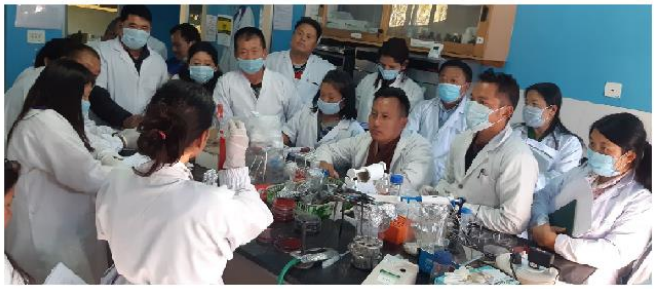
རྒྱལ་ཡོངས་སྐོར་རྩོད་གསོ་བའི་ཞེས་བ།
ROYAL GOVERNMENT OF BHUTAN
Ministry of Agriculture and Forests
Department of Livestock
NATIONAL CENTRE FOR ANIMAL HEALTH

ANNUAL REPORT 2020-21





LABORATORY SRVICES UNIT

PABX: +975-2-351083/351093; Fax: +975-2-351095; Post box: 155
Website: www.ncah.gov.bt; Email: ncah2013@gmail.com

*Toll free number **1244** for any queries/reporting on livestock and poultry diseases*

TABLE OF CONTENTS

1. BACKGROUND

The Laboratory Services Unit (LSU) is one of the technical units under the National Centre for Animal Health, Serbithang; functions as the national veterinary referral laboratory in the country. It is mandated with providing referral laboratory services besides it's routine diagnostic services. The unit has the capacity for advanced diagnostic tests such as Enzyme-linked immunosorbent assay (ELISA), Fluorescent antibody test (FAT) and molecular assays for emerging and re-emerging infectious disease like Foot and Mouth Disease (FMD), Highly Pathogenic Avian Influenza (HPAI), Classical Swine Fever (CSF), African Swine Fever (ASF), Brucella, Porcine Respiratory & Reproductive Syndrome (PRRS), Rabies, Porcine Circovirus Type 2, Capripox, Lumpy Skin Disease (LSD).

The laboratory is also equipped with real time Polymerase chain reaction (PCR) technology. The unit has Bio-safety level 2 plus facilities for safe handling of high-risk pathogens. In addition, the unit is also responsible for monitoring and evaluating Bio-safety activities in the veterinary laboratories in the country. The unit is also responsible for coordinating collaboration of advance level diagnostic research with international reference laboratories and institutes. It is also mandated to carry out laboratory-based surveillances/researches. The lab is also the national referral lab for the Antimicrobial Resistance (AMR) in animal health.

2. MANDATES

The main mandates of the Laboratory Services Unit are:

1. Providing referral veterinary laboratory diagnostic services to the clients
    - Provide routine veterinary laboratory diagnostic services, support clinical services, animal health programs and One-Health activities in the country;
    - Serve as the national referral laboratory for diagnosis of animal diseases in the country
2. Major Livestock Disease Surveillance/Survey
    - To lead/coordinate and conduct laboratory-based animal health research activities in the country
3. Coordination and implementation of Biosafety and Bio-security programs
    - Implement and monitor bio-safety measures and good laboratory practices in all veterinary laboratories in the country
4. Strengthening and enhancement of laboratory diagnostic capacities
    - To serve as focal laboratory for antimicrobial resistance monitoring in animals in the country
    - To participate in regional proficiency testing for specific diagnostic methods
    - To technically backstop regional, satellite and district laboratories in the country

- Introduction of new diagnostic tests/upgradation of diagnostic tests for the emerging and re-emerging diseases in the country
- To liaise, collaborate and establish efficient laboratory networks with the outside agencies like National Food Testing Laboratory, Bhutan Agriculture and Food Regulatory Authority; Clinical Laboratory, Jigme Dorji Wangchuck National Referral Hospital; Royal Centre for Disease Control, Department of Public Health; and Wildlife Clinic, Nature Conservation Division, Department of Forests and Park Services;
- To liaise, collaborate and establish efficient laboratory networks with the international reference laboratories such as OIE and WHO Referral Laboratories;

5. Laboratory skill enhancement
    - To develop human resource capacity by conducting the diploma course in laboratory technology in collaboration with other relevant institutions.
    - Conduct refresher course and up-gradation courses for laboratory technicians

3. HUMAN RESOURCES CAPACITY

The followings are the available human resource in the Laboratory Services Unit as on 30th June 2021 (Table 1).

Table 1: Over all human resource capacity of LSU during 2020-21

Specialization	**Sections**	**Number**
Animal Health Specialist- II (Veterinary Pathologist)	Pathology	1
Sr. Laboratory Officer	Microbiology/Molecular biology; Bio-safety & Bio-security/Biochemistry & toxicology	2
Sr. Laboratory Technician	Parasitology/Serology & Virology /Bacteriology	3
Laboratory Technicians	Serology & Virology/Hematology/Bio-chemistry& Toxicology/Pathology	4
Laboratory Attendant	General	1
Total		**11**

3.1 Bacteriology Section

Background

The section provides routine diagnostic services for microbial diseases (bacteria & fungi) in the livestock through culture & identification. The section also has capacity for second stage bio-chemical tests and identification of important bacterial pathogens like *Salmonella, B. anthracis, Campylobacter spp. E. coli, Staphylococcus aureus* etc.

Human resources

The section is manned by the following staffs:

1. Ms. Puspa Maya Sharma, Sr. Laboratory Officer
2. Mr. Tenzinla, Sr. Laboratory Technician
3. Ms. Tshewang, Dema, Laboratory Technician

Diagnostic capacities

The section has the following diagnostic capacities:

- Bacterial culture and identification using sheep blood agar, MacConkey agar and other selective media and various bio-chemical tests;
- Fungal culture and identification using Sabouraud's dextrose agar;
- Staining techniques - Grams, Giemsa, Methylene blue, Ziehl-Neelsen/Acid fast, Leishman, Lactophenol, Spore staining and Capsule staining;
- Species identification of important bacterial pathogens in Bhutan – *Salmonella sp., E. coli, Staphylococcus spp., Bacillus anthracis, Clostridium sp., Pasteurella, Pseudomonas sp., Erysipelas rhusiopathiae, Brucella sp., Aeromonas hydrophila* and *Streptococcus sp.*
- Enumeration of bacteria - total aerobic count by pour plate technique and spread plate technique, total coli count by pour plate technique and spread plate technique, Most Probable Number (MPN) technique;
- Detection of *Mycobacterium species* by acid-fast technique;
- Agglutination tests: Slide agglutination test (SAT), Tray agglutination test (TAT) and Micro-titer plate agglutination test (MAT);
- Detection of mastitis in milk samples through the California mastitis test (CMT), Cell count and White side test (WST);
- Antimicrobial susceptibility test (AST), disk diffusion method; ESBL detection method
- Intra-dermal test for bovine tuberculosis (TB) using purified protein derivatives (PPD).

3.2 Biochemistry and Toxicology Section

Background

The section conducts basic tests for clinical bio-chemistry in serum and also qualitative analysis of urine to support the clinical diagnosis. The section also conducts basic toxicological tests especially, screening of important mycotoxins in the animal feeds.

Human resources

The Bio-chemistry and Toxicology section has the following staff:

1. Dr. NK Thapa, AHS II
2. Ms. Dechen Wangmo, Sr. Laboratory officer
3. Ms. Ugyen Pema, Laboratory Technician

Diagnostic capacities

The following are the diagnostic capacities available in this section:

- Rapid tests for Aflatoxin in animal feed
- Quantitative estimation of mycotoxins (Aflatoxin, Ochratoxin, Fumonisin) in animal feeds;
- Mineral estimation for Ca, Mg and P in the serum;
- Qualitative urine analysis;
- Qualitative and quantitative bio-chemistry;

3.3 Haematology Section

Background

The section conducts basic haematological tests to support clinical diagnosis in the animals. In addition, the section also carries out examination of blood parasites like microfilaria and *Trypanosomes* etc.

Human resources

The Haematology section is manned by the following staffs:

1. Dr. NK Thapa, AHS-II
2. Ms. Tshewang Dema, Laboratory Technician

Diagnostic capacities

The hematological parameters and tests commonly conducted in this section are:

- Haemoglobin estimation (Hb);
- Packed Cell Volume (PCV);
- Total Red Blood Cell Count (TRBCC);

- Total White Blood Cell Count (TWBCC);
- Differential Leukocyte Count (DLC);
- Erythrocyte Indices – MCV, MCHC and MCH;
- Erythrocyte Sedimentation Rate (ESR);
- Wet film examination for blood parasites like microfilaria and trypanosome;

3.4 Molecular biology Section

Background

The section performs tests on both routine basis and also on the samples referred by the Regional/District/Satellite Laboratories in the country. The section, conducts test for both emerging and re-emerging diseases in the country like, Classical swine fever, African swine fever, Capripox, Porcine Respiratory and Reproductive Syndrome (PRRS) etc.

Human resources

The section is manned by the following officials:

1. Ms. Puspa Maya Sharma, Sr. Laboratory Officer
2. Ms. Dechen Wangmo, Sr. Laboratory Officer
3. Ms. Kelzang Lhamo, Laboratory Technician

Diagnostic capacities

The section has capacities for conducting the molecular tests as follows:

- Real-time PCR for AI Type A, (H5, N1, H7, N8) FMD, CSF, ASF, PRRS (EU and NA), Pigeon Paramyxovirus (PPMV) and ND;

3.5 Serology Section

Background

This section is equipped with advanced diagnostic facilities such as ELISA, SAT etc.

Human resources

The Serology section is manned by:

1. Mr. Dawa Tshering, Sr. Laboratory Technician

Diagnostic capacities

The diagnostic capacities available in this section are:

- Antibody ELISA for FMD, Brucellosis, Rabies, ND, IBD, CSF, Infectious bovine rhinotracheitis (IBR), Leptospirosis, Contagious Bovine Pleuropneumonia (CBPP), Contagious

Caprine Pleuropneumonia (CCPP), Porcine reproductive and respiratory syndrome (PRRS), Johne's Disease (JD), Avian leucosis complex (ALC) and Peste des petits ruminants (PPR);
- Antigen ELISA for CSF and PPR;
- Typing ELISA (sandwich) for FMD

3.6 Virology Section

Background

This section is equipped with basic rapid tests and also advanced diagnostic facilities such as Fluoresce Antibody test (FAT) etc

Human resources

The Virology section is manned by:

1. Mr. Purna Bahadur Rai, Sr. Laboratory Technician

Diagnostic capacities

The section has capacity for diagnosis of various disease as follows:

- Rapid antigen detection tests for Avian Influenza type A, H5, Newcastle disease (ND) virus, Infectious Bursal Disease (IBD), Foot and Mouth Disease (FMD) and Rabies.
- FAT for diagnosis of Rabies

3.7 Parasitology Section

Background

The section carries out basic parasitological tests for routine diagnostic services for parasitic disease and recommends control guidelines and advisory services to the government livestock farms, dzongkhags and private livestock agencies. It also provides other professional backstopping to RLDCs, SVLs and DVHs/DVLs. Besides the routine activities, the section regularly conducts research and surveillance pertaining to parasitic diseases in collaboration with government farms, RLDCs and the Dzongkhags. The section is also responsible to provide refresher/in-service courses for field staffs and trainings to the farmers with regard to parasitic diseases and control programs.

Human resources

The Parasitology section is currently manned by the following staff.

1. Ms. Ugyen Pema, Laboratory Technician
2. Ms. Tshewang Dema, Laboratory Technician

Diagnostic capacities

The following are the lists of diagnostic tests that are being conducted by the section:

- Identification of parasites through direct technique;
- Identification of parasites through qualitative tests (Sedimentation and Floatation methods);
- Identification of parasites through quantitative tests (Stoll method);
- Urine sedimentation test for nematodes;
- Skin scraping examination using 10% KOH digestion method;
- Blood parasite examination;
- Pepsin digestion test;
- Fecal culture (simple tube method, culture tube method, Baermann's method);
- Tick identification (stereo-zoom method);
- Post-mortem recovery of helminths, post mortem worm count;
- Microfilaria identification from blood (modified Knott's method);
- Worm staining & preservation;
- ELISA for Fasciola;
- Isolation and identification of Taeniid eggs from faeces and soil samples

3.8 Pathology Section

Background

The section has Post mortem and Histo-pathology section which provides necropsy and histopathological diagnosis.

