. The presence of ethanol and methanol in the samples were also quantified. The mean ethanol concentration in the samples was20.9±4.2 % (v/v). The highest ethanol concentration was 32.34% v/v and lowest was 13% v/v. The mean methanol concentration was 2.06±1.3% v/v, where the highest concentration was 8.9% v/v and lowest was 0.38 v/v respectively**(Figure 6).**

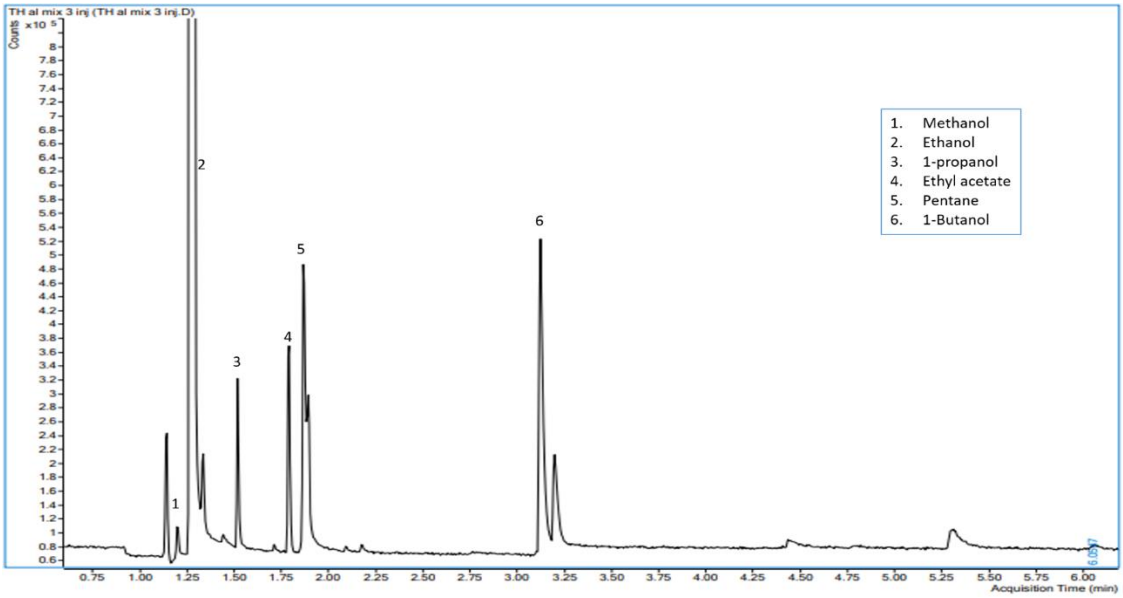


Figure 6: Separation and identification of toxic alcohols

The figure 5 illustrates the different chemical constituents present in an *ara*samples. Each peak represents a compound; however, due to the high ethanol content in the samples, the ethanol peak (Peak 2) is much larger than minor components. One important finding in this method development study is the use of the HP-5MS 5% phenyl Methyl Siloxcolumn thatprovided an excellent peak shape for other challenging analytes of interest. Several other toxicants were also found in analysis of Ara samples including ethanol, methanol, ethylene glycol, toluene, 1-pentanol,isobutyl acetate, 1-butanol, 2,3 butanediol, propylene glycol, ethyl lactate, 1-butanol 3-methyl.

Separation and identification of pesticides

Samples from the National Plant Protection Center (NPPC) were received for the analysis and identification of hazardous pesticides in the bio-weedicide. This was analyzed to uncertain the risk of importing hazardous pesticides in the country. A profiling of different pesticides in the samples were conducted. The results indicate that there was absence of any hazardous pesticides in the sample.

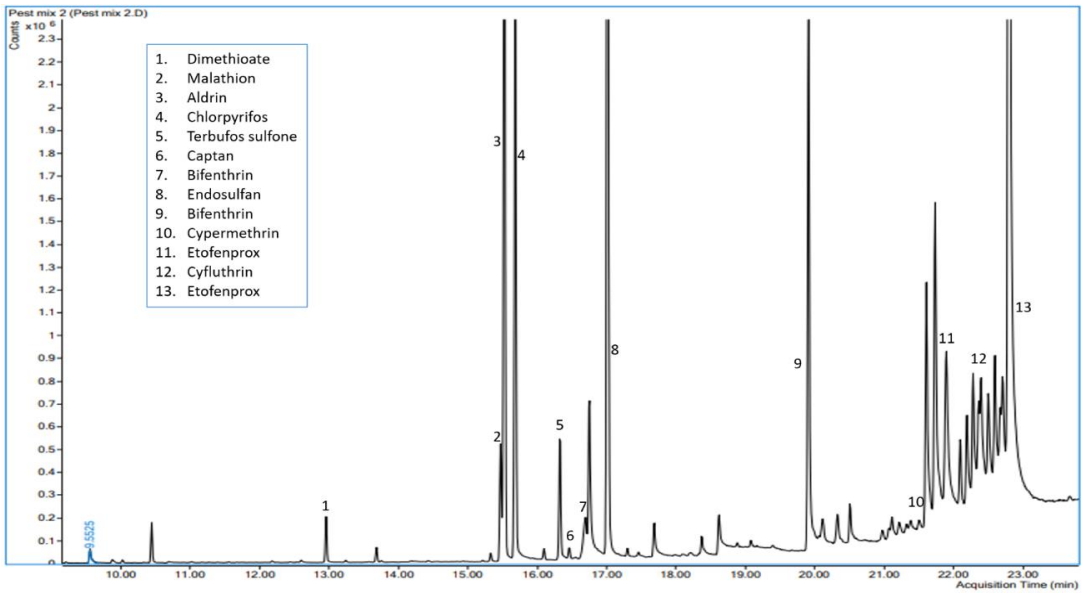


Figure 7: Separation and identification of pesticides

Discussion

The main aspect in the toxicological analysis of a given specimen is to detect, identify and quantify the toxicants (5). Thus the analytical methods applied in toxicology must be of high quality to ensure reliable results. False analytical findings may lead to severe consequences including the wrong diagnosis of cases, unjustified punishment of defendants or false verdicts in civil or administrative procedures (6).

To ensure an accurate, precise, reliable and correct analytical finding, all methods applied must be continuously monitored including method development and validations. This report presents the attempts of routine applications followed in our laboratory that are integrated with the quality control. Furthermore, the steps in method development including the selection of sample preparation and chromatographic techniques are highlighted.

GC-MS is considered as the gold standard technique for the identification of drugs of abuse in laboratories due to its sensitivity and specificity (7). Consequently, for the analysis of drugs of abuse, we separated a total of 8 drugs namely, Methamphetamine, Ketamine, Tramadol, Diazepam, cocaine, nordazepam and temazepam in our laboratory. For the identification, a full scan mode was selected that gave us a good separation of confirmation ions and an accurate analytes's retention time.

In the current scenario of the country, the number of youths getting involved in drug abuse have increased. The Poison Information and Toxicology Laboratory in collaboration with Bhutan Agriculture and Food Regulatory Authority (BAFDA) is analyzing a number of illicit drugs (amphetamines, caffeine, cocaine), alkaloids and amines (detromethorphan), narcotics and analgesics (codiene, morphine), hallucinogens (LSD), antidepressant, sedatives (barbiturates) and marijuana) depending on the circumstances of the crime/accident, and the observations of the physical appearance of the victim/suspect.

Toxic alcohols mainly methanol and ethylene glycol cause fatal poisoning around the globe (8). Therefore, toxicology laboratory must be capable of identifying a wide range of possible toxic chemicals in this samples. A simple dilute and shoot method were developed for the analysis of these toxicants in our laboratory. We observed a liner response of the chemical constituents in the samples, and a total of 12 compounds can be separated and identified respectively.

Analysis of pesticides residues in food and other environmental commodities have become essential requirements for consumers, producers and food quality control authorities (9, 10). As pesticides residues above the maximum permissible limits are a concern due to its implications on human health (11). For the purpose, an excellent separation and analysis of 13 pesticides were achieved with good peak shape, resolution and reproducibility.

Conclusion

Various extraction and analytical methods are employed in identification of the exogenous substances present in the samples individually. Development of various methods of analysis for different samples using GC possesses a huge challenge. However, our toxicology laboratory has employed different methods for the analysis of unknowns and blind toxicological tests.

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Evaluation of Sensitivity and Specificity of SARS-COV-2 antigen test kits used in Bhutan for detection of Omicron variant

Adeep Monger, ThinleyJ amtsho, Vishal Chettri, KelzangWangdi,Sonam Wangchuk

Royal Center for Disease Control

Introduction

The diagnosis of SARS CoV-2 virus has been very pertinent in overall management of COVID-19 cases worldwide (1). The real-time reverse transcriptase polymerase chain reaction (RT-PCR) for amplification of nucleic acids to detect direct pathogen-specific Virus is the gold standard for the diagnosis of the disease (2,3). However, the RT-PCR test often takes a longer time (up to 6-8 hours) to declare the results which delay implementation of intervention measures in the healthcare system (3). Moreover, in a resource limited country, only few health centers are able to provide RT-PCR testing services.