Human resources

The section has the man power as follows:

1. Dr. NK Thapa, Animal Health Specialist – II
2. Ms. Pasang Bida, Laboratory Technician
3. Mr. Tenzinla, Sr. Laboratory Technician

Diagnostic capacities

The section is responsible for following diagnostic capacities:

- To conduct post-mortem examination and diagnosis in animals like poultry, ruminants, canine, feline, equine, swine species and wild animals including reptiles and fish;
- To perform histo-pathological examination and diagnosis through processing and examination of slides (H&E, Grams, ZN, pigment staining and pearls staining);
- To perform immuno-histochemistry
- To conduct post-mortem examination and diagnosis in the wild life

3.9 Biosafety and Biosecurity Section

Background

The section is mandated to implement and monitor bio-safety measures and good laboratory practices in all veterinary laboratories in the country. Thus, this section is an aide-de-section for all other sections.

Human resources

The human resource in this section is as follows:

1. Ms. Dechen Wangmo, Sr. Laboratory Officer

Main activities

The section is responsible for the following:

- Planning, Coordination and Implementation of Biosafety and Bio-security plans
- Technical monitoring of Biosafety and Biosecurity measures
- In house training on biosafety and Biosecurity
- Reporting and Monitoring
- Sample referral to collaborating laboratories
- Compilation of routine and research laboratory test kits, reagents, consumables procurement
- Monitoring on the functionalities of the equipment

4. ACHIEVEMENTS

4.2 Overall Achievements of LSU

During the fiscal year, 2020-2021, a total of 3552 numbers of various diagnostic samples were received or collected and 8843 laboratory tests were performed for routine tests, disease outbreaks, disease screening, surveillance and researches (Table 2).

Table 1: Summary of sample received and test performed during FY 2020-21

Section	Samples Received	Tests conducted	Remarks
Toxicology	75	332	
Bio-Chemistry	43	70	
Parasitology	928	1350	
Clinical path/Haematology	235	740	

Bacteriology/Mycology	572	3,557
Post Mortem	111	108
Histopathology	580	1096
Serology	819	1022
Virology	54	71
Molecular	210	497
Total	**3,552**	**8,843**

4.2 Section Specific Achievements

4.2.1 Bacteriology Section

About 572 different types of samples were received/collected and 3,557 different tests were conducted. The detail of the samples tested in the bacteriology section is as shown in Table 3.

Table 3: Sample and test performed in Bacteriology & Mycology section

Type of specimen	**Specimen Received**	**Types of tests**	**Tests conducted**
a. Bacteriology			
Organs	155	Culture	1052
Froth swab	7	Gram stain	234
Lung swab	13	Motility	222
Cloacal swab	123	BC test	1192
Vaginal swab	10	Sensitivity test	280
Ear swab	23	Leishman stain	3
Ocular swab	37	Methylene Blue Stain	6
Other swabs	26	Inoculation test	214
Milk	50	Isolate archival	242
Caeca	40		
Abscess	1		
Blood	2		

Urine	5		
PT samples	23		
b. **Mycology**			
Skin scraping	55	Culture	57
Feed	2	Lactophenol Cotton Blue Stain	**57**
Total	**572**		**3557**

Significant findings

Significant findings include *Moraxella* in yaks, *Salmonella* in swine, *Closrtidium perfringens* in avian and swine, *Escherichia fergusonii* in wild pig, ESBL producing bacteria (*E. coli*, *Serratia* and *Proteus*) from clinical samples, *Malassezia* and *Rhizopous* in canines.

4.2.2 Pathology Section

A total of 111 animal carcasses, 557 tissue samples and 23 biopsy samples were processed and 1204 types of tests were conducted in pathology section in Table 4.

Table 4: Samples and tests performed in the Pathology section

Type of specimen	**Number**	**Test type**	**Number**
Tissue, organs	557	Histopathology- H and E Staining	1073
Biopsy	23	Giemsa staining	18
Carcass	111	Methylene Blue	5
		Post-mortem/Necropsy	108
Total	**691**		**1204**

Significant findings

Significant findings include Histomoniasis, IBD, Avian Leukosis Complex, canine Distemper, Canine parvo viral infection etc.

4.2.3 Parasitology Section

A total of about 928 samples were received/processed and 1350 tests were performed by the section. The details of tests performed by this section are shown in Table 5.

Table 5: Sample and test performed in Parasitology section

Type of specimen	Number	Test type	Number
Faecal samples	297	Direct examination, Sedimentation, Stoll's dilution, Floatation	666
Dog environmental samples	75	Floatation/Sieving technique by using 1:1 sugar solution.	53
Soil Samples	419	Floatation/Sieving technique by using zinc sulphate (1:1)	419
Intestinal content	34	Direct smear	34
Skin scrapping	172	10% KOH digestion	172
Liver, caeca impression smear	3	Giemsa staining	3
Diaphragm muscle	2	Pepsin digestion for *Trichinella*	2
Cyst Sample	1	Direct examination	1
Total	**928**		**1350**

During the year, the section commonly detected parasitic infestations through the microscopic detection of eggs of *Fasciola, Paramphistomes, Strongyles* in bovine and *Taeniid* in stray dogs, *Ascarids* and Tape worm in poultry.

4.2.4 Haematology Section

Basic haematological tests were also conducted to support the clinical diagnosis in the animals. About 235 samples were processed and 740 different tests were conducted. Details of samples and tests conducted in these sections are presented in Table 6.

Table 6: Sample type and the tests conducted in Clinical pathology/Hematology section

Type of specimen	Number	Test type	Number

Blood smear	11	PCV	149
Whole blood	149	Hb	149
		DLC	160
		TRCC	77
		TWCC	77
		Knott's test	64
		Direct smear examination	64
Total	**160**	**Total**	**740**

4.2.5 Bio-chemistry & Toxicology Section

In the Toxicology section, 75 feed samples were screened against Aflatoxins. Serum biochemistry was performed in 40 samples. In addition, 3 urine samples were tested to assess the renal health of the animals. Details of samples and tests conducted in this section are presented in Table 7.

Table 7: Sample type and the tests conducted in Bio-Chemistry & Toxicology section

Type of specimen	Number	Test type	Number
Feed	75	Aflatoxin	332
Serum	40	Mineral bio-chemistry	104
Urine	3	Urine biochemistry	30
Total	**118**		**466**

Significant findings

About 21 feed samples were detected with Aflatoxin. The sera samples from the goats at Paro quarantine showed high levels of calcium and phosphorous. Urine analysis revealed the presence of protein and Nitrite indicating renal ailment in the animal.

4.2.6 Serology Section

The section received/collected 819 samples and carried out 1022 different types of tests as described below in table 8.

Table 8: Sample type and the tests conducted in Serology section

Type of specimen	Numbers Received	Type of tests conducted	Number of tests conducted	Remarks
Serum	819	PPR ELISA	61	
		SAT Mycoplasma	24	
		SAT Salmonella	24	
		Rapid test FMD NSP	26	
		FMD Serotype O ELISA	50	
		FMD Serotype A ELISA	11	
		FMD Serotype Asia I-ELISA	26	
		RBT Brucella	150	
		Brucella ELISA	91	
		Porcine Circovirus Type-2	2	
		IBR ELISA	89	
		BVD ELISA	89	
		RAPINA	379	
Total	**819**		**1022**	

Significant findings

Important findings in serology includes *Brucella* antibody from cattle in NJBC, Samtse, and CRC, Wangkha; CSF in pigs; CCHF in goats; IBD, *Mycoplasma* and *Salmonella* in poultry; and PPR in Takin. The section also performed proficiency testing for RBT for Brucellosis for four RLDCs.

4.2.7 Virology Section

The section received/collected 54 samples and carried out 71 different types of tests as described below in table 9.

Table 9: Sample type and the tests conducted in Virology section

Type of specimen	Numbers Received	Type of tests	Tests conducted
Brain	13	FAT	12
Swab	41	FMD NSP(Rapid)	47
		Rapid AI	24
		Rapid NDV	27
		Rapid IBD	4
		Canine parvovirus	1
		CDV	3
		CPV	1
Total	**54**		**71**

Significant findings
Important findings in virology section were Rabies, FMD and Canine Parvovirus.

4.2.8 Molecular Diagnostic Section

The section received/collected 210 samples and carried out 497 different types of tests as described below in Table 10.

Table 10: Sample type and the tests conducted in Molecular biology section

Type of specimen	Numbers Received	Type of PCR tests	Tests conducted
Organ/swabs/bone marrow	91	PRRS-Eu & NA	31
Ocular/nasal	46	CSFV	39
Tissue biopsy	6	ASF	58

Whole blood		MCCP	59
Tracheal/cloacal swab	11	PPMV	8
		Capripox	59
Epithelial/Secretions	26	FMD	44
Lung swab	6	AI	57
Cloacal swab	6	NDV	10
PT panel	18	PPR	59
		HS	63
		LSD	10
Sub Total	**210**		**497**

Significant findings
Important findings in molecular biology include *Capripox* in Takin, Goral, Goats, LSD in cattle and ASF in stray pigs and CSF in wild pigs.

4.2.9 Biosafety & Biosecurity Section

a. Laboratory Auditing at LSU and BPU

The section coordinated the internal auditing of the LSU and BPU by forming a team. The laboratory auditing was carried out during the month of June 2021 and the main objectives of the auditing were as follows:

- To determine overall compliance of biosafety measures practiced
- To conduct a follow-up on previous recommendations.
- To ensure any new regulatory requirements of the laboratory are followed.
- To monitor the system
- To verify compliance of the quality management system, including SOPs and internal laboratory biosafety policies

The team was formed from LSU and BPU and in order to avoid biasness, the auditing was carried out on each other's laboratory.

The main findings in the LSU included the following:

- Lack of colour coded bins (Currently the bins used are labelled only, it is preferrable to use colour coded bins).

- Spill kits not available in almost all the sections
- Non disposal of waste bins.
- Floor /work bench not kept clean.
- Temperature log chart not recorded by all the sections.

The main findings in the BPU included as follows:

- No Color-coded Bins.
- Spill Kits not available.
- Need to maintain SOPs for all equipment and also for ingredient preparation (Measurements).

The recommendations were provided for improvement in the future.

b. Biosafety & biosecurity monitoring at RLDC Wangdue

Dechen Wangmo & NK Thapa

One of the main mandates of the section is implementation of the biosafety measures in the laboratories in the country. The biosafety and biosecurity activities followed in the laboratories is also needs to be monitored.

During the month of June 2021, RLDC Wangdue was visited by the section. During inspection, it was observed that their laboratory section has strictly implemented all required and recommended biosafety measures**.** The staffs were also found to be trained and well aware of Good Laboratory Practices. However, there were few issues related to inadequate no. of staffs whereby out of three staffs one is already on medical leave which is hampering their daily biosafety monitoring.

Two lab personnel have to handle every sections wherein they have to test daily routine samples and simultaneously attend field emergency cases. Therefore, it is hampering their daily monitor and coordinating the biosafety protocols in their lab.

As per the observations, the following recommendations were made:

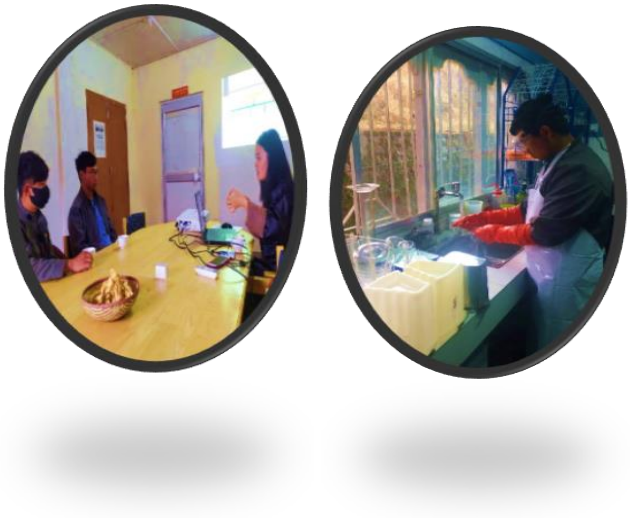
- RLDC, Wangdue needs to appoint a Biosafety coordinator/focal person in the centre so that this officer will work closely with the NVL biosafety team in regards to biosafety information sharing. NVL can assist the centre in implementing and strengthening the Biosafety practices in their centre.
- It was observed that the laboratory setup is not up to Biosafety standard protocol but still there can be some adjustment done dividing the sections based on risk factor involved.
- There is need for standard operating procedures for all new equipment installed. In addition, equipment log chart is to be maintained to ensure the proper functioning of equipment and also to keep record of the user.
- There is need for incident/accident report form, which should be maintained every day.
- Thermometer is needed for every fridge so that proper temperature for different test kits can be maintained as a result the quality of test kits will be maintained too.
- Sharp disposal bin should be placed in the lab.

- It is highly recommended to purchase the closed toe shoes and water proof shoes, instead of the open toe shoes or cloth type to prevent chemical spill on foot.

c. Fresher Training on Cleaning and Maintenance of Laboratory Glassware

Dechen Wangmo & NK Thapa

A one-day Fresher training on Cleaning Laboratory Glass wares and Safety Handling was conducted at National Veterinary Laboratory, Serbithang for the lab Attendant of Laboratory Services Unit and Biological Production Unit on 1st April, 2021. The main objectives of the training were to ensure proper cleaning of lab glassware in order to avoid interference during analysis which is also one of Good Laboratory Practices (GLP) and Quality Control in the laboratory and also to train on safe handling practices during cleaning and maintenance.



The training program was conducted through video clips on HOW TO WASH LAB GLASSWARES? and HOW TO OPERATE AUTOCLAVE? Followed by presentation on types of glass wares in Laboratory, usage of PPE, safe handling Glassware. And also, the participants were sensitized on the newly developed maintenance log sheet and SOP on Cleaning Glassware and Maintenance. Finally, the training Program ended with discussions related to issues faced by lab attendants and distribution of lab consumables required for cleaning the lab glass wares and safety gears.

d. Immunization of Laboratory personnel against Rabies

Dechen Wangmo & NK Thapa

Safeguarding the health of the laboratory workers against important zoonosis is one of the main mandates of the section. As the laboratory personnel gets exposure to Rabies virus due to frequent handling of Rabies samples from the animals, it is very essential to get immunized against the disease. Hence, immunization program was conducted for the staff at NCAH. The immunization of the staff was carried out by the team from Jigme Dorji Wangchuk National Referral Hospital

(JDWNRH) and the blood collection was also done by the Royal Centre for Disease Control (RCDC) before vaccination to check the immune status of the staff.
In addition to the laboratory staff, the staff from DPM, NCD and Jangsa Animal Trust were also immunized. There were total of 41 blood samples collected for RAPINA test, out of which 22 blood samples were positive to protective antibody titre.
The AR vaccine doses were administered according to recommendation in the manufacturing leaflet. The dose schedules recommended were as follows (0,7 & 21) which was as follows; 1st dose was administered on 11th May followed by 2nd dose on 18th May and 3rd dose on 1st June,2021. The serum will again be collected after 60 days of the first dose to assess the antibody level.

5. ESTABLISHMENT AND STRENGTHENING OF LABORATORY DIAGNOSTIC CAPACITY

5.25.1 Introduction of new tests

During the financial year 2020-21, the following new diagnostic technologies for important diseases were established:

a) Establishment of Cell culture facilities- installation of the CO_2 incubator and trial conducted.

b) Bacterial diagnostics techniques

- Isolation and identification of *Streptococcus equi*, *Campylobacter spp*, *Actinomyces spp*, *Moraxella spp*, *Escherichia fergusonii*, *Serratia rubideae*, *Proteus mirabilis*, *Clostridum perfringens* and *Enterococci*
- Antimicrobial sensitivity testing for *S. aureus*, *Escherichia fergusonii*, *Serratia rubideae*, *Proteus mirabilis*, *Campylobacter*, *Salmonella*, *Clostridum perfringens*
- Detection of extended spectrum beta lactamases producing bacteria- *E. coli*, *Serratia rubideae* and *Proteu mirabilis*

c) Mycology diagnostic techniques-Isolation and identification of *Malassezia spp*

d) Molecular diagnostics techniques-Real time PCR- For *Capripox*, *Pasteurella multocida*, *Mycoplasma*, PPR, LSD

e) Serological diagnostic techniques-*Porcine circovirus 2*

5.3 Laboratory Quality Assurance

5.2.1 Asia Pacific Regional Proficiency Testing

a. Molecular diagnostics

The avian diseases PCR panel for 2020 proficiency testing consisted of 15 and 18 gamma irradiated samples that were sent to each participating laboratory with instructions to test the samples using their standard diagnostic real-time PCR for Avian disease PT panel (Influenza A, H-type PCR and Avian paramyxovirus-1) and the swine disease PT panel (CSF, PRRS, ASF and SIV). National Veterinary Laboratory reported results for Influenza A matrix, H5, N1. The table and figure below reveals the results obtained by NVL. D1 is the code for Bhutan

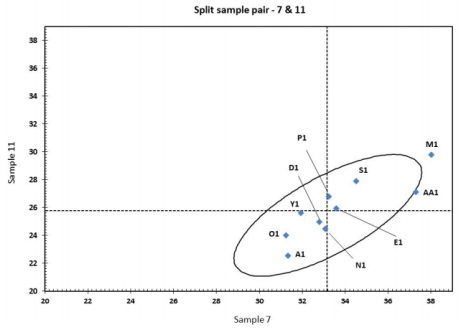
Table1: Comparison based on the mean of the reported Ct value AIV, matrix assay, real time PCR results.

No.	Agent	Type	A1	D1	E1	L1	M1	N1	O1	P1	S1	Y1	AA1	Median
1	A/Chicken/Philippines/0938-1/2017	H5N6	29.2	27.7	32	33.8	32.2	28.9	28.1	28.2	33	30	30	30
2	A/Chicken/Nepal/S-105-TS/2017	H5N8	31.4	33.8	34.6	36.2	38.6	33.8	30.8	34.7	34.4	35.9	31.6	34.4
3	P/Chicken/Hoa Binh/A508/2014	APMV-1	45	Und.	no Ct	0	0	0	45	45	Und.	36.6	Und.	-
4	A/Duck/Japan/AQ-HE29-52/2017	H7N9	32.5	35	34.8	0	41.7	38.4	31.7	35.2	35.6	39.5	32.2	35.1
5	A/Chicken/Nepal/LAL-407-CL/2019	H5N1	32.1	Und.	34.5	0	41.2	36.7	32.8	36.4	34.6	36	38.3	36
6	A/Chicken/Nepal/SUR-515-CB/2019	H9N2	29.6	34.6	32.7	38	37	34.3	31.1	35.1	33.5	-	32.4	33.9
7	A/Quail/Myanmar/SP232/2015	H5N1	31.3	32.8	33.6	0	38	33.1	31.2	33.2	34.5	31.9	37.3	33.2
8	P/Chicken/Philippines/16-0055-I/2016	APMV-1	45	Und.	no Ct	0	0	0	45	35.9	Und.	-	Und.	-
9	A/Duck/Japan/AQ-HE29-52/2017	H7N9	32.3	Und.	34.7	0	0	0	32.1	35.7	36.1	36.9	34	34.7
10	A/Chicken/Nepal/K-112-TS/2017	H9N2	31.7	35.8	no Ct	39	41.6	37.3	31.3	34.7	35.6	36.8	33.3	35.7
11	A/Quail/Myanmar/SP232/2015	H5N1	22.5	25	25.9	30.8	29.8	24.5	24	26.8	27.9	25.6	27.1	25.9
12	Negative		45	Und	no Ct	0	0	0	45	45	Und.	36	Und.	-
13	A/Chicken/Myanmar/1528/2017	H5N1	28.4	36	32.1	40.4	37.6	32.8	29.9	34.1	30.6	32.9	32.6	-*
14	P/Chicken/Philippines/16-0055-I/2016	APMV-1	45	Und	no Ct	0	0	0	45	36.6	Und.	-	Und.	-
15	A/Environment/Myanmar/SP443/2017	H9N2	32	36	34	37.5	41.3	35.7	30.5	35.7	37.3	37.8	32.6	35.7

Paired sample analysis

Table 2: AIV matrix assay- spilt sample pair 7 and 11, A/Quail/Myanmar/SP232/2015 H5N1, clade 2.3.4.2

Laboratory	Results		Between-Laboratory	Within-Laboratory
	Sample 7	Sample 11	Z-Score	Z-Score
A1	31.3	22.5	-1.44	0.76
D1	32.8	25.0	-0.26	0.07
E1	33.6	25.9	0.26	-0.07
M1	38.0	29.8	2.77	0.35
N1	33.1	24.5	-0.33	0.64
O1	31.2	24.0	-1.02	-0.39
P1	33.2	26.8	0.42	-0.94
S1	34.5	27.9	1.15	-0.84
Y1	31.9	25.6	-0.32	-1.05
AA1	37.3	27.1	1.75	1.80



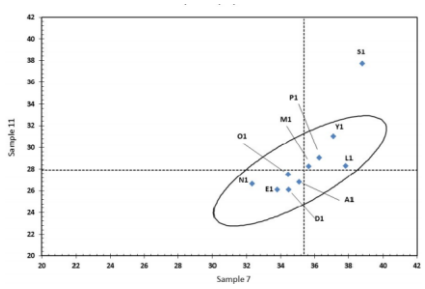
The between-laboratories and within-laboratories Z-scores are for the related pair, sample 7 and 11. A Z-score between 0 and =2 is acceptable. A Z-score between 2 and 3, or -2 and -3 is questionable

AIV matrix real time PCR assay- Youden plot for split sample pair 7 and 11

Table 3: AIV H5 real time PCR-Within and between laboratory Z-score analyses for split sample pair 7 and 11

Laboratory	Results		Between-Laboratory	Within-Laboratory
	Sample 7	Sample 11	Z-Score	Z-Score
A1	35.1	26.8	-0.27	0.68
D1	34.5	26.1	-0.61	0.76
E1	33.8	26.1	-0.78	0.26
L1	37.8	28.3	0.82	1.57
M1	35.7	28.3	0.25	0.06
N1	32.3	26.7	-1.03	-1.16
O1	34.5	27.5	-0.25	-0.28
P1	36.3	29.1	0.61	-0.06
S1	38.8	37.8	3.49 §	-4.43 §
Y1	37.1	31.0	1.34	-0.87

The between-laboratories and within-laboratories Z-scores are for the related pair, sample 7 and 11. A Z-score between 0 and =2 is acceptable. A Z-score between 2 and 3, or -2 and -3 is questionable



AIV H5 real time PCR assay Youden plot for split sample pair 7 and 11

5.2.2 EQASIA external quality assurance in Asia

a. Bacteriology Diagnostics

The aim of the EQAsia project is to improve the Quality of Bacteriology Diagnostics for Antimicrobial susceptibility testing (AMR) in the Asian region. The EQAsia project is supported by the Fleming Fund (UK Aid Programme).

The microbiology laboratory at national veterinary laboratory received proficiency test panels consisting of 22 unknow samples (11 PT for *E. coli* and 11 PT for *Salmonella*). The samples were cultured and identified against *E. coli*, *Salmonella* and antimicrobial susceptibility tests. The results obtained by NVL is referred as "Obtained Test Strain ID".

Table 1: Showing results of PT for *E. coli* obtained test strain verses expected test strain

Strain	Obtained Test strain ID	Expected Test Strain ID
E EQASIA 21.1	*E. coli*	*E. coli*
E EQASIA 21.2	*Non E. coli*	*Non E. coli*
E EQASIA 21.3	*E. coli*	*E. coli*
E EQASIA 21.4	*E. coli*	*E. coli*
E EQASIA 21.5	*E. coli*	*E. coli*
E EQASIA 21.6	*Non E. coli*	*Non E. coli*
E EQASIA 21.7	*E. coli*	*E. coli*
E EQASIA 21.8	*E. coli*	*E. coli*

E EQASIA 21.9	*E. coli*	*E. coli*
E EQASIA 21.10	*Non E. coli*	*Non E. coli*
E EQASIA 21.11	*E. coli*	*E. coli*

All the eight *E. coli* isolated were tested for antimicrobial susceptibility tests against six antibiotics via disk diffusion method. The antibiotics used were Ampicillin, Cefepime, Ceftazidime, Nalidixic acid, Gentamicin and Tetracycline. All five antibiotics test results were interpreted correctly for eight *E. coli* except for two *E. coli* where Ceftazidime were interpreted as intermediate but expected results was resistance. Out of five ESBLs only one was detected. ESBL production by resistance strains were also tested and one out of five were correctly identified as ESBLs producing *E. coli*.