There are numerous points of care antigen tests (Ag), that are quick, cheap and can be performed outside of the laboratory and do not require highly trained staff (4). No specialized laboratory equipment is needed and the results can be acquired in a minimal time of 15 minutes. The ideal diagnostic target for antigen test is the Nucleocapsid protein (NP) due to its expression and relative abundance (5). Due to these advantages, the antigen test plays a significant role in surveillance, patient management, and public health decision making for COVID-19 response[4].

In Bhutan, there are only six out of 184 healthcare centers that provide RT-PCR services (6,7). The health centers without the RT-PCR facilities perform antigen tests for the diagnosis of the disease to facilitate quicker decisions for all medical processes and other public health interventions. However, there is a need for careful test selection of different brands of antigen test for its potential role in the diagnosis of COVID-19 due to its varying sensitivity and specificity as compared to the RT-PCR test (8). This is further aggravated with the emergence of SARS-CoV-2 new variants of concern like Alpha, Gamma, Beta, Delta, and now Omicron that has rapidly spread across the world including Bhutan, since it was first reported by South Africa on 26th November 2021. Unlike other SARS-COV- 2 variants of concern, Omicron has numerous mutations both in the spike protein as well as in the nucleocapsid (9). These factors

warrant if the currently used antigen test kits can still detect the omicron variant.This study evaluated the analytical sensitivity and specificity of the currently on use antigen test kits used in Bhutan by comparing its real time performance in the field simultaneously to RT-PCR test.

Methods

Study design

A retrospective study was conducted on RT-PCR and antigen test results recorded in the Royal Center for Disease control (RCDC)'s COVID-19 Integrated Influenza Surveillance system during the Omicron outbreak between 28.01.2022 and 6.02.2022 for case detection and monitoring.

As per the surveillance protocol of the Ministry of Health, Bhutan, the samples were collected from populations in the outbreak affected places and from positive patients kept in various isolation facilities. The swab samples were taken simultaneously; nasopharyngeal swab for antigen test and oropharyngeal swab for RT-PCR test. Patient details were collected using the Healthusing Health Facility System database andantigen-positivee samples were confirmed by RT-PCR as per the testing protocol. All antigen and RT-PCR results including the sample ID, Positive/Negative and the C_t values were extracted from RCDC's COVID-19 Integrated influenza surveillance system database into MS excel.

Test assay

RT-PCR was performed on all the oropharyngeal samples collected. Briefly, 200 µL of samples were extracted using the spin-X viral DNA/RNA extraction kit (SD Biosensor) and RT-PCR were performed using nCOV Real-Time Detection kit (SD Biosensor) accordingly to the manufacturer's instructions. A sample with $C_t \leq 36$ were interpreted as SARS-CoV-2 positive.

Antigen tests were conducted using the Standard Q COVID-19 Ag test kit (SD Biosensor) on the nasopharyngeal samples. Samples collection and testing were carried out by trained health professionals in the respective isolation and test sites. The collected samples were inserted into the extraction buffer and about three drops of extracted samples were applied into the sample cassette and read the result within 15-30 minutes. Appearance of both the purple colored in control line and test line was interpreted as positive (11).

Data analysis

The data was extracted from the COVID-19 integrated influenza surveillance system database and exported to MS excel. Data analysis was carried out in MedCalc Software version 20.027. The sensitivity, specificity and predictive values were expressed as percentages. The confidence interval used for sensitivity and specificity was "exact" Clopper-pearson CI and the standard logit confidence intervals for predictive values.

Results

A total of 476 clinical swab samples were tested using both antigen and RT-PCR tests simultaneously during the outbreak period. Of these, 76 (15.9 %) samples were positive, 11 (2.3%) inconclusive and 389 (81.7%) negatives by RT-PCR with a cut off C_t value of $\leq$ 36. Therefore, only 465 positive and negative samples were included for the analysis.

Among 76 RT-PCR positive samples, 54 of the samples were positive by antigen test giving a sensitivity of 71.0 % (95.0 % CI 59.5-80.8). In contrary, of the 389 negative RT-PCR results, 4 were positive by antigen test and 22 samples were negative on antigen test. Thus, the specificity of the antigen test kits was 98.9 % (95.0% CI 97.3-99.7). The positive and negative predictive values were 93.1% (95.0% CI 83.4-97.3) and 94.5 % (95.0% CI 92.4-96.1), respectively **(Table 1).**

Table 1: Comparison of antigen to RT-PCR results with cut off C_t value of ≤36

SARS-COV 2	**RT-PCR Positive**	**RT-PCR Negative**	**95% confidence interval**
Antigen Positive	54	4	
Antigen Negative	22	385	
Sensitivity (%)	71.0		59.5-80.8
Specificity (%)	98.9		97.3-99.7
Positive predictive value (%)	93.1		83.4-97.3
Negative predictive value (%)	94.5		92.4-96.1
Accuracy (%)	94.4		91.9-96.3

Sensitivity based upon cut off C_t values generated by different samples were performed. The sensitivity of antigen test increased with increasing viral load in the sample. The sensitivity and specificity for samples $C_t \leq 25$ were 94.2 % (95.0% CI 80.8-99.3) and 94.0% (95.0% CI 91.4-96.0), respectively. Similarly, for samples with $C_t \leq 30$ - 26, the sensitivity and specificity were 85.7% (95.0% CI 73.7-93.6) and 97.3 (95.0% CI 95.3-98.6), respectively **(Table 2)**.

Table 2: Performance of antigen test according to the RT-PCR C_t values

Characteristics	$C_t \leq 25$ as RT-PCR positive	$C_t \leq 30$ - 26 as RT-PCR positive
Prevalence (%)	7.3 (35/476)	11.7 (56/476)
Sensitivity (%)	94.2	85.7
95% CI	80.8-99.3	73.7-93.6
Specificity (%)	94.0	97.3
95% CI	91.4-96.0	95.3-98.6
Positive predictive value (%)	55.9	81.3
95% CI	46.4-65.0	70.6-88.7
Negative predictive value (%)	99.5	98.0
95% CI	98.1-99.8	96.3-98.9
Accuracy (%)	94.0	95.9
95% CI	91.5-96.0	93.7-97.5

Discussion

To respond the current on-going COVID-19 pandemic, substantial amount of testing is required as per the surveillance protocol for early case detection and contain the infection through stringent public health measures. Although the preferred test is RT-PCR, forbut for a resource limited country including Bhutan, RT-PCR facilities are fewer in number. Thus, an alternative antigen tests for the detection of SARS-COV-2 that are easy, cheap, quick and robust are commonly used. Nevertheless, different antigen test kits are available with varying sensitivity

and specificity. This is further aggravated with continuous mutations of the original Wuhan strain, thereby raising concerns on the reliability of the antigen test kits.

The present study showed that the existing antigen test kits used in Bhutan have an overall sensitivity and specificity rate of 71.0 and 98.9% respectively. Similar results were reported in a meta-analysis with the sensitivity of 70.0 and 98.0% (10,11). The manufacturer of the current antigen test kit reported an overall sensitivity of 97.1% (95.0% CI 90.0-99.6%) for $C_t \leq 25$ and 90.7% (95.0% CI 84.6-94.9%) for $C_t \leq 33$. These discrepancies may bedue to pre-analytical factors such as performing tests in controlled laboratory settings, collection of nasopharyngeal samples and tests being performed by highly trained personnel often involvedoften been involved in test development. Furthermore, other synergistic factors include the tests beingbeen conducted under real-life circumstances such as extreme weather conditions (high and low temperature), different proceduresof sample collection by different people deployed for outbreak response, might influence the kit's performances.

Rapid antigen test can detect the active infection in an individual when the viral load is comparatively high. Similarly, our findings revealed that the sensitivity increased from 70.0% to 94.2% with increase in viral load in the samples ($C_t \leq 25$, ≤ 36-26). This was in concordance with studies reported by Perez et al, and also reported by Brummer et al in their meta-analysis (10,12). Of note, the patients with high viral loads ($C_t \leq 25$ or $>10^6$ genomic virus copies/ml) usually appear in the pre-symptomatic phase of the infection (1-3 days) and early symptomatic phases of the illness (within the first 5-7 days of illness) thereby, providing opportunity for early diagnosis (13). However, patients with lower viral load, usually during the early period of the infection (1-2 days) and latter (5-7 days) are likely to have false negative results in the antigen test. Hence, it is recommended that antigen test should be complemented by RT-PCR along with more detailed clinical manifestations and proper judgements.