Table 2: Showing results of PT for *Salmonella* obtained test strain verses expected test strain

Strain	Obtained Test strain ID	Expected Test Strain ID
S EQASIA 21.1	*Salmonella*	*Salmonella*
S EQASIA 21.2	*Salmonella*	*Salmonella*
S EQASIA 21.3	*Salmonella*	*Non-Salmonella*
S EQASIA 21.4	*Salmonella*	*Salmonella*
S EQASIA 21.5	*Salmonella*	*Salmonella*
S EQASIA 21.6	*Salmonella*	*Salmonella*
S EQASIA 21.7	*Non-Salmonella*	*Non-Salmonella*
S EQASIA 21.8	*Salmonella*	*Salmonella*
S EQASIA 21.9	*Non-Salmonella*	*Non-Salmonella*
S EQASIA 21.10	*Salmonella*	*Salmonella*
S EQASIA 21.11	*Salmonella*	*Salmonella*

All the eight *Salmonella* were identified correctly except one which was incorrectly identified as *Salmonella* spp. All *Salmonella* spp isolated were tested for antimicrobial susceptibility tests against six antibiotics via disk diffusion method. The antibiotics used were Ampicillin, Cefepime, Ceftazidime, Nalidixic acid, Gentamicin and Tetracycline. All six antibiotics test results were interpreted correctly for eight *Salmonella*. ESBL production by resistance strains were also tested and two out of two were correctly identified as ESBLs producing *Salmonella*. Out of five ESBLs only one was detected.

5.2.3 Proficiency testing on Rose Bengal test for screening of Brucellosis in animals

Dr. RB Gurung, Dr. NK Thapa & Kelzang Lhamo

Conducted by National Veterinary Laboratory, National Centre for Animal Health, Serbithang as a part of National External Quality Assurance System (NEQAS) for regional laboratories (2020-21)

Introduction

Proficiency testing (PT) is a part of laboratory quality assurance system (QAS) to ensure a test procedure consistently produces quality result. Proficiency testing along with various other components of QAS such as record keeping, quality control, training, evaluation, calibration, monitoring, taking corrective actions and competency assessment will contribute to quality management of laboratory performance. Staff performing test must be qualified, their competency documented, trained in the areas of specific requirement, should be able to perform intended test and evaluate result. Proficiency testing samples are sent to participating laboratories for a specific testing method and results are reported to the coordinating laboratory for analysis. The coordinating laboratory then collates the results and ranks participating laboratories based on their testing performance. Details of performance of participating laboratories shall be anonymous. This anonymity allows participating laboratories to identify their laboratory and compare performance with other laboratories. The coordinating laboratory shall individually convey performance of each participating laboratory with details of their strength, weakness and recommendation.

The disease: Brucellosis

Brucellosis is an infectious zoonotic disease caused by bacteria of the genus Brucella. It is a zoonosis found throughout the world with major implications both in the field of public health and farming economy. Usually, Brucellosis in cattle is caused by *Brucella abortus*, *Brucella suis* in swine and *Brucella melitensis* in sheep and goats. Brucellosis cause abortion or birth of weak calves and infertility. Brucellosis is commonly transmitted to susceptible animals by direct contact with infected animals or with an environment contaminated by discharges from infected animals. As the disease primarily localizes in the udder and/or reproductive organs of animal, the milk,

aborted foetuses, placental membranes, fluids and other reproductive tract discharges of an infected animal are highly contaminated with infectious Brucella organisms. The disease may also spread when wild animals or animals from an affected herd are introduced into Brucellosis-free herds. The general rule is that Brucellosis is carried from one herd to another by an infected or exposed animal.

Brucellosis poses serious public health risk when humans are infected. Human infection with Brucella organisms usually occurs through occupational contact with discharges from infected animals, particularly through calving, but also through slaughtering processes or ingestion of unpasteurised dairy products. In humans the main signs of infection are undulant fever with serious and frequent complications during chronic evolution.

The test: Rose Bengal Test (RBT)

The Rose Bengal Test (RBT) is a rapid slide-type agglutination assay performed with a stained *B. abortus* suspension at pH 3.6–3.7 and plain serum. Serum (25-30 µl) is mixed with an equal volume of antigen on a white tile or enamel plate to produce a zone approximately 2 cm in diameter. The mixture is rocked gently for 4 minutes at ambient temperature (20°C ± 3°C) and then observed for agglutination (1). Any visible reaction of agglutination is considered to be positive. The test is sensitive, especially in vaccinated or infected animals and positive samples should be retested by a confirmatory test such as the complement fixation test (CFT) or enzyme-linked immunosorbent assay (ELISA). False-negative reactions may occur and can be detected by retesting animals at intervals over a period of at least 3 months.

Importance of proficiency testing

From the data collected during proficiency testing, laboratory managers can identify staff that may require further training. This, therefore leads to more consistent working practices throughout the laboratory. Regular testing also keeps the team focused on how routine procedures should be carried out. Proficiency testing can provide an opportunity to further educate staff in the potential areas of testing. Training allows staff to appreciate their contribution to the output quality of their laboratory. Staff can then also appreciate their role in the success of their laboratory. Anyone who is trained and is regularly involved in routine testing needs to be included in proficiency testing.

Brucella RBT-PT for fiscal year 2020-21

During the fiscal year 2020-21, as a part of national external quality assurance (NEQAS) in laboratory test performance, the National Veterinary Laboratory, National Centre for Animal Health, Serbithang organized a round of proficiency testing with regional animal health laboratories at Regional Livestock Development Centres on Rose Bengal Test for screening Brucellosis in cattle. The main objective of this PT was to assess the performance of different

regional laboratories in screening of Brucellosis in cattle using RBT. Details of coordinating and participating laboratories in RBT-PT are as follows:

1. Coordinating laboratory
    a. National Veterinary Laboratory, National Centre for Animal Health, Serbithang
2. Participating laboratories
    a. Regional Livestock Development Centres, Kanglung
    b. Regional Livestock Development Centres, Tsimasham
    c. Regional Livestock Development Centres, Wangdue
    d. Regional Livestock Development Centres, Zhemgang

Proficiency testing panel

Twenty serum samples collected and archived at National Veterinary Laboratory, National Centre for Animal Health, Serbithang were identified. These samples were tested with RBT and ELISA to determine the level of agglutination reactivity in RBT and optical density (OD) values in ELISA, respectively. Accordingly, samples were classified as negative or positive. Depending on the level of reactions among positive samples (n = 6), they were further classified as + (weak positive), ++ (positive) and +++ (strong positive). Panel also included a set of negative samples (n = 14), one positive control (PC) and one negative control (NC). Antigen (Rose Bengal-stained *B. abortus* suspension) was also supplied along with panel of serum samples.

Test procedure

Following steps are the guide to perform RBT at each participating laboratory.

- Bring antigen and test or panel sera to room temperature (20°C ± 3°C)
- Pipette 25 µl of panel serum and place on agglutination plate (white smooth tile) leaving about 4 cm distance between each serum sample
- Similarly, pipette 25 µl of positive and negative control serum and place on the agglutination tile
- Pipette 25 µl of RBT antigen and place next to each serum sample and controls. The antigen and serum should not be mixed while placing on the tile
- Once all antigen and serum samples are placed on tile, start mixing with clean tooth pick in a circular fashion to develop a uniform border line of the mixture.
- Match stick also can be used for this purpose. When match sticks are used, use only its tail end.
- Ensure the mixture of antigen and sample does not mix with other set of sample and antigen
- Set timer as soon as mixing is started

- Ten samples can be tested at one go to minimise delay in time between the addition of antigen to the first and last serum
- Hold the plate and oscillate gently for about 4 min; 20-25 oscillation is good to mix the antigen and serum properly
- Read the results under bright light depending on the level of reaction (agglutination or no agglutination) (Table 1)
- Read the results within 10 min after mixing of antigen and serum
- Read the results of control sera first, then the panel sera
- Record result in the provided form (Appendix 1)
- Negative and positive control serum should be used for each batch of panel serum tested

Table 1: Distinction of degrees of reaction

Reactivity	Description	Interpretation
0	No agglutination, no flakes (Negative)	N
+	Barely perceptible agglutination. May be doubtful (Weak positive)	D or WP
++	Fine agglutination, definite flakes and some clearing (Positive)	P
+++	Coarse clumping, definite clearing (Strong positive)	SP

Note: 0, N (Negative); +, D/WP (Doubtful/Weak positive); ++, P (Positive); +++, SP (Strong positive)

Analysis

Collation of RBT results

The participating laboratories were coded as laboratory code 1, 2, 3 and 4 to maintain the anonymity of test results among all participating laboratories. The test results of all participating laboratories were collated and compared with the results of coordinating laboratory.

The results were collated as reported by participating laboratories. (Table 2)

Table 2: Collated RBT result of coordinating and participating laboratories

Sample	NVL, NCAH		LAB 01		LAB 02		LAB 03		LAB 04	
	Result	Intpn	Result	Intpn	Result	Intpn	Result	Intpn	Result	Intpn
1	+++	P	+++	SP	0	N	+	P	+	P
2	0	N	0	N	0	N	0	N	0	N
3	0	N	0	N	0	N	0	N	0	N
4	+++	P	+++	SP	0	N	++	P	++	P
5	0	N	0	N	++	P	0	N	0	N
6	0	N	0	N	+	D	0	N	0	N
7	0	N	0	N	0	N	0	N	0	N
8	0	N	0	N	++	P	0	N	0	N
9	++	P	+	P	0	N	0	N	+	P
10	++	P	++	P	0	N	++	P	+	P
11	0	N	+	P	0	N	0	N	0	N
12	0	N	0	N	++	P	++	P	0	N
13	0	N	+	P	0	N	0	N	0	N
14	0	N	0	N	+	D	0	N	0	N
15	+++	P	++	P	0	N	+	P	++	P
16	0	N	+	P	+	D	0	N	0	N
17	0	N	0	N	++	P	0	N	0	N
18	0	N	0	N	+	D	0	N	0	N
19	++	P	++	P	+	D	0	N	+	P
20	0	N	0	N	+	D	0	N	0	N

Analysis of test result

The sensitivity and specificity of tests have been calculated as below:
Sensitivity=TP/(TP+FN)
Specificity=TN/(TN+FP)

- TP= True positive
- TN=True Negative
- FP= False positive
- FN= False negative

Analysis of test results is shown in Table 3.

Diagnostic sensitivity

Two laboratories (Lab 1 and 4) correctly identified all true positive samples as positive. Therefore, their diagnostic sensitivity is 1.00. Laboratory 2 correctly identified only one true positive as positive, thus had diagnostic sensitivity of 0.20. Laboratory 3 correctly identified 4 true positives as positive, thus had diagnostic sensitivity of 0.70.

Diagnostic specificity

Only one laboratory (Lab 4) correctly identified all true negatives as negative, thus had diagnostic specificity of 1.00. One laboratory (Lab 1) correctly identified 11 true negatives as negative, thus had diagnostic specificity of 0.80. Lab 2 and 3 correctly identified 5 and 13 true negatives as negatives, thus had diagnostic specificity of 0.40 and 0.90, respectively.

Only one laboratory (Lab 4) correctly identified all true positives and true negatives as positive and negative achieving diagnostic estimates of 1.00 for both sensitivity and specificity.

An ideal test is the one with diagnostic estimates of 1.0 (sensitivity and specificity). Unfortunately, there is no commercial test available with diagnostic estimate as 1.0. The diagnostic estimates reported here for all participating laboratories and coordinating laboratory are only relative estimates. Owing to the small number of samples the PT results here showed the diagnostic estimates of 1.0. Under the situation of testing large number of samples the diagnostic estimates shall drop below 1.0. However, these estimates are useful in recognizing the strength and weakness in the testing capacity of each laboratory and provide directions for improvement.

Laboratory 1

a. Did not have issue in identifying true positive samples as positive irrespective of samples having different intensity of agglutination reactions
b. Had some difficulty in identifying true negative sample as negative
c. There is a need to improve on reducing the rate of false positive test result

Laboratory 2

a. The lab had sensitivity of 0.20 and specificity of 0.40
b. Had difficulty in identifying true positive samples as positive (only one sample detected as positive)
c. Had issues in identifying true negative samples as negative
d. Detected very high false positive.
e. There is a need to improve on RBT process and interpretation

Laboratory 3

a. Had some difficulty in detecting true positive and true negative samples. However, with this can be improved through guidance and practice

Laboratory 4

a. The laboratory produced excellent results by correctly identifying all true positives and true negatives as positive and negative.
b. The lab needs to continue with such quality of tests.

Overall analysis found that most of the laboratories still had some difficulties in determining the intensity of reactions in positive samples. This is the skill that will be acquired only through regular practice. All laboratories need to improve in this area over the period of time.