During the current outbreak of the highlyof highly transmissible Omicron variant, antigen testing was conducted to facilitate case management and immediate public health response. The healthcare workers and other frontliners were also screened with antigen test to limit onward nosocomial infection for the surge in outbreak. Smith et al., reported that, with two rounds of well-timed antigen screening (4-5 days delay in screening rounds), up to 75.0% of infections

were prevented, compared to 47.0% with routine RT-PCR testing alone (14). Current surveillance protocols in the country mandates for routine screening at biweekly intervals to reduce outbreak probability. Therefore, opportunities exist to revisit the current protocol for effective public health impact as antigen testing has faster diagnostic turnaround time, decent performance and tends to outweigh reduced sensitivity relative to RT-PCR test.

This study has several limitations; Firstly, it is a retrospective study that has been conducted in a single institution. Secondly, the evaluation of sensitivity according to the days post symptoms onset were not conducted due to the study's ethical limitations and the sample size was comparatively low compared to other studies. Consequently, this present study should not be extrapolated to other available Antigen test kits in the market. Nevertheless, this is the first type of study conducted in the country and our findings indicate that these tests should be used with proper clinical judgement before making important public health decisions.

Conclusion

The current antigen test kit's overall sensitivity, specificity, positive predictive value and negative predictive value were of 71.0%, 98.9%, 93.1% and 94.5% respectively. Notably the sensitivity improved with an increase in viral load ($C_t \leq 25$ with a sensitivity of 94.2%). Although antigen is less sensitive than RT-PCR tests, it is still useful and supplement compensates with faster diagnostic turnaround time to detect individuals during the pre-symptomatic and early symptomatic phase of the illness with high viral loads. However, to provide clinical and public health intervention utility in the country where the antigenwhere antigen test is used, other epidemiological and clinical judgements should be considered.

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National External Quality Assessment Scheme (NEQAS) of malaria microscopy among the participating laboratories in Bhutan

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National Malaria Reference Laboratory

Introduction

Globally, malaria remains a major public health challenge. In 2021, there were an estimated 247 million cases of malaria worldwide with an increase of 2 million cases compared with 2020 (1). The World Health Organization (WHO) African Region continues to account for 95.0% of malaria cases and 96.0% of malaria deaths (1). Bhutan has been a low endemic country for malaria and most of the cases are recorded in the southern districts.Bhutan started the fight against malaria with the support from the global fund for AIDS, Tuberculosis and Malaria (GFATM) and WHO (2).

In 2022, Bhutan detected 11 malaria cases. Of these, five cases were detected from non-national individuals who sought health services at our health facilities and six were Bhutanese. Among the six cases, the Vector Borne Disease Control Program (VDCP) in consultation with the malaria technical advisory group classified three imported and three introduced cases aligned to WHO malaria elimination framework. (3)

Early diagnosis and prompt effective treatment is the basis for the management of malaria and in reducing the disease burden. The WHO recommends all clinical manifestations, suspicious cases of malaria to be confirmed with parasitological test either by microscopy or Rapid diagnostic tests (RDT). Both microscopy and RDTs should be supported by a quality assurance programme (4).

The National Malaria Reference Laboratory (NMRL) was established in 2019 at Royal Centre for Disease Control (RCDC) as a reference laboratory to oversee the quality assurance on malaria diagnosis and provide technical assistance to VDCP. From 2019 until date, NMRL has conducted four rounds of malaria microscopy panel testing. The primary role of NMRL is to conduct the National External Quality Assessment Scheme (NEQAS) for malaria diagnosis. It includes blinded rechecking, panel testing, monitoring and supervision. Blinded rechecking is an

important component of effective quality assurance (QA) whereby participating laboratories send all positive slides and 10% of negative slides to the reference laboratory. It indicates whether a participating laboratory is providing accurate results and can detect major deficiencies in laboratory performance due to level of competence, poor internal quality control (IQC) and provide feedback report with necessary corrective action for quality improvement.

In panel testing, malaria slides with undisclosed results were sent to participating laboratories for identification. Inter-laboratory comparisons on each participating laboratories are compared, and feedback reports provided. Additionally, NEQAS guidelines and Standard Operating procedure (SOPs) for malaria diagnosis are prepared aligned with WHO Standard (5).

A country in an elimination phase may have low malaria incidence and a low positivity rate, resulting in a gradual decrease in the skills of malaria microscopists for participating laboratories, and a lower level of competency may be a consequence of fewer positive blood slides or a less frequent occurrence of positive malaria slides in the laboratory. Therefore, NEQAS provides periodic QA for malaria diagnosis in sustaining and maintaining high competency level in microscopy skills and improving IQC which will provide supporting documents for malaria elimination and certification by the WHO.

Methodology

For proficiency testing, participants include one national referral hospital, two regional referral hospitals, 48 district hospitals, and eight primary health centers from across the country.

Panel slide preparation

The panel consists of 5 slides comprising both thick and thin blood film of malaria positive and negative smear. As part of the positive panel, EDTA blood samples were obtained from positive patients and prepared and processed according to national SOPs as well as WHO guidelines on malaria microscopy quality assurance (5).Thick and thin blood films were prepared on single slides. Only thin films were fixed with methanol and were stained with Giemsa pH 7.2. The stained slides were covered with coverslip using DPX mount.

Shipment and timeframe

Slides packed in slides mailers and request letters packed separately were shipped in protective envelopes in accordance with UN 3373 recommendations. The web-based system automatically accepts the participating laboratories' results within the time frame and gets close when the closing date is reached. Participating laboratories are not able to submit their results after the closing date. The time frame is set to 40 days from the date of shipment made.

Reporting

Participating laboratories were asked to examine the slides by maintaining proficiency and report the diagnosis as (Malaria "Yes" or "No"), *plasmodium* species identification, stages identification and parasite density. For positive slides, presence of *plasmodium* species, and parasite density were assessed as well as the presence of asexual (trophozoite and schizont) and sexual (gametocytes) results were examined and validated by NMRL staff (6, 7). To avoid variation in parasite count, each microscopist count parasite densities and if the discrepancy difference is less than 20%, then the parasite density is used as the true value for calculating the ± 50% parasite density range.

Data analysis

After the closing date, all examined data were captured in the online database system (mNEQAS). Level of performance on specificity, sensitivity, parasite detection; malaria species identification, malaria stages identification and parasite density were assessed against pre-determined results of the reference laboratory.

Results

A total of 300 panel slides were distributed to 60 participating laboratories. Of the 300 panel slides, 180 (60.0%) were positive and 120 (40.0%) were no malaria parasite seen (NMPS) slides. From the total participating laboratories, 55 participating laboratories have examined the panel with a response rate of 91.7 % and the remaining five participating laboratories fail to participate in this panel round (8.3%)**(Figure 1).**

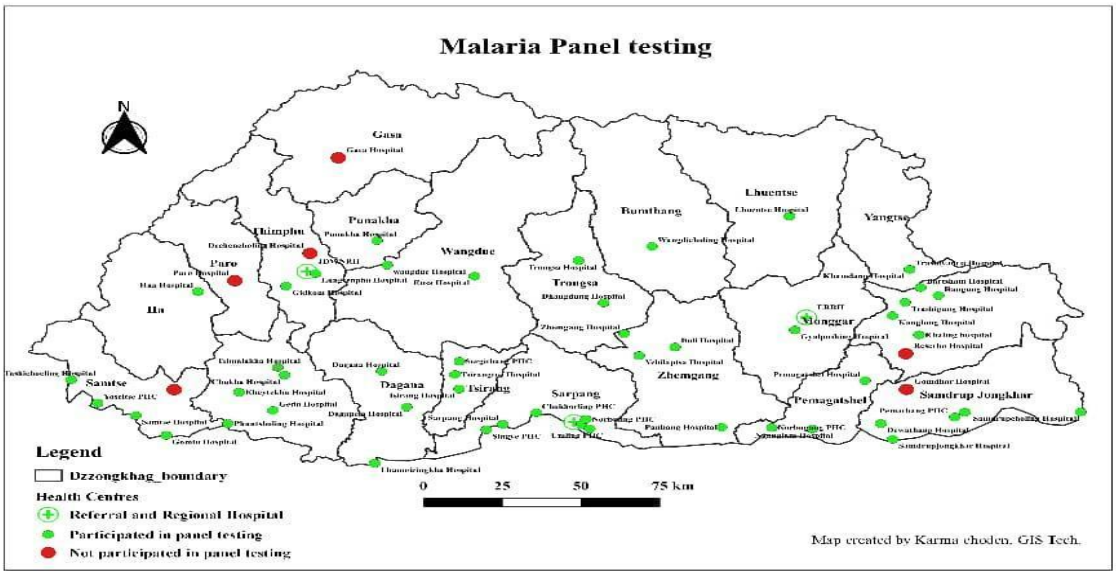

Figure 1: Panel reporting by health facilities.