Table 3: Calculation of diagnostic estimates (sensitivity and specificity)

Sl no	Parameters	LAB 01	LAB 02	LAB 03	LAB 04
1	True positive	6	1	4	6
2	True negative	11	5	13	14
3	False positive	3	9	1	0
4	False negative	0	5	2	0
5	Sensitivity	1.00	0.20	0.70	1.00
6	Specificity	0.80	0.40	0.90	1.00

Note:

1. Diagnostic sensitivity: Probability of a positive test given that the animal has the disease
2. Diagnostic specificity: Probability of a negative test given that the animal is well

Conclusion

The result analysis was performed based on the ability of participating laboratory to correctly identify true positives as positive and true negatives as negative. Owing to the small size of PT panel, analysis on the ability of laboratories to correctly identify different intensities of reaction among positive samples were not performed. Although RBT is a sensitive test, it is also a highly subjective test in terms of result interpretation. Performing and interpreting RBT requires high level of experience. Therefore, performing this test and accurately interpreting result can be gained only regular practice. This applies to all the participating laboratories and work towards improving their performance. Through this PT it appeared that at least in one laboratory RBT was performed who did not have experience. It is recommended that future PT on RBT should be performed by laboratory staff who has prior experience.

Reference

1. Morgan W.J.B., MacKinnon D.J., Lawson J.R. & Cullen G.A. (1969). The Rose Bengal plate agglutination test in the diagnosis of brucellosis. Vet. Rec., 85, 636– 641.

Appendix 1: Result recording sheet

TEST RESULT REPORTING FORMAT FOR PT ON BRUCELLA RBT

	Lab name	Your laboratory name	
	Lab code	Mentioned in the cryo box of PT panel	
		RBT	
		Test performed on:	Test performed date
		Antigen supplier:	NCAH, Serbithang
	Tube ID:	Result	Interpretation (N/D/P/SP)
PC		+	P
NC		0	N
1			
2			

3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
1: Enter the raw results (0, +, ++, +++) depending on the intensity of the reaction			
2: N = Negative; Positive (P/WP) for + or doubtful; Positive (P) for ++ and Strong positive (SP) for +++			

FOR THIS PROFICIENCY TESTING, REPORT ONLY NEGATIVE (N), POSITIVE (P) AND STRONG POSITIVE (SP). IF POSSIBLE "D" FOR DOUBTFUL	
Comments:	
Name and designation of the official performing test	Signature

5.2.4 Proficiency testing on parasitological techniques conducted by RLDC Wangdue, collaborated by NVL

Dr. NK Thapa, Dr. RB Gurung, Ugyen Pema, Tenzinla, Sangay Tenzin

Conducted by RLDC Wangdue in collaboration with National Veterinary Laboratory, National Centre for Animal Health, Serbithang as a part of National External Quality Assurance System for District Veterinary Laboratories (2020-21)

Introduction

Proficiency testing (PT) is a part of laboratory quality assurance system (QAS) to ensure a test procedure consistently produces quality result. PT along with various other components of QAS such as record keeping, quality control, training, evaluation, calibration, monitoring, taking corrective actions and competency assessment will contribute to quality management of a laboratory. Staff performing test must be qualified, their competency documented, trained in the areas of specific requirement, should be able to perform intended test and evaluate result. PT samples are sent to participating laboratories for a specific testing method and results are reported to the coordinating laboratory for analysis. The coordinating laboratory then collates the results and ranks participating laboratories based on their testing performance. Details of performance of participating laboratories shall be anonymous. This anonymity allows participating laboratories to see trends in their own testing performance and to compare with other laboratories. The coordinating laboratory shall individually convey performance of each participating laboratory with details of their strength, weakness and recommendation.

Importance of proficiency testing

From the PT results, laboratory managers can assess the competency and identify staff that may require further training. This therefore leads to more consistent working practices throughout the laboratories. Regular testing also keeps the team focused on how routine procedures should be carried out. PT can provide an opportunity to further educate staff in the potential areas of testing. Training allows staff to appreciate their contribution to the output quality of their laboratory. Staff

can then also appreciate their role in the success of their laboratory. Anyone who is trained and is regularly involved in routine testing needs to be included in PT.

Sedimentation and Floatation technique

Majority of trematode eggs are too large and heavy to float in the flotation fluids normally used for nematode eggs. However, they sink to the bottom of a faecal/water suspension which is the basis of the faecal sedimentation technique. Important trematodes include *Fasciola, Dicorcoelium, Amphistomes* and *Paramphistomes*.

The simple test tube flotation technique is a qualitative test for the detection of nematode and cestode eggs. Common floatation fluids include salt/sugar solutions. The method is useful for preliminary surveys to establish which parasite groups are present in the samples.

PT on basic parasitological techniques for the Four District Laboratories under RLDC Wangdue in collaboration with NCAH (2020-21)

During the fiscal year 2020-21, as a part of national external quality assurance (NEQAS) in laboratory test performance, Regional Livestock Development Centre (RLDC), Wangdue in collaboration with the National Veterinary Laboratory (NVL), National Centre for Animal Health (NCAH), Serbithang organized a round of PT with the district veterinary laboratories (DVLs) under the jurisdiction of RLDC, Wangdue on basic parasitic identification techniques in the cattle coprological samples. The main objective of this PT was to assess the performance of different DVLs in identification of various helminth eggs in cattle. Details of coordinating and participating laboratories in PT are as follows:

3. Conducting Laboratory
    a. Regional Livestock Development Centre, Wangdue
4. Collaborating laboratory
    a. National Veterinary Laboratory, National Centre for Animal Health, Serbithang
5. Participating laboratories
    a. Dzongkhag Veterinary Laboratory, Dagana
    b. Dzongkhag Veterinary Laboratory, Tsirang
    c. Dzongkhag Veterinary Laboratory, Gasa
    d. Dzongkhag Veterinary Laboratory, Wangdue

Proficiency testing panel

The coprological samples were collected from the cattle nearby Punakha, Wangdue and examined at NCAH. The panels were prepared based on the findings by the technician of RLDC Wangdue and the NCAH. Based on the findings, different panels were prepared positive control (n=8), (*Dicrocoelium*-2nos, *Fasciola hepatica*-4nos. *Paramphistomes*-1no, *Strongyles*-1no.) and 2 nos. negative controls (Table 1).

Test procedure

All the laboratories carried out the test procedure was as per the SOP on Parasitology developed and distributed by the NCAH.

Table 1: Details of the panels identified at NCAH

Sample I/D	Sample PT result	Interpretation
1	200 EPG *Dicrocoelium*	P
2	200 EPG *Strongyle*	P
3	300 EPG *Dicrocoelium*	P
4	100 EPG *Paramphistome*	P
5	100 EPG *F. hepatica*	P
6	500 EPG *F. hepatica*	P
7	300 EPG *F. hepatica*	P
8	400 EPG *F. hepatica*	P
9	Negative	N
10	Negative	N

Analysis

The results were interpreted as positive (P) irrespective of the EPGs. If different parasites were detected than the control, they were rated as false positive (FP). However, if any other helminths were detected in addition to the required helminths in the PT sample, the result were considered positive.

Collation of PT results

The participating laboratories were coded as laboratory code P, Q, R & S to maintain the anonymity of test results among all participating laboratories. The test results of all participating laboratories were collated and compared with the results of conducting laboratory (Table 2).

Analysis of test result

The sensitivity and specificity of tests have been calculated as below:

Sensitivity=TP/TP+FN

Specificity=TN/TN+FP

TP= True positive

TN=True Negative

FP= False positive

FN= False negative

Diagnostic sensitivity:

Positive samples: All the four labs did not detect all the 8 true positives (Lab P- 2, Lab Q-4, Lab R-3, Lab S-3), Hence, the diagnostic sensitivity was estimated as 0.3, 0.5, 0.4 and 0.4 respectively.

Diagnostic specificity:

All the four laboratories could detect all the 2 true negative samples as negative thus resulting into estimated diagnostic specificity of 1.0.

An ideal test is the one with diagnostic estimates of 1.0 (sensitivity and specificity). Unfortunately, there is no diagnostic test available with diagnostic estimate as 1.0. The diagnostic estimates reported here for all participating laboratories and coordinating laboratory are only relative estimates. However, these estimates are useful in recognizing the strength and weakness in the testing capacity of each laboratory and provide directions for improvement.

Laboratory P

d. Did have issue in identifying true positive samples as positive
e. Did not have difficulty in identifying true negative sample as negative however, had detected very high false negative (perhaps confused with the helminth eggs)
f. There is a need to improve on reducing the rate of false positive test result

Laboratory Q

f. The lab could detect at least 4, true positives as positive out of 8
g. Had no issue in identifying true negative samples as negative however, had high false negatives
b. There is a need to improve on both detecting the true positive

Laboratory R & S

a. The lab could detect only 3, true positives as positive out of 8
b. Had no issue in identifying true negative samples as negative however, had high false negatives
c. There is a need to improve on both detecting the true positive.

Conclusion

Since, such PT for parasitology is being conducted for the first time in the country, there is a need for improvement in preparation of PT samples and also the assessment of the results of the participating laboratories. In subsequent PTs, the laboratories conducting PT will improvise the methods as per the need.

The result analysis was performed based on the ability of participating laboratory to correctly identify true positives as positive and true negatives as negative. It is based on the experience and expertise of the technicians in identifying the different helminth eggs. Technicians of each participating laboratories should compare their results with the PT result of the conducting laboratory and also with other laboratories and try to improve their skills.

Table 2 The results were collated as reported by participating laboratories

Sample I/D	PT sample result	Interp	Lab P		Lab Q		Lab R		Lab S	
			Results	**interp**	**Results**	**inter**	**Results**	**interp**	**Results**	**interp**
1	200 EPG Dicrocoelium	P	Negative	N	Negative	N	Fasciola sp	N	100 EPG Trichuris sp	N
2	200 EPG Strongyle	P	200 EPG Strongyle	P	Negative	N	Negative	N	Negative	N
3	300 EPG Dicrocoelium	P	200 EPG Strongyle	N	F.hepatica	N	Negative	N	300 EPG B.coli	N
4	100 EPG Paramphistome	P	100 EPG Strongyle	N	F.hepatica	N	Fasciola sp	N	500 EPG Fasciola sp	N
5	100 EPG F. hepatica	P	100 EPG Fasciola	P	F.hepatica	P	Strongyle sp	N	200 EPG Fasciola,100 EPG B.coli	P
6	500 EPG F. hepatica	P	500 EPG Amphistome	N	F.hepatica	P	Fasciola sp	P	Negative	N
7	300 EPG F. hepatica	P	300 EPG Amphistome	N	F.hepatica	P	Fasciola sp	P	300 EPG Fasciola,100 EPG Strongyloides	P
8	400 EPG F.hepatica	P	200 EPG Amphistome,400 EPG,Strongyle	N	F.hepatica	P	Fasciola sp	P	300 EPG Fasciola sp	P
9	Negative	N	Negative	N	Negative	N	Negative	N	Negative	N
10	Negative	N	Negative	N	Negative	N	Negative	N	Negative	N
			FP	0		0		0		0
			TP	2		4		3		3
			TN	2		2		2		2
			FN	6		4		5		5
		Sensitivity	**TP/TP+FN**	0.3		0.5		0.4		0.4
		Specificity	**TN/TN+FP**	1		1		1		1

5.3 Upgrading diagnostic capacity at RLDC, Wangdue

Kelzang Lhamo & Purna Bahadur Rai

As a part of technical backstopping, assistance was provided to RLDC Wangdue for installation of newly procured equipment for serological diagnosis. Team comprising of the following visited the lab on 29th June 2021 to assist in installation of the equipment as per the request from RLDC, Wangdue:

1. Mr. Purna Bdr Rai, Sr. LT
2. Ms. Kelzang Lhamo, LT

Serological equipment

RLDC, Wangdue purchased following equipment in their Centre as follows:

1. Wellwash Microplate Washer (Thermo fisher Scientific)
2. Multiskan FC Microplate Photometer (Thermoscientific)

a. Microplate washer

1. Read the Manual
2. Assembled Microplate washer with power cable, wash buffer jar and aspirate jar.
3. Fixed the wash head
4. Operated the System/Software/steps
    - Wash
    - Aspirate
    - Dispense
    - Soak
    - Layout
5. Trial Run was carried out with distil water
6. It is recommended to shut down the Wellwash at the end of each working day

Figure 1. Microplate Washer

b. Microplate Reader

1. Read the Manual
2. Assembled Microplate reader with power cable and USB cable.
3. Unpacked the filter wheel and checked whether it is clean and undamaged.
4. Fixed the filter into filter slot.
5. Performed the operational check.
6. Demonstrated on how to set plate layout, filter measurement and to read the OD.
7. Demonstrated on how to transfer OD values from ELISA reader to PC.
8. Data Analysis

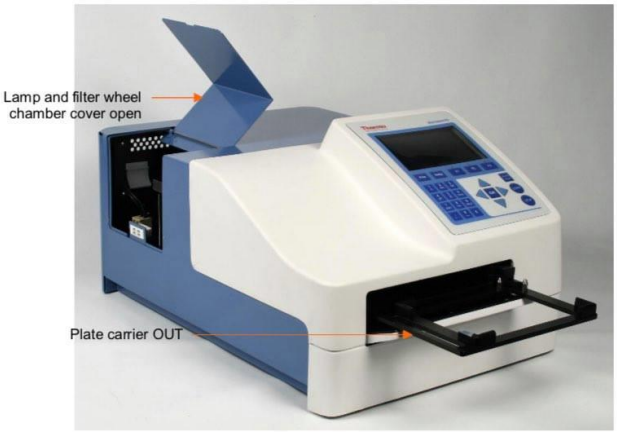


Figure 2. Multiskan FC microplate Reader

Recommendations

Finally, the team advised the staff at RLDC, Wangdue to seek further assistant if any issues are encountered in the future during their machine operation. Also, can refer troubleshooting column in the manual for any servicing or maintenance. The team instructed to print the equipment manual for future uses which were inside pen drive.

Conclusion

With the installation of the Microplate washer and the Microplate photometer, the diagnostic facility and the capacity of the RLDC is being upgraded. The lab can carry out the ELISA tests for important livestock diseases for the region.

5.4 Laboratory test standardization at RLDC Kanglung

Dawa Tshering and Purna Bahadur Rai

Standardization and demonstration on ELISA test against *Mycobacterium Bovis* antibody test was conducted at RLDC, Kanglung on 22-6-21 to 24-6-21.

Test principle

-To detect the presence of antibody against *Mycobacterium bovis* in bovine serum or plasma samples.

- Antibody detection ELISA test with 97%-98% specificity.

Participants.

-4 technicians from RLDC Kanglung were demonstrated on how to perform ELISA test and hands on training in within three-day training with the guidance of Vets. from RLDC.

- A total of 979 samples tested. Samples were collected from whole 6 Eastern Dzongkhags.

Findings

Table 1 Results

Sl.no	Dzongkhag	Total sampled and tested.	No. of positive	Percentage (%) positive
1	Monggar	290	14	4.82%
2	Lhuntse	150	4	2.66%
3	Trashiyangtse	106	1	0.94%
4	Trashigang	259	5	1.93%
5	Samdrupjongkhar	91	1	1.01%
6	Pemagatsel	83	1	1.2%

5.5 Samples referred to International Laboratories

During the year various samples were referred outside to international laboratories despite of lockdown and restricted travels (Table 11).

Table 11: Sample referred to international laboratories

Species	**Sample type**	**Samples referred to**	**Numbers**	**Remarks**
Rodents	Tissue Sample	AFRIMS, Bangkok	295	From Tsirang & S/jongkhar for molecular detection of vector borne diseases
	Ear Samples		59	
	Parasites		59	
	Whole Blood (FTA)		59	
		Sub-total	*472*	

Takin	Swab in UTM	Animal Production and Health Laboratory, Vienna; Austria	4	Gene sequencing of Capripox
		-do-		
		-do-		
		-do-		
Serow	Swab in UTM		3	
Goral	Swab in UTM		2	
Caprine	Swab in UTM		4	
	Sub-total		*13*	
Mix Species	*E. coli* isolates	The Doherty Institute, University of Melborne, Australia	76	Whole genome sequencing
	TOTAL		**561**	

5.5 Technical Assistance in diagnosis of COVID-19

During the COVID-19 mass screening in three regions viz. Phuentsholing, Thimphu and Paro, National Centre for Animal Health under One health program deployed a team of 4 laboratory staffs in assisting the national COVID lab testing team in RCDC.



The members were allotted in Sample reception/distribution team, Data management/ Reporting team, Sorting /pooling team, Extraction Team and Master Mix & Data Analysis Team.

5.6 Collection of dog samples from quarantined owners at paro for COVID testing

LSU collected the samples from the dogs of quarantined owners at Paro for COVID testing. The samples were tested at RCDC to rule out the COVID in the pet animals of the people who were quarantine.

Date	Owner	Station	Species	Specimen	Test	Remarks
1/6/2021	Quarantine Station	Paro	Canine	O/Nasal swab	COVID-19-Negative	Tested at RCDC, Serbithang
9/6/2021	Quarantine Station	Paro	Canine	O/Nasal swab	COVID-19-Negative	Tested at RCDC, Serbithang
16/6/2021	Quarantine Station	Paro	Canine	Nasal swab	COVID-19-Negative	Tested at RCDC, Serbithang
23/6/2021	Quarantine Station	Paro	Canine	O/Nasal swab	COVID-19-Negative	Tested at RCDC, Serbithang

6. DISEASE SURVEILLANCE AND ANIMAL HEALTH RESEARCH

6.1 Genotype analysis of *Bacillus anthracis* strains circulating in Bhutan

Puspa Maya Sharma, Nirmal Kumar Thapa, Tenzinla

1. Introduction

Anthrax caused by *Bacillus anthracis* is primarily a disease of herbivores. The bacteria *Bacillus anthracis is* spore forming, a Gram positive, rod- shaped and the spore forms are highly resistant to extremes of heat, cold, pH, desiccation, chemicals (and thus to disinfection), irradiation and other such adverse conditions. Therefore, the spore forms are the predominant phase in the environment and it is very largely through the uptake of spores that the animals contact anthrax.

The disease is one of the most important causes of mortality in cattle, sheep, goats, horses and pigs worldwide. The disease is enzootic in most countries of Africa and Asia, a number of European countries and countries/areas of the American continent and certain areas of Australia. In Bhutan, the disease is reported sporadically every year from some parts of the country (Figures 1 and 2. There was one human case occurred in 2010.

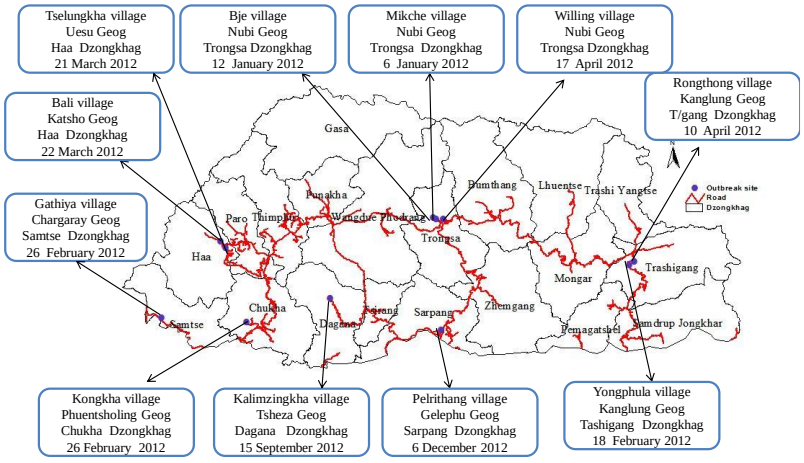


Figure 1: Geographic location of anthrax outbreaks in animals in Bhutan during 2012.

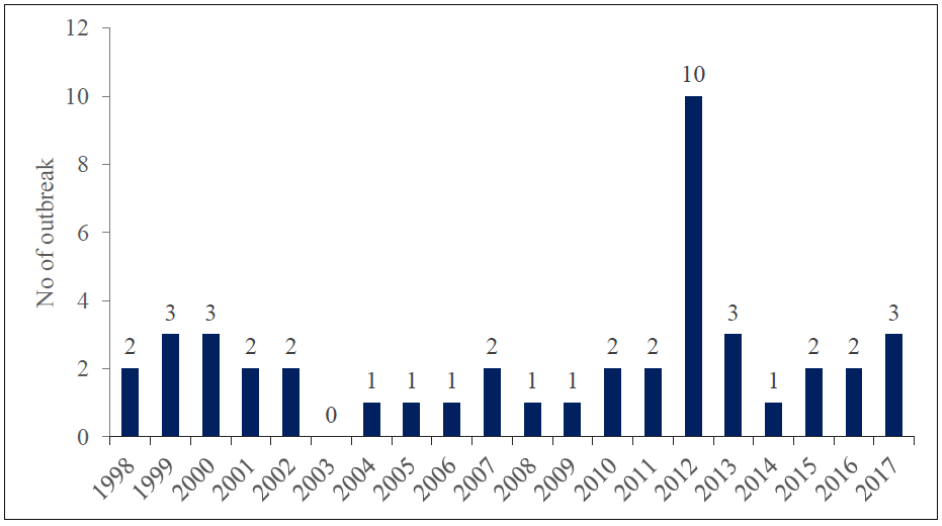


Fig.2 Number of anthrax outbreaks in animals in Bhutan (1998-2017)

Through the genetic study, *B. anthracis* isolates from the 2010 outbreak in cattle in Zhemgang, central Bhutan was found to be part of the multilocus variable-number tandem repeat (MLVA) analysis B1 lineage (genotype 83) and canonical single-nucleotide polymorphism subgroup B.Br.001/002. The B lineage is less widespread and primarily associated with South Africa, but it has been reported in parts of the United States, Europe, and Asia, including the Caucasus region. Although anthrax has been reported sporadically from different parts of the country, no detailed studies has been conducted to understand the circulation and identification of anthrax strain and environmental and other associated risk factors for the occurrence of anthrax in Bhutan. Therefore, the main objectives of this study are to:

1) Identify anthrax strain circulating in different agroecological zones in Bhutan

2) Identify risk factors and map the risk of anthrax outbreaks at human-animal- environmental interface

2. Methodology

2.1 Study areas

The anthrax outbreak location in animals between 2010 and 2017 identified from DPCU database were validated and burial sites located. Three soil samples were collected from burial sites and control sites respectively, table 1.

Table 1: Soil samples collected from outbreak areas.

SI.No	Name of owner	Village	Sub-district	District	Species affected	No. of Cases	Burial soil (Nos)
1	Stray	BCCL Colony	Sampheling	P/ling	Cattle	1	3
2	Breeding Bull/RNR	Lower Ghathia	Sangacholing	Samtse	Cattle		3
3	Stray	Samtse Town (Post Office)	Samtse	Samtse	Cattle		3
4	Wangchuk (Shukho)	Peling	Tseza	Dagana	Bovine	1	3
5	Gangchu	Kalizingkha	Tseza	Dagana	Bovine		3
6	Tshering Wangdi	Patsaling Maed	Patsaling	Tsirang	Bovine	10	3
7	Dorji	Kagtong	Ngangla	Zhemgang	Bovine	1	3
8	Tenzin Rabgay	Kagtong	Ngangla	Zhemgang	Equine	1	3
9	Dorji Kencho	Kagtong (pongchaling	Ngangla	Zhemgang	Equine	1	3
10	Sangay Pindu	Kagtong (pongchaling	Ngangla	Zhemgang	Bovine	1	3
11	Unknown	Uesu	Tshelungkha	Haa	Bovine	1	3
12	Unknown	Puduna	Samar	Haa	Bovine		3
13	Sangay Wangdi	Sheripam	Silambi	Mongar	Cattle	5	3
14	Sangay Wangdi	Sheripam	Silambi	Mongar	Cattle		3
15	Leki Chezom	Sheripam/Yari	Silambi	Mongar	Cattle	5	3
16	Pema	Rawabi/Yomey	Maenbi	Lhuntse	Cattle	5	3

2.2 Clinical samples

If the cases are encountered in animals during the study period, the samples (blood smear and tissue samples) from the clinical cases or the carcasses will be collected for culture and isolation of the organism.

2.3 Laboratory analysis

A total of 96 soil samples were collected from both burial and control sites. Out of 96 samples, 60 samples were processed and cultured for *Bacillus anthracis*. Sheep blood agar and BACARA plates were used for culture and identification of *Bacillus anthracis* typical colonies. Suspected colonies were observed under microscope and PCR performed.

3. Results

No *Bacillus anthracis* resembling colonies were identified from sheep blood agar. Only hemolytic colonies resembling *Bacillus cereus* were identified.