A total of 136 laboratory staff including laboratory officers, laboratory technicians and malaria technicians have participated from various hospital labs and reported their panel results. Out of 136, 69.8% (n=95) microscopists have examined and reported on time and the rest 30.1% (n=41) reported late**(Figure 2).**

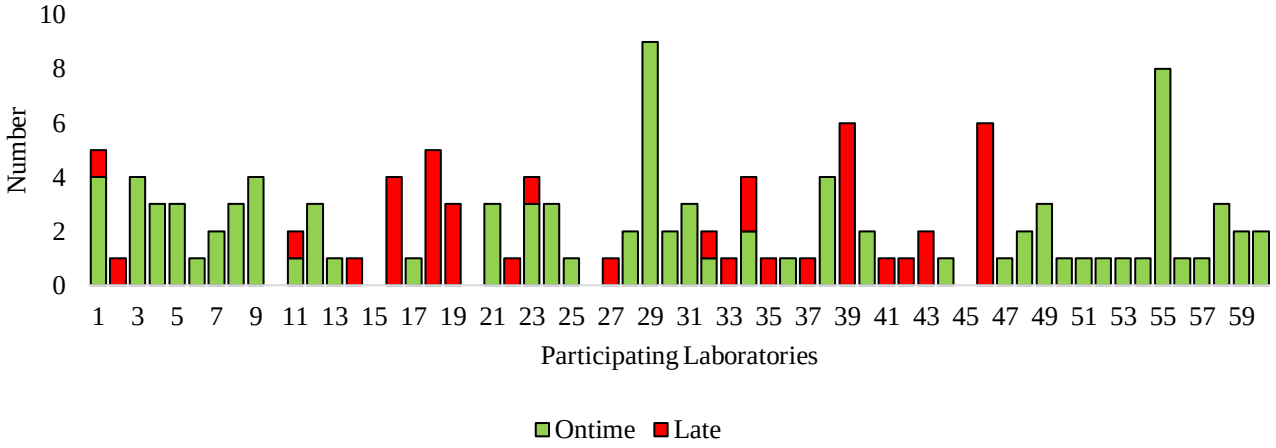

Figure 2: Panel reporting timeliness on malaria microscopy.

Accuracy of results for malaria parasite detection

Of the total 680 slides examined by 136 microscopists, overall, 588 (86.4%) were correctly reported. Among the 408 positive slides, 351 (86.03%) were correctly detected as positive and

the rest (13.9%) were reported as false negative. From 272 malaria negative slides, 237 (87.1%) were correctly detected as NMPS and the rest reported as false positive (12.8%) **(Figure 3).**

For more detailed information, Annexure 1 shows the performance of each participating laboratory on each parameter and key metrics were defined in annexure 2.

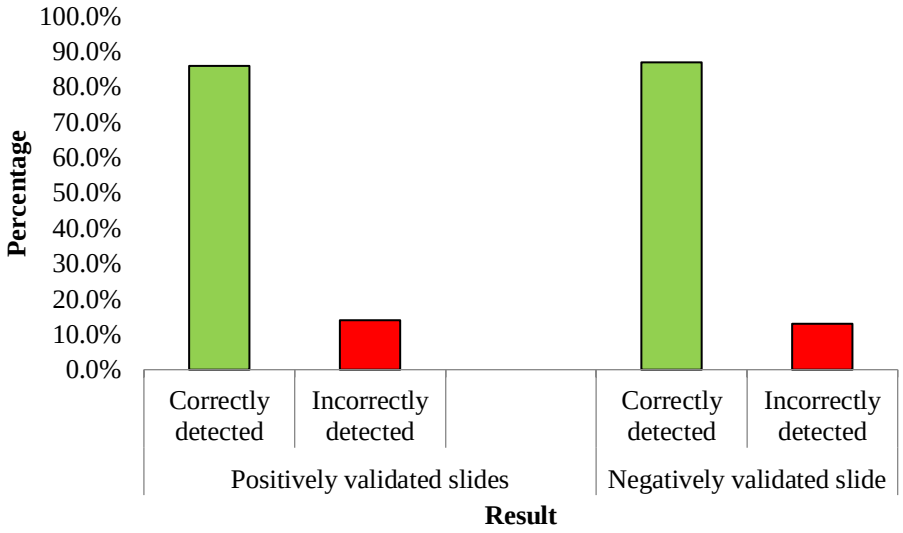

Figure 3: Proportion on detection and identification malaria parasite

Plasmodium species identification

For the positive panel, a total of 136 (33.3%) slides were for *Plasmodium falciparum* (Pf) species and 272 (66.7%) slides were for *Plasmodium vivax* (Pv) species. Out of 351 positive reported slides, the proportion of correct detection for Pf and Pv were 114 (83.8%) and 167 (61.4%) respectively. Six microscopists failed to identify Pf, and 52 microscopists failed to identify Pv and reported NMPS. Additionally, 16 Pf slides were misread as 15 Pv slides and 1 mix infection as PfPv. Likewise, 52 Pv slides were misread and identified as 48 Pf and 4 mix infection of Pf- Pv respectively (**Figure 4**). The misreading of the positive slides as negative is considered as a major error.

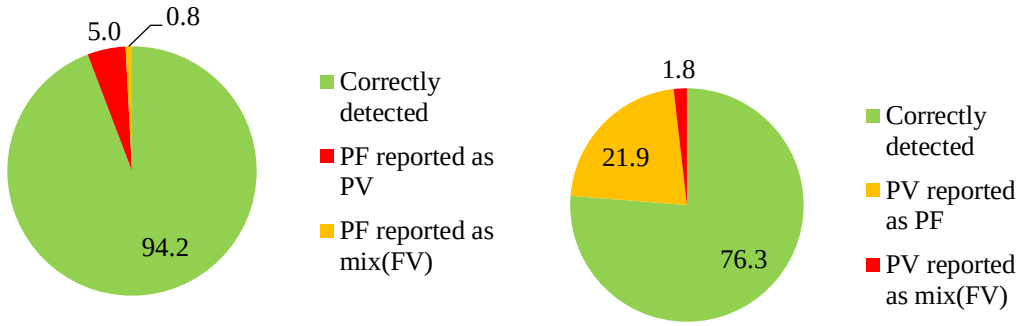

Figure 4: Percentage of plasmodium parasite identification and misidentification

Plasmodium stages identification

Of 136 Pf slides containing trophozoite, 103 (75.7%) were correctly identified as trophozoite, 7 (5.1%) identified as trophozoite and schizont and the rest were discordance results. Among 24Pf slides, microscopists failed to identify parasite stages and reported 6 false negative, 15 identification errors and 3 not reported. Of 272 Pv slides with trophozoite and gametocyte form, 81(29.7%) correctly identified all forms of malaria stages and rest were reportedas discordance results (**Table 1**).

Table 1: Comparison of plasmodium species stages reported by participating laboratories with results obtained at reference laboratories

Malaria parasite stages identification	Reference laboratory	
Participating laboratories	**T**	**TG**
***Trophozoite* (T)**	132[a]	43[b]
***Schizont* (S)**	2[c]	13[d]
***Gametocyte* (G)**	1[c]	3[e]
TS	12[f]	25[g]
TG	7[h]	14[a]
SG		1[i]
TSG	12[f]	16[j]
FN	53	5
***Identification Error* (IDE)**	49	15
***Not Reported* (NR)**	4	1
Total	272	136

T: Trophozoite; S: Schizont; G: Gametocyte; FN: False Negative; IDE: identification error ; NR: not reported

Codes: a: denote correct identification of all stages, b: denote gametocyte missed; c: denote only schizont and gametocyte reported in slide containing trophozoite; d: denote trophozoite & gametocyte missed; e: denote trophozoite missed; f: denote trophozoite/schizont/gametocyte reported in slide containing trophozoite; g: denote trophozoite and schizont reported in slide containing trophozoite and gametocyte; h: denote trophozoite and gametocyte reported in slide containing trophozoite; i: denote schizont and gametocyte reported in slide containing trophozoite and gametocyte; J: denote all stages reported in slide containing trophozoite and gametocyte

Quantification of parasite density

Parasite density is determined as the ratio of parasite to white blood cell (WBC) in thick blood film. Parasite were counted against 200 WBCs and if parasite count is less than 100 after counting 200 WBCs, further parasite count is performed against 500 WBCs. Parasite density (parasite /ul of blood) is calculated as follows;

$$Parasite\ Density = (No.of\ parasites\ counted/No.of\ WBC\ counted)X\ 8000$$

Panel slides with varied parasite density including low and high parasitemia were included to test the parasite counting skills of the participating laboratories. To obtain a score, responses must be within ±50% of the reference count. For this round of panel test assessment, the range of accuracy (±50% of the reference count) for the 3 positive slides were 144 (35.29%) and the rest reported out of range of the reference count.(8, 9)

For 350/408 (87.8%) malaria positive slides reported, 146 slide (41.7%) reported out of range with parasite density in 57(16.3%) slides lower than the reference count, 67 (19.1%) slides with higher parasite density from the reference count, 64 (18.3%) slides with parasite identification error, and 55 (15.7%) slides reported false negative. In 21 slides (6.0%), few participating laboratories failed to perform the parasite count (Table 2).