4. Conclusion

The possibility of culturing *Bacillus anthracis* from soil is very low due to multiple factors like low or unavailability of spores in samples, heavy environment contaminants, wrong identification of burial site, etc. Hence, the study will focus more on clinical samples like blood, blood-stained soils, tissue or swabs from anthrax suspected cases.

5.5 Case report on mortality of wild boar due to *E. fergusonii*

N.K Thapa, Puspa M Sharma, Tenzinla, Dr. RB Gurung

1. Introduction

Following a call from the Royal Palace, Samtenling and the command to perform post-mortem of a dead pig (wild boar), the team of officials from National Centre for Animal Health (NCAH), Serbithang and National Veterinary Hospital (NVH), Motithang immediately visited the site to conduct post-mortem and sample collection.

2. History

It was informed that the animal was found dead with bleeding from nostrils and salivation on the evening of 29/5/2021. There were about 17 numbers of wild pigs excluding one dead (11 piglets, 5 females, 1 male) as reported.

3. Post-mortem findings

Externally, bleeding from the nostrils and frothy salivary discharge from the mouth were noted however, no external injuries were observed. In the digestive tract, no signs of irritation were observed in the internal lining of digestive tract which usually occurs during oral consumption of poisons. Stomach contained partially digested grass material and grains and no unusual odour noted from the stomach contents (usually in rat poisoning case with zinc phosphate, the stomach contents emit the smell of garlic).

4. Laboratory analysis

Sl. no	Agent examined	Test method	Result
A.	**Molecular analysis**		
1	African Swine Fever (ASF)	RT-PCR	Negative
2	Classical Swine Fever (CSF)	RT-PCR	Negative
3	Porcine Reproductive and Respiratory Syndrome (PRRS)	RT-PCR	Negative
B.	**Bacterial culture**		
4	*Escherichia coli*	API-20	Positive
5	*Escherichia fergusonii*	API-20	Positive
C.	**Parasitology**		
6	Trichinella	Pepsin digestion	Negative
7	Other gut worms	Sedimentation	Negative
D.	**Toxicology**		
8	Maize aflatoxin	Rapid and ELISA	Negative
E.	**Forensic poison analysis**		
9	Organophosphates (Diazinon, Chlorpyrifos, Parathion)	GC-MS	Negative
F.	**Metal**		
10	Zinc	ICP-OES	Insignificant

5. Conclusion

The samples were tested against major viral diseases of pig, common parasites, toxins in feed, forensic poison and metals. None of these agents were found to be involved in the cause of the death of the pig. However, bacterial infection due to *E. fergusonii* is implicated responsible for

the death of the pig. Commensal *E. coli* was also detected that are usually found also in normal animal. Insignificant or non-toxic level of zinc was also detected in gut content.

5.6 Investigation of Porcine circovirus-associated disease (PCVAD) at National Nucleus Pig Breeding Centre (NNPBC) Yusipang

NK Thapa, Purna B Rai & Ugyen Pema

1.Introduction

The National Nucleus Pig Breeding Centre is located at Yusipang. The farm was started during 2016 for maintaining the great grandparents (GGP), grandparents (GP) and parent stock (PS) to supply the genetically improved piglets and PS. The farm supplies piglets to the farmers for breeding and fattening purpose.

As per the request from NNPBC, Yusipang vide letter no. (13) DoL/NNPBC/2020-21/232 dated 20/4/21, the investigation of disease was carried out on 27/04/2021 in the farm by the following staff of NCAH, Serbithang:

1. Dr. NK Thapa, AHS II
2. Mr. Purna B Rai, Sr. LO
3. Ms. Ugyen Pema, LT

The farm had reported of preweaning mortality and still birth for some time.

2. General Observation of the farm

During the visit the farm had following stock of animals:

a. Stock

Particulars	**Stock**
1 Boar & Dry sow shed	23
2.Gestation shed	22
3. Farrowing Shed	69
4. Nursery shed	79
5. Replacement shed	50
TOTAL	**243**

b. Clinical observations of the affected animals

On investigation and as per the history, various ailments were noted as abortion, still birth, foetal mummification and also bluish ear tip in the females and adult; weakness and skin

lesions in the piglets. The details are tabulated in the laboratory findings. Appropriate samples were collected and screened for various diseases.

Fig.1 Bluish colour in the ear tips

Fig.2 Skin lesion

c. Hygiene:

- It was observed that the water supply restoration was on full swing
- Foot dips are well maintained at the entrance of the farm and each shed except replenishment shed where, the disinfectant neds to be replaced regularly.
- The floor of the sheds appeared cleaned however; the washing of the walls also is advised.
- Currently, most of the sheds are occupied half or more than half only. It is advisable to maintain space, use alternate cage for the animals.

4. Laboratory findings

Samples were tested for various diseases as follows:

a. Clinical signs with lab findings

Sl. No.	**Tag No.**	**Signs/history**	**Samples collected**	**Remarks**
A	**Farrowing Shed**			
1	700161, LR	Abortion history, repeat breeder, passing Yellowish coloured Urine	Vaginal swab	*Actinomyces sps.*
2	304464	Black coloured	Vaginal swab	*Escherichia coli*

		discharge from vagina, History of fetal mummification, Abortion		
3	700161 LR	Abortion history, repeat breeder	Vaginal swab	*Actinomyces sps.*
B.	**Nursery Shed**			
	1763, 701305, 701308, 701330, 501087, 501089, 701356, 70172	Skin lesions on skin and at the base of the Ear	Skin scrapping	701356- *Rhizopus sps* 701372- *Aspergillus fumigatus*
C	**Dry Sow**			
1	701187	Vaginal discharge, all piglets died.	Vaginal swab	*Streptococcus sps.*
D	**Replacement Shed**			
1	701277	Off feed, bluish ear, Limping	Serum	Antibody against *Porcine circovirus type 2*
2	701299	Bluish ear	Serum	Antibody against *Porcine circovirus type 2*

				Actinomyces sps. from vaginal swab

b. Summary of Screening of Important diseases

	Diseases screened			
Tag No.	**PRRS-EU(PCR)**	**PRRS-NA (PCR)**	**Porcine circovirus type 2(Ab ELISA)**	**Mycoplasma (AB ELISA)**
701299	Negative	Negative	Positive	Negative
701277	Negative	Negative	Positive	Negative

5. Diagnosis

Detection of antibody against Porcine circovirus type 2 indicates the exposure of the animals to virus. The animals are not being vaccinated against PCV2. Hence, the syndrome can be diagnosed as porcine circovirus-associated disease (PCVAD) tentatively.

6. Discussion

Porcine circovirus type 2 (PCV2) is the primary causative agent of several syndromes collectively known as porcine circovirus-associated disease (PCVAD). PCV2 can be transmitted mainly by oro-nasal contact with infected feces, contact with infected urine, or directly with infected pigs. PCV2 is shed in respiratory secretions, oral secretions, urinary secretions, and feces in both clinically affected as well as in infected but apparently healthy pigs. PCV2 can also be transmitted vertically (from the sow to the piglets) through the placenta causing persistently infected piglets at birth. PCV2 is also shed in semen and in experimental studies seminal virus shedding was detected as early as 5 days post-inoculation. Shedding in naturally infected boars appears to be low and sporadic.

The agent implicated as creating the greatest risk is porcine reproductive and respiratory syndrome virus (PRRSV). Other agents include porcine parvovirus (PPV) and *Mycoplasma hyopneumoniae*. However, currently, PRRS and *Mycoplasma hyopneumae* has not been detected in the farm.

The most significant manifestation of PCVAD is the multisystemic syndrome. This disease affects pigs between 5 and 16 weeks. Clinical signs of PCVAD include wasting with progressive weight loss, lethargy, dark-colored diarrhea, lymphadenopathy, and paleness or jaundice. Early signs of reduced weight gain, ill-thrift, pale skin, and rough hair coat often go unnoticed or are

misdiagnosed. Later signs include dyspnea, tachypnea, anemia, diarrhea, and jaundice. Pigs can also have coughing and gastric ulceration, which most likely contributes to the anemia.

PCV2-Associated Enteritis-This syndrome affects piglets from 8 to 16 weeks old. Affected piglets have diarrhea, unthriftiness, retarded growth, and increased mortality.

PCV2-Associated Pneumonia-It affects pigs from 8 to 26 weeks old and is associated with multiple pathogens. The clinical signs include decreased rate of growth, decreased feed efficiency, anorexia, fever, cough, and dyspnea.

PCV2-Associated Reproductive Failure-typically affects gilt. The clinical signs include increased abortion, still births, fetal mummies, and preweaning mortalities.

PCV2-Associated Neuropathy-pigs born with congenital tremors and a nonsuppurative meningoencephalitis located in the brain. naturally occurring neurologic disease characterized by opisthotonus, nystagmus, and convulsions was associated with PCV2 infection in pigs ranging from 6 to 8 weeks old.

Most of the syndromes described in the literatures as above are concurrent with the symptoms observed or recorded in the NNPBC Yusipang.

7. Conclusion

The signs and symptoms recorded in the farm is consistent with PCAVD. Further, the presence of antibody in the animals indicates the exposure of animals to the virus. Currently symptomatic treatment should be carried out with improve in hygiene and management. Further, screening of animals will be carried out subject to availability of the kits. The farm should enhance the biosecurity of the farm and improve hygiene management. Avoid overcrowding in the shed (keep one empty cage in between), if possible and routine disinfection of the sheds.

6.4 Detection of multi-drug resistant and extended-spectrum beta-lactamase producing *Enterobacteriaceae* in Thimphu, Bhutan.

Puspa Maya Sharma, Tenzinla, Nirmal Kumar Thapa, Meena Devi Samal

Enterobacteriaceae group of bacteria are Gram-negative, facultative anaerobes and non-sporing bacilli. These bacteria have become one of the most important causes of nosocomial and community-acquired infections. They can cause urinary tract, respiratory tract, bloodstream and wound infections. Increasing rates of antimicrobial resistance in Enterobacteriaceae have become a worldwide problem (Murray et al., 2005; Paterson et al., 2006). Beta-lactam drugs such as extended-spectrum penicillin's, cephalosporins, monobactams, carbapenems, fluoroquinolones (e.g., ciprofloxacin) and aminoglycosides (e.g., gentamicin) are among the most prescribed antibiotics to treat infections caused by Enterobacteriaceae. The most important mechanism of resistance to beta-lactam antibiotics involves the production of beta-lactamases (especially extended-spectrum beta-lactamases) that inactivate beta-lactam antibiotics and this continue to be the prominent cause of β-lactam antibiotics resistance among Enterobacteriaceae worldwide (Paterson, 2005; Pitout 2008). Among Enterobacteriaceae,

ESBLs have been found mostly in *Klebsiella spp* and *E. coli* as well as in other Enterobacteriaceae families such as *Enterobacter spp, Proteus spp, Citrobacter spp, Morganella spp, Providencia spp., Salmonella spp* and *Serratia spp* (Jacoby GA etal., 1991; Tzelepi E et al.,2000).

Today, the most important ESBLs belong to the CTX-M family of enzymes [Cantón et al., 2012]. In the EU, CTX-M-1 is the most frequently reported ESBL subtype in E. coli originating from food producing animals (poultry, cattle and pigs) and food [EFSA J 9, 2011]. In contrast, in Asia, CTX-M-2, CTX-M-14 and CTX-M-15 are found in cattle and pigs [Ewers, 2015]. Congruently, an earlier study revealed the presence of ESBL CTX-M-15 producing *E. coli* from pig breeder farms in two different regions Gelephu and Lingmethang in Bhutan (Sharma et al., 2017). However, little is known about the magnitude of ESBL producing Enterobacteriaceae in Bhutan. Hence, assessing ESBL producing Enterobacteriaceae in the local scenario is necessary to understand the prevalence, spread and the disease burden in the country. Therefore, this study aimed to detect MDR and ESBL producing Enterobacteriaceae from different clinical specimens received at National Veterinary Laboratory, National Centre for Animal Health.

Methods

Study setting

All the clinical specimen received by the microbiology laboratory at national veterinary reference laboratory for the period July 2020 to June 2021 were processed for pathogen identification and antibiotic susceptibility test using a standard gram positive and gram-negative antibiotic panels.

Culture and identification

All specimens received by the laboratory were cultured on sheep blood agar and MacConkey agar. The isolates were identified by standard microbiological laboratory methods and API test kits (Biomerieux). Antibiotic susceptibility and ESBLs confirmatory tests were done using the pure isolates as per the CLSI guidelines 2016. The isolates were preserved in BHI with 20% glycerol at -80°C for future testing and reference.