Table 2: NEQAS parasite density count reports

Reference laboratory	**Participating laboratories' parasite density**							
Parasite density	Correct	Incorrect	Higher count	Lower count	Identification error	False negative	Not reported	Total
±50% reference count	144	146	67	57	64	55	21	554

Discussion

The accuracy of malaria microscopy diagnostic testing is key for early diagnostic and prompt treatment (EDPT) in improving malaria case management. Accuracy of malaria microscopy and factors associated with inaccurate microscopy reported by participants in district health facilities were assessed.

The current report showed poor performance of malaria microscopy in all levels of health facilities, however, data on the performance of panel testing is still limited as we are not able to

compare the past participating laboratories performance due to in consistent participation for this activity from the participating laboratories.

Overall finding and challenges

1. Irregular participation in panel testing

Out of 60 participating laboratories, there was no response from five labs. Some of the reasons cited were lack of human resource, misplacing of slides etc.

2. Late submission of report

Usually the non endemic malaria sites were late reporter.

2. False positive reporting

This type of error is considered as a minor error whereby the slides are misread as positive slides. This will result in unnecessary admission and treating the patient thereby increasing the risk of anti-malaria resistance and treatment failure.

3. False negative reporting

This type of error is considered as a major error whereby the positive slide is misread as a negative slide. Under-diagnosis of malaria is a concern as patients will not receive treatment and if they undergo severe malaria, it may be fatal. Moreover, the chain of disease is not interrupted; there are chances of any malaria outbreak in the locality.

4. Malaria parasite identification and stages concordance

Few of the participating laboratories find difficulties in identifying and differentiating malaria parasite stages (i.e., Trophozoite, Schizont and Gametocyte). In most of the panel slides, microscopists have missed examining schizont and gametocyte stage.

5. Parasite Density Determination:

From the findings, participating laboratories face difficulties quantifying the parasite density using the formula as most of the parasite density value was not within ±50% range count determined by the reference laboratory.

Area of improvement:

Declining competency skills in malaria microscopy might be due to utilization of RDT as the routine method including blood film preparation and staining. The malaria RDT can be used in situations such as during emergencies time, mass screening and during busy routine schedule.

All the passive cases have to be detected by microscopy and if any doubtful finding, use RDT to resolve the finding.

The microscopist must examine the positive slide if available while examining routine slides to get familiar and update on malaria parasite morphology. To improve malaria microscopy competency, participating laboratories must participate in panel testing provided by reference laboratory where staff get a chance to examine positive slides of both plasmodium falciparum and plasmodium vivax.

The microscopist must examine and practice performing parasite count if positive slide available in their laboratory to improve skills on PC determination and refer SOPs for parasite density determination on how to calculate and perform the test.Since Bhutanis in the elimination phase, each sites must give equal importance to NEQAS.

Conclusion

Overall, the present report showed that laboratory professionals need improvement in malaria microscopy with the majority getting less than 90% score. Training and supervisory visit from referral lab is critical. Only few participating laboratories had good performance in malaria parasite detection, species identification and determination of parasite density. Therefore, to fill the gaps, NEQAS activities should be conducted regularly for continuous assessment of quality performance. Additional NEQAS methods such as blinded rechecking and supervisory visits must supplement the panel testing

Reference

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Panel Testing for Bacteriology and Antimicrobial Resistance Across One Health Sector

Kinley Gyem, Sonam Pelden, Dorji Tshering, Rinzin Wangchuk, Kinley Penjor, SangayDorji, RinzinWangdi, Jigme Tenzin and BirdilalPhuyel

Enteric and Invasive Disease Laboratory

Background

External Quality Assessment (EQA) is a systematic assessment conducted by an external organization, where proficiency test items are sent to participating laboratories and their responses are evaluated. It is a tool that measures the testing process (the analytical phase), including the quality of results, generated by the laboratory [1]

The National External Quality Assessment Scheme (NEQAS) for Bacteriology and Antimicrobial Resistance (AMR) was launched in May 2021 as an educational tool that enables participants to examine, monitor and improve their own laboratory methodologies and personnel performance by allowing comparison of results between the microbiology laboratories. With laboratories participating from the human, animal, and food testing sectors, the program aims to strengthen the NEQAS services across the One Health sector. The program is organized bi-annually and supported by the Fleming Fund project, Ministry of Health, Bhutan.There were six laboratories participating in the scheme. The aim of this program is to monitor the performance of the quality of identification and Antimicrobial Susceptibility Testing (AST) produced by the participating laboratories (PLs) and identify underperformers and accordingly design methods to enhance their performances.

Methodology

NEQAS Participating Sites

A total of six laboratories, four clinical laboratories, an animal health center lab, and the food testing laboratory were members of the scheme (**Figure 1**). The laboratories are identified by codes and each code is confidential and known only by the corresponding laboratory and the organizers.

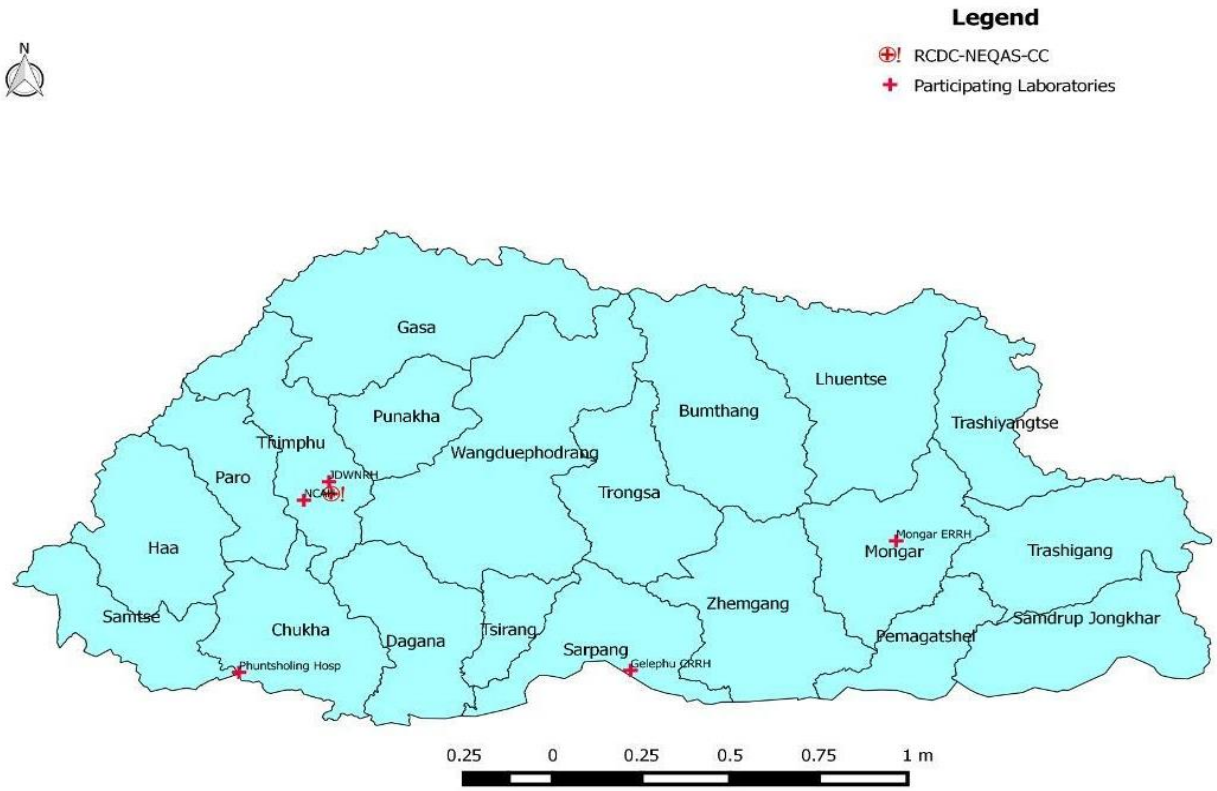


Figure 1. Participating laboratories of the NEQAS PT

Proficiency Testing (PT) Panel

The PT used for every exercise was compatible with the epidemiology of the resistance phenotypes of species under surveillance at the Royal Center for Disease Control (RCDC). From the National Biorepository for Human Health (NBCH) stationed within the center, a panel of two to three species of bacteria was re-cultured, lyophilized, and shipped to the PLs following the triple packaging system for biological substances. The PT samples were identified, characterized, and tested for antimicrobial susceptibility by two staff respectively, and confirmed the concordance of the test as indicated for each PT before shipment to the PL. To check for the integrity of the PT sample (a part of the IQC measure), a batch of the lyophilized specimen was prepared and sent along with the PT specimen to one of the PLs to be shipped back to the NEQAS-CC after two to three weeks of PT receipt at their lab. This QC specimen is re-tested for viability and AST confirming the concordance of obtained to the expected result. The PT samples were dispatched to the PLs biannually as reflected in **Table 1**.

Table 1: Overview of the PLs and PT round

PT round	Pilot study	Round 1	Round 2	Round 3
Dispatched date	August 2021	Nov 2021	June 2022	Nov 2022
No. of PT samples dispatched	2	3	3	3
PT samples	*E-coli*	*Aeromonas hydrophilia*	*Pseudomonas aeruginosa*	*Salmonella enteritidis*
	Salmonella typhimurium	*Shigella flexneri*	*Salmonella typhimurium*	*Aeromonas hydrophilia*
		Staphylococcus aureus	Blank (No growth)	*Shigella sonnei*
Site	JDWNRH	JDWNRH	JDWNRH	JDWNRH
	CRRH	CRRH	CRRH	CRRH
	ERRH	ERRH	ERRH	ERRH
	NCAH	NCAH	NCAH	NCAH
			P/LING	P/LING
			BAFRA	BAFRA

Procedure

Prior information was made and hands-on sensitization of the NEQAS web-based reporting to all the PLs was conducted before the initiation of the PT exercise. Protocols and all relevant information were provided to allow access to all the necessary information at any time. The participants were recommended to store the lyophilized strains in a dark, dry, and cool place until the performance of identification and AST. PLs were advised to perform identification and AST of the PT panels according to the methods routinely applied in their laboratory. Participants were encouraged to submit serotyping results for the *Salmonella* and *Shigella* strains.

The reference values for interpreting Kirby-Bauer disk diffusion results are in accordance with current zone diameter breakpoint values developed by CLSI (M100, 31st Ed. and VET06, 1st Ed.)[2]. Participants were encouraged to test as many as possible of the antimicrobials listed in the web system[3], but always considering their relevance to the laboratory's routine work. Interpretative criteria referring to disk diffusion are listed in the web-based system and allow for the obtained results to be interpreted into categories as Resistant (R), Intermediate (I), or Susceptible (I). All PLs were invited to enter the obtained results into the NEQAS web-based reporting system [3].

Scoring for Identification and ABST

Results in agreement with the expected interpretation in the case of bacterial identification are scored "1" (**correct identification**), "0.5" (**Partially correct identification**), and "0" (**Incorrect identification**); And AST is scored '3' (**correct**), '2' (**Incorrect with minor deviation**), and '1' (**Incorrect with major deviation**) and while results completely deviating from the expected interpretation are scored as either '0' (**incorrect**) as reflected in the score evaluation matrix (**Table 2**)(4), (5).

Table 2: Score evaluation matrix

Scores		Obtained Interpretation		
		Susceptible	Intermediate	Resistant
Expected Interpretation	**Susceptible**	3	2	1
	Intermediate	2	3	2
	Resistant	0	2	3

0	Incorrect: Very major
1	Incorrect: Major
2	Incorrect: Minor
3	Correct

Results

Participation

Among the six laboratories participating in this PT scheme, for the pilot testing and Round 1 scheme, Two PLs could not join due to the unavailability of the lab, and another was late to join the scheme.

Identification of bacterial cultures

Conventional standard microbiological methods using biochemical tests were used for bacterial identification. For PT identification, almost every PL failed to correctly identify one of the given PT panels in all the rounds. Three laboratories reported "No growth" from one panel each. Serotyping of the *Salmonella and shigella* is a voluntary component of this scheme and hence many PLs have not serotyped the panels, only two PLs correctly assigned the serogroup for

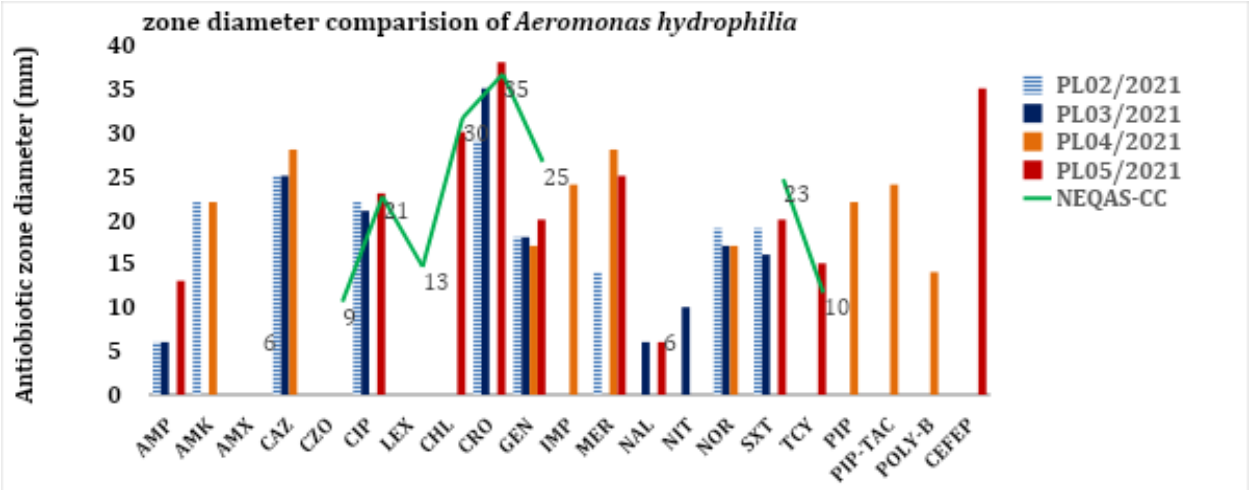
salmonella and three laboratories could precisely serotype for *shigella* panels. In case of no antisera for serotyping, serogroup could still be reported and further evaluated, meaning that serotype and serogroup were separately assessed in this scheme. One PL failed to submit the round 2 result and failed the PT study (**Table 2**).

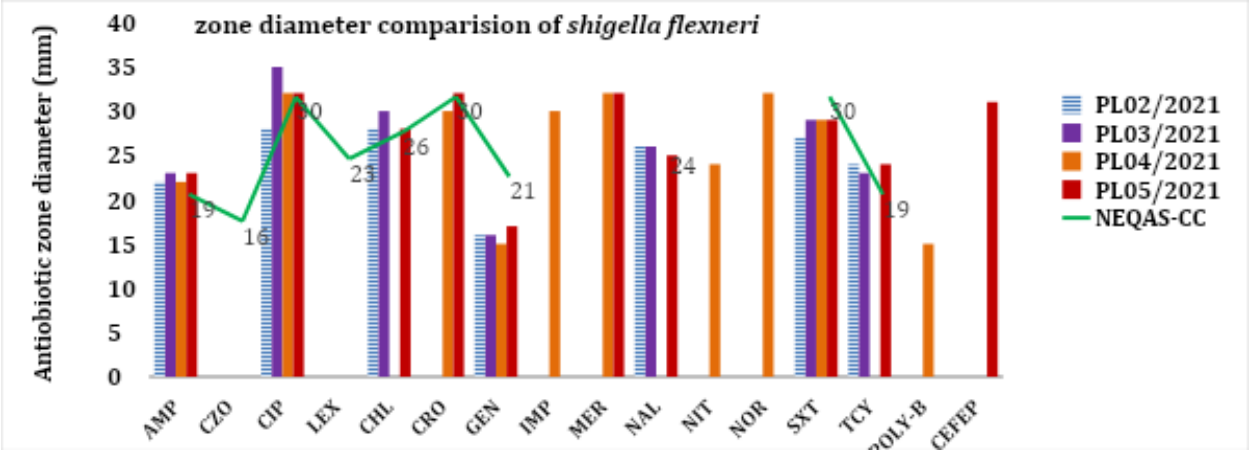
Table 2: Identification score of PT samples for all PT rounds

Lab Code	Round 1			Round 2			Round 3		
	Expected	Obtained	Score	Expected	Obtained	Score	Expected	Obtained	Score
PL01	Couldn't join the Scheme			*P. aeruginosa*	*P. aeruginosa*	1	*S. enteritidis*	*Salmonella sp*	0.5
				S. typhimurium	*Salmonella sp*	0.5	*A. hydrophilia*	No growth	0
				Blank	No growth	1	*S. sonnei*	*Shigella sp*	0.5
PL02	*A. hydrophilia*	*Pseudomonas sp*	0	*P. aeruginosa*	*P. aeruginosa*	1	*S. enteritidis*	*Salmonella sp*	0.5
	S. flexneri	*Shigella sp*	1	*S. typhimurium*	*Salmonella sp*	0	*A. hydrophilia*	No growth	0
	S.aureus	*S. aureus*	1	Blank	No growth	1	*S. sonnei*	*Shigella sp*	0.5
PL03	*A. hydrophilia*	*A. hydrophilia*	1	*P. aeruginosa*	*P. aeruginosa*	1	*S. enteritidis*	*S.typhi*	0.5
	S. flexneri	*S. flexneri*	1	*S. typhimurium*	*Salmonella sp*	0.5	*A. hydrophilia*	No growth	0
	S.aureus	*S. epidermidis*	0	Blank	No growth	1	*S. sonnei*	*Shigella sp*	0.5
PL04	*A. hydrophilia*	*Vibrio sp*	0	*P. aeruginosa*	*P. aeruginosa*	1	*S. enteritidis*	*S. enteritidis*	1
	S. flexneri	*S. flexneri*	1	*S. typhimurium*	*Salmonella sp*	0.5	*A. hydrophilia*	*A. hydrophilia*	1
	S. aureus	*S. aureus*	1	Blank	No growth	0	*S. sonnei*	*S. sonnei*	1
PL05	*A. hydrophilia*	*A. hydrophilia*	1	*P. aeruginosa*	*P. aeruginosa*	1	*S. enteritidis*	*S. typhimurium*	0.5
	S. flexneri	*S. sonnei*	0	*S. typhimurium*	*Salmonella sp*	0.5	*A. hydrophilia*	*A. hydrophilia*	1
	S.aureus	*S. aureus*	1	Blank	No growth	1	*S. sonnei*	*S. flexneri*	0.5
PL06	Couldn't join the Scheme			*P. aeruginosa*	Result not submitted	Failed	*S. enteritidis*	*S. enteritidis*	1
				S. typhimurium			*A. hydrophilia*	No growth	0
				Blank			*S. sonnei*	*S. sonnei*	1

Antimicrobial Susceptibility Testing

The PLs ABST results were analyzed and scores are rated based on excellent concordance if the reported categorization agreed with the coordinating center's interpretation. There was a good consensus of reported results for ABST and reported within the range as per the working guideline, although few deviations of zone interpretation are reported. The major observation was the incorrect classification of ceftazidime and meropenem reported values. In round 1, the expected value of these beta-lactam groups of third-generation cephalosporin and carbapenem antibiotics was reported as "Susceptible" and "Intermediate", when the expected value is 6mm (Resistant). The detailed comparison of ABST for all the rounds is illustrated in **Figures 2, 3 and Table 3**. The performance score for bacterial identification and ABST for all rounds among the PLs is reflected in **Figure 4**.





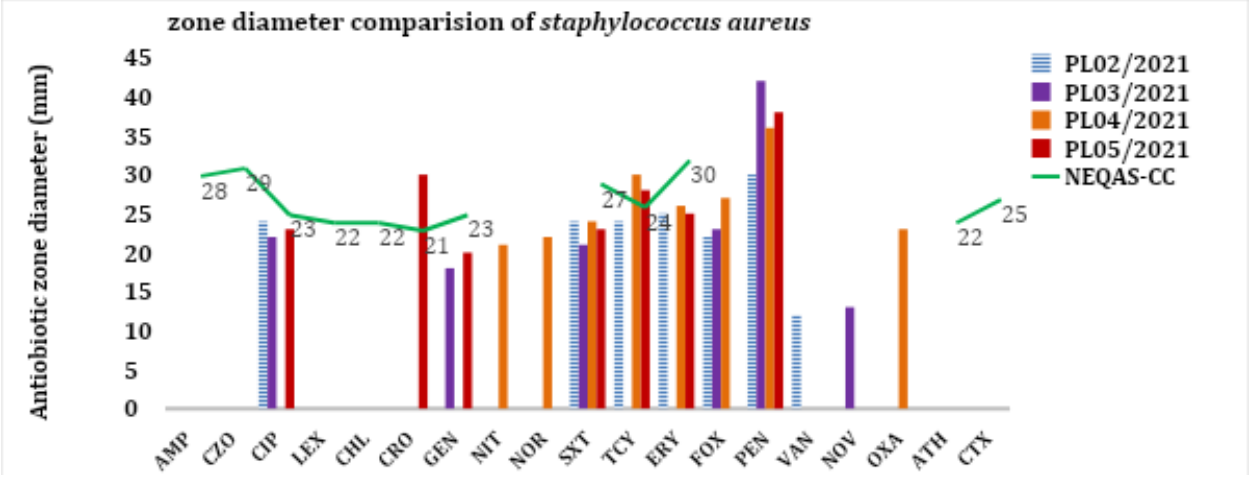


Figure 2. Zone diameter comparison of PT panels in Round 1

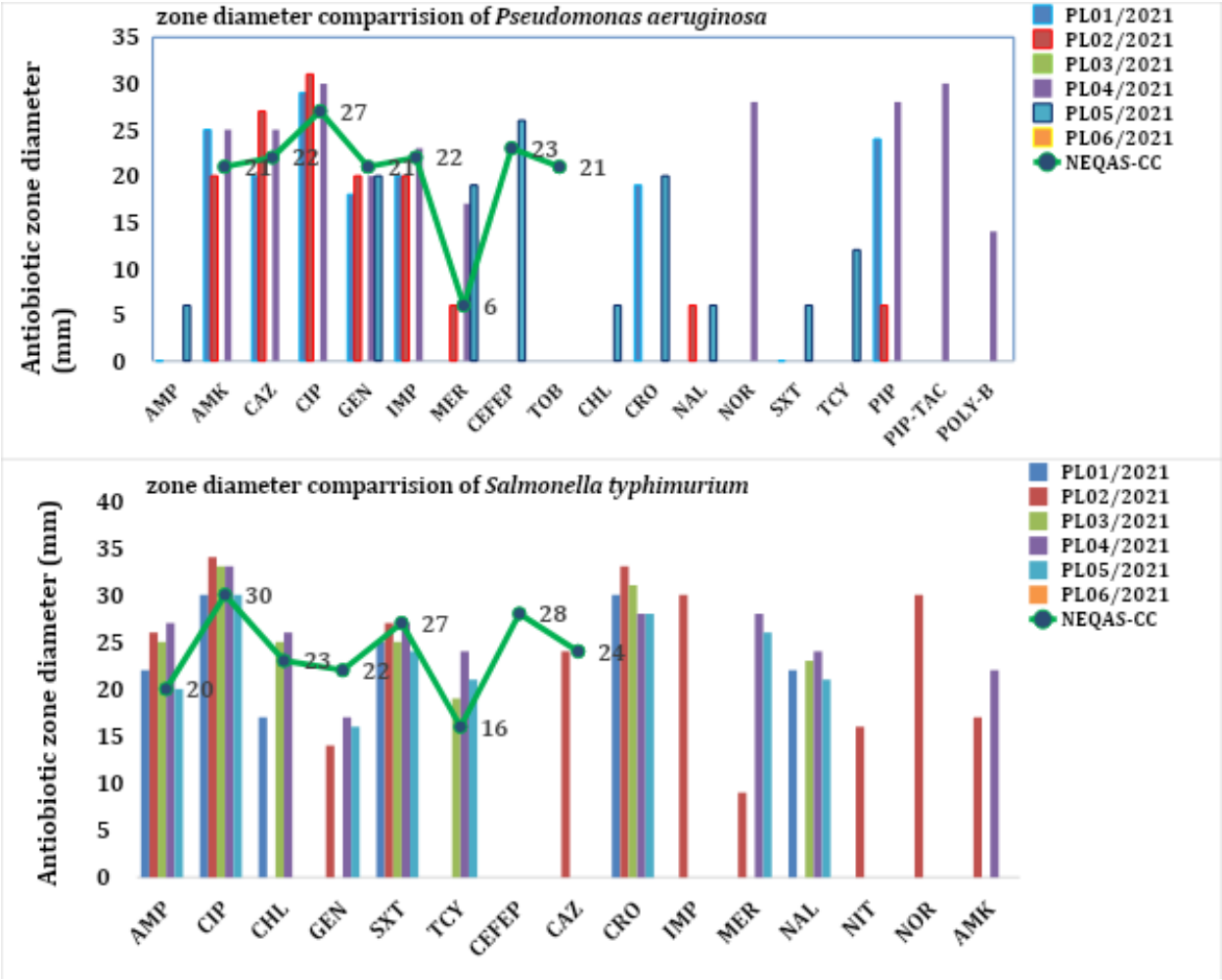


Figure 3: Zone diameter comparison of PT panels in Round 2

Table 3: ABST score of the PT panels in round 3 (extracted from the web-based system)

Site	Sample ID	Expected identification	Obtained identification	ABST Score
PL01/2022	BAC223016	*Salmonella enteritidis*	*Salmonella species*	9/21
	BAC223017	*Aeromonas Hydrophilia*	No Growth	0/36
	BAC223018	*Shigella species*	*Shigella species*	15/45
Total Score				**24/102**
PL02/2021	BAC223001	*Salmonella enteritidis*	*Salmonella species*	12/21
	BAC223002	*Aeromonas Hydrophilia*	No Growth	0/36
	BAC223003	*Shigella species*	*Shigella species*	26/45
Total Score				**38/102**
PL03/2021	BAC223004	*Salmonella enteritidis*	*Salmonella typhi*	21/21
	BAC223005	*Aeromonas Hydrophilia*	No Growth	0/36
	BAC223006	*Shigella species*	*Shigella sonnei*	40/45
Total Score				**61/102**
PL04/2021	BAC223007	*Salmonella enteritidis*	*Salmonella enteritidis*	21/21
	BAC223008	*Aeromonas Hydrophilia*	*Aeromonas Hydrophilia*	25/36
	BAC223009	*Shigella species*	*Shigella sonnei*	38/45
Total Score				**84/102**
PL05/2021	BAC223010	*Salmonella enteritidis*	*Salmonella typhimurium*	20/21
	BAC223011	*Aeromonas Hydrophilia*	*Aeromonas Hydrophilia*	18/36
	BAC223012	*Shigella species*	*Shigella flexneri*	26/45
Total Score				**64/102**
PL06/2022	BAC223013	*Salmonella enteritidis*	*Salmonella enteritidis*	15/21
	BAC223014	*Aeromonas Hydrophilia*	No Growth	0/36
	BAC223014	*Shigella species*	*Shigella sonnei*	30/45
Total Score				**45/102**

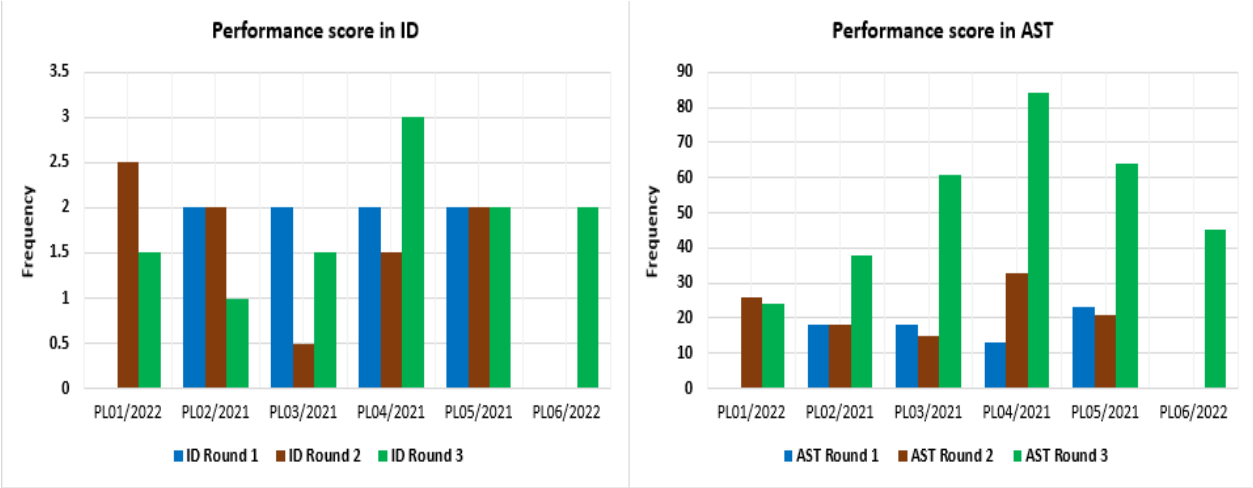


Figure 2: Performance score comparison over time among the PLs

Discussion

The overall performance of the PLs indicates that there was good performance among the PLs in all rounds except for round 2, which probably could be due to the slow shift in work focus right after the uplifting of the pandemic lockdown. The PLs showed high proficiency in correctly identifying PT panels to the genus level. Three PLs showed a limited capacity to correctly identify the target species reported "No growth" for the "*Aeromonas hydrophilia*" panel. These submitted results also suggest that there might be poor handling and maintenance of the PT panels, as some reported data possible contamination or inviable strains. Serotyping of *Salmonella* and *shigella* was also a component with voluntary participation, however, a score is assigned for the correctly serotyped panels. Of those, three PLs submitted data for both serogroup and serovars. Based on the results, it is notable that some PLs could only identify certain serogroups. This could be due to limited technical capacity, but also a lack of antisera supply.

There was a good consensus on the reported results for ABST and reported within the range as per the working guideline, although few deviations of zone interpretation are reported among the PLs. The web-based system helped suggest the PLs test the relevant antibiotics for the provided PT panels and those tested was scored accordingly.

For the incorrect classification of ceftazidime and meropenem values, a systemic performance of internal quality control including testing of reference strains must be implemented to warrant the

improvement of laboratory performance. According to the CLSI recommendation, the quality of laboratory performance is determined by indicating the accuracy and precision of data produced by an individual laboratory.

Conclusion

Accurate pathogen identification is crucial, especially in a clinical setting, classification by serotyping is essential for surveillance, source tracking, and outbreak detection. There is a need to assess the bacterial retrieving process and associated factors for some of the pathogens and provide guidance and appropriate training if required. Proper storage and maintenance of the test strains are recommended. IQC measures should be strengthened at every step, as deviating results among the panels imply invalidation of the reported results.

For the misinterpretation of the zone diameter values, it is recommended that PLs should review their individual performance and revisit all areas where they did not achieve the intended results. The observation that some participants are reporting Resistant (R), Intermediate (I), and Susceptible(S) in cases where their guidelines do not define such a category indicate that methods are not always strictly adhered to, and participants should review their reporting practices.

References

[1] “EQARG”.

[2] Clinical Laboratory Standards Institute (CLSI) logoClinical Laboratory Standards Institute (CLSI), “CLSI Publishes M100—Performance Standards for Antimicrobial Susceptibility Testing, 31st Edition.”

[3] RCDC, “http://192.168.100.25/COMMONNEW/dashboard/dashboard.php.”

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Annexures

Annexure 1: Health facilities wise 4th round panel testing score for Malaria.

Lab No	Sensitivity	Specificity	Infection Detection	Species Identification	Stage Identification	Density Calculation	Average Score
1	100	100	100	67	50	33	75
2	100	100	100	100	83	33	86
3	100	0	60	100	67	33	60
4	100	100	100	100	100	67	94
5	100	100	100	33	17	33	64
6	67	100	80	0	0	0	41
7	67	100	80	67	50	67	72
8	100	100	100	100	100	67	94
9	100	100	100	100	100	100	100
10	100	100	100	100	83	67	92
11	33	50	40	33	33	0	32
12	100	100	100	67	67	67	83
13	100	100	100	100	100	100	100
14	100	100	100	100	83	100	97
15	67	50	60	67	50	33	54
16	100	100	100	67	50	67	81
17	100	100	100	100	100	67	94
18	100	100	100	100	100	67	94
19	67	100	80	67	67	33	69
20	100	100	100	100	100	67	94
21	100	100	100	100	100	67	94
22	100	100	100	67	50	33	75
23	100	100	100	100	83	0	81
24	100	100	100	100	100	67	94
25	67	100	80	67	50	0	61
26	100	100	100	100	100	67	94
27	100	100	100	100	83	67	92
28	100	100	100	100	83	0	81
29	100	100	100	67	67	33	78
30	67	100	80	67	33	0	58

31	100	100	100	100	100	67	94
32	100	100	100	67	50	0	69
33	67	100	80	33	17	33	55
34	67	50	60	67	50	0	49
35	100	100	100	67	50	67	81
36	67	100	80	67	50	33	66
37	100	100	100	67	50	67	81
38	67	100	80	67	67	33	69
39	100	100	100	67	50	33	75
40	100	50	80	67	50	33	63
41	100	50	80	33	33	0	49
42	100	100	100	100	83	0	81
43	33	0	20	33	17	0	17
44	100	100	100	100	100	100	100
45	100	100	100	100	100	100	100
46	67	50	60	67	50	33	54
47	67	100	80	67	33	0	58
48	67	100	80	67	50	0	61
49	100	100	100	100	100	67	94
50	67	50	60	67	50	67	60
51	67	50	60	67	33	33	52
52	67	50	60	67	33	0	46
53	67	100	80	67	67	0	63
54	67	100	80	67	67	33	69
55	100	100	100	100	83	100	97

Annexure 2: Definitions

Sensitivity: Sensitivity of the panel testing is proportion of true positive among the panel slides

Specificity: Proportion of the panel slides which are true negative among the panel slides

Malaria parasite detection: This refers to assessment of the slides with positive and negative findings

for the presence of malaria parasite.

Concordance of species identification: refers to assessment of the results for identification of each

species present in the positive slides

Concordance of stages identification: refers to assessment of the results on identification of the sexual and asexual stages of Plasmodium present in the positive slides

Concordance of parasite density determination: refers to assessment of the results on parasite density in positive

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