Antibiotic susceptibility testing and screening for potential ESBL-producing isolate

Antibiotic susceptibility testing was carried out by the disk diffusion method and the results were interpreted as susceptible, intermediate or resistant according to CLSI guideline 2016. Muller Hinton agar plates were inoculated with 0.5 McFarland turbidity inoculums and antimicrobial disks applied to the plate. The antibiotic disks used were ampicillin (AMP:10 µg), gentamicin (GEN:10 µg), tetracycline (TET:30µg), Ceftriaxone (CTR: 30 µg), sulfamethoxazole-trimethoprim (SXT: 3.75/ 1.25 µg). The antibiotic disks used were from Oxoid ltd. An Enterobacteriaceae isolate was considered as MDR if it was non-susceptible to three or more drugs from different classes/groups of antibiotics [Magiorakos, 2011]. The isolates that showed an inhibition zone size of ≤22 mm with ceftriaxone (30 µg) were considered as potential ESBL-

producer (screening ESBL positive) and were selected for confirmation for ESBLs production using CDT as recommended by CLSI guideline 2016.

Confirmation of ESBLs with combination disc test

A disc of ceftazidime (30 µg), and cefotaxime (30 µg), and ceftazidime + clavulanic acid (30 µg/ 10 µg) and cefotaxime (30 µg) + clavulanic acid (30 µg/10 µg) was placed at appropriate distance on MHA plate inoculated with a bacterial suspension of 0.5 McFarland turbidity standards and incubated overnight (18–20 h) at 37 °C. An increase in the inhibition zone diameter of > 5 mm for a combination disk versus ceftazidime or cefotaxime disk alone was confirmed as ESBLs production.

Quality control

Quality control was performed using ATCC 25922 *E. coli* standard strain to check the quality of culture media and antibiotics disks.

Results & Discussion

Three MDR and ESBL producing Enterobacteriaceae were detected from clinical samples from companion animals (cat and dog) and aquatic duck referred from National Veterinary Hospital, Thimphu. The isolates were identified as *Serratia rubidaea* (cat), *Proteus mirabilis* (dog) and *E. coli* (duck). The detailed description of specimen is mentioned in table 1.

Table 1: Details of specimen received and bacteria isolated

Bacteria	Host species	Specimen	Location
E. coli	Duck	Intestine	Samtenlng, Thimphu
Serratia rubidaea	Cat	Blood	Babesa, Thimphu
Proteus mirabilis	Dog	Urine	In patient at National Veterinary Hospital, Thimphu

The antibiotic profile of different ESBL organisms are tabulated below (table 2).

Table2: The antibiotic profile of MDR bacteria

Bacteria	AMP	TCY	SXT	GEN	CTR
E. coli	R	R	R	S	R
Serratia rubidaea	R	R	R	R	R
Proteus mirabilis	R	R	S	I	R

All the isolates were found to be MDR with susceptibility to only Gentamicin for *E. coli* and Sulfamethoxazole Trimethoprim for *Proteus mirabilis*. However, *Serratia rubidae* was found resistant to all the antibiotics tested. The isolates will be referred to University of Melbourne for characterization of resistant genes.

Conclusion

The detection of above MDR/ESBLs producing organisms in the various animals in the country is significant from the public health point. It is also important from the animal health perspective since, they were isolated from the clinical cases and also from postmortem case of duck. The investigation of such MDR /ESBL producing organisms shall be continued for understanding the antibiotic profile and also the clinical significance in the animals. It could also be undertaken as one health studies in future with other agencies.

6.5 Detection of Classical Swine Fever in wild piglets

NK Thapa, Tenzinla, Kelzang Lhamo, Puspa M Sharma

During the month of June, three piglets of wild pigs were found dead in the Samtenling palace forest area. The carcass was submitted to LSU for post-mortem examination. Since, the carcass was fully decomposed and infested with maggots, post-mortem examination could not be conducted. However, the bone marrow samples were collected and tested for African swine fever, Classical Swine Fever and Porcine Respiratory and reproductive Syndrome by real time PCR. The samples were negative to ASF and PRRS. However, it tested positive to CSF indicating the cause of death as Classical Swine Fever. Control measures were advised. However, vaccination of the wild pig population may not be possible at this stage. If required, we may have to explore the possibilities in future in collaboration with Nature Conservation Division, Department of Forests and Park Services.

6.6 Prevalence and characterization of *Staphylococcus aureus* cultured from raw milk from government farms in Bhutan

Puspa M Sharma, Tenzinla, Bindu Parajuli, Nirmal Kumar Thapa

Introduction

Staphylococcus aureus is a pathogen associated with serious community and hospital-acquired diseases. It has low nutritional requirements and widely exists in nature. *S. aureus* is widely associated with food poisoning in many developed countries like China and USA (Wu et al., 2018; Scallan et al.,2011). In addition *S .aureus* is a one of the leading sources of intra-mammary infections in dairy cows (Dufour et al., 2012; Zecconi and Scali, 2013). It is reported that 10–40% of the mastitis cases are caused by *S. aureus* in China and other countries (Kateete et al., 2013; Basanisi et al., 2017; Liu et al., 2017).

S. aureus associated food poisoning in humans and similarly mastitis in animal is caused by those isolates possessing virulence factors (Hennekinne et al., 2012). This bacterium produces wide range of factors, for example toxic shock syndrome toxin-1 (TSST-1), staphylococcus

enterotoxin (SE), and Panton-Valentine leukocidin (PVL). SEs is regarded as the major cause of *S. aureus* associated food poisoning (Bergdoll et al., 1981; Hennekinne et al., 2012).

S. aureus often develops antibiotic resistance. Isolation of single or multiple-drug resistant *S. aureus* strains from food, the environment, and clinics has been constantly reported (Gould et al., 2012; Rasigade et al., 2014). The ability of *S. aureus* to form biofilms helps the bacterium to survive in hostile environments within the host and is considered to be responsible for chronic or persistent infections (Costerton et al., 1999). The ability of some strains to synthesize biofilms could increase their pathogenicity since established biofilms can tolerate antimicrobial agents, thus making the bacterium extremely difficult to eradicate (Zmantar et al., 2010).

In Bhutan, milk and milk products are consumed on a daily basis by individual families. Further, the method of milk and milk products consumed in the country possesses a high risk of transmitting the pathogenic bacteria from animals to humans. Currently, there are no study conducted to characterize *S. aureus* in country. Hence, this study aims to estimate the prevalence of *S. aureus* from raw milk from dairy cows from three government breeding farms v.i.z. National Jersey Breeding Centre, National Brown Swiss Farm and Regional calf rearing centre, Wangkha located at three different districts and describe the characteristics of the isolates. The study will also provide baseline for further studies on the control and prevention of *S. aureus* contamination in raw milk of dairy cows affected with mastitis.

Material and Methods

Sampling

From June 2020 to June 2021, a total of 180 (45 individual cows * 4 teats) raw milk samples were collected from cows from three government breeding farms. Milk collection process was performed after cleaning the teats, initial streams of milk discarded and teat tips cleaned with cotton balls moistened with 70% alcohol. Milk samples from individual teats were collected in sterile container and shipped to National Veterinary Laboratory, NCAH in chilled condition for culture and identification.

Phenotypic Identification and AST

A 20ul of milk samples were taken and plated onto SBA and MacConkey agar. The plates were incubated at 37°C for 24 hours. The beta hemolytic colonies, appearing as gram positive cocci, catalase negative were identified. The pure colonies were tested for coagulase activity using rabbit plasma. Those isolates positive for coagulase activities were preserved in BHI with 20% glycerol at -80°C. The isolates were shipped to Doherty & Melbourne University for whole genome sequencing.

Antibiotic susceptibility tests were also performed to all putative *S. aureus* isolates at NVL, NCAH using disk diffusion method as per CLSI protocol.

Results & Discussion

Highest prevalence of *S. aureus* of 43.75% was detected at Regional Calf rearing center (7/16) followed by Brown Swiss cattle farm, Bumthang with 31.1% (14/45) however, no isolate was

detected at National Jersey Breeding Centre (0/15), Samtse. The high prevalence of *S. aureus* at RCRC Wangkha could be due to low sample size compared to Brown swiss cattle farm (Table 1).

All positive isolates were shipped to Australia for whole genome sequencing and identification of virulence factors.

Table 1: Farm wise *S. aureus* isolated

Name of Farm	**Total animals sampled (n)**	**Nos. of cows with *S. aureus***	**Percentage**
Brown Swiss Cattle Farm, Bumthang	45	14	31.1%
Regional Calf Rearing Centre, Wangkha	16	7	43.75%
National Jersey Breeding Centre, Samtse	15	0	0%

The antibiotic susceptibility tests were performed at NVL against five antibiotics. Of the 14 *S. aureus* isolates tested, resistance was most frequently observed to penicillin 64% (9/14) followed by tetracycline 21% (3/14). No resistance was observed towards erythromycin, gentamycin and Trimethoprim/sulfamethoxazole, (Figure 1).

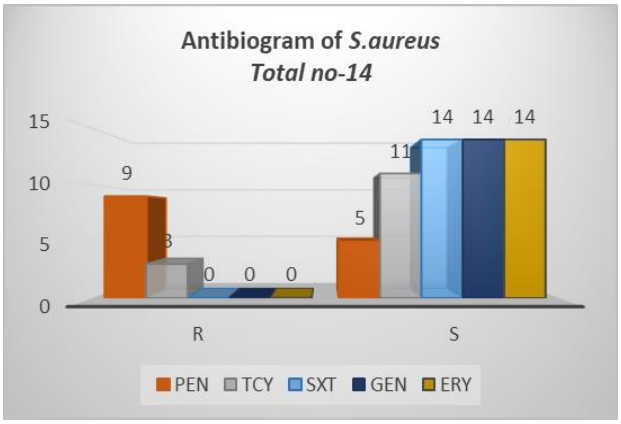


Figure1: Antibiogram of *Staphylococcus aureus* against to penicillin, tetracycline, Trimethoprim/sulfamethoxazole, gentamycin and erythromycin.

Highest resistance was observed against Penicillin 64% followed by Tetracycline 21%, this could be due to the frequent use of these drugs in the farm as they are readily available in the centers.

Conclusion

Detection of *S. aureus* at high level in 2 farms indicate the significance of the bacteria associated with the mastitis cases in the animals. However, only after the genetic characterization the

strain will be known. However, alarming levels of resistance is observed to Penicillin and Tetracyclines. The absence of the organism at NJBC could eb due to following of proper hygiene and management practiced at the farms.

Reference

1. Dai J, Wu S, Huang J, et al. Prevalence and Characterization of *Staphylococcus aureus* Isolated from Pasteurized Milk in China. *Front Microbiol*. 2019;10:641. Published 2019 Apr 2. doi:10.3389/fmicb.2019.00641
2. Wu S, Huang J, Wu Q, Zhang F, Zhang J, Lei T, Chen M, Ding Y, Xue L. Prevalence and Characterization of *Staphylococcus aureus* Isolated from Retail Vegetables in China. Front Microbiol. 2018 Jun 14;9:1263. doi: 10.3389/fmicb.2018.01263. PMID: 29963025; PMCID: PMC6011812.
3. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis. 2011 Jan;17(1):7-15. doi: 10.3201/eid1701.p11101. PMID: 21192848; PMCID: PMC3375761.

6.7 Detection of African Swine Fever in stray pigs at Phuntsholing

During the month of May 2021, many scavenging pigs were found dead at Phuntsholing sewerage area. At some places, the remains of the carcass were spotted. The carcasses spotted were in decomposed state hence, bone marrow was submitted to LSU for diagnosis. Molecular diagnosis was carried out for African swine fever, Classical Swine Fever and Porcine Respiratory and reproductive Syndrome by real time PCR. The samples tested positive to African swine fever.

The RRT team collected and sent the blood and serum from the sick animals. The samples again tested positive to ASFV. The outbreak of ASF was confirmed following the laboratory results and stamping out operation was carried out. In addition, the surveillance were carried out in the surrounding areas.

6.8 Detection of Lumpy Skin Disease (LSD)

The first suspected case of LSD was reported from Langchenphu gewog under Samdrup Jongkhar on the 21 August 2020 in six cattle. Confirmation of the outbreak could not be done as the country was under lockdown. In addition, there was no diagnostic capacity at NCAH then. Second suspected case of LSD was reported from Tashicholing, Samtse on 22nd September 2020. The National Centre for Animal Health received four samples from Samtse on 4th October and confirmed the outbreak on the next day. The disease was confirmed by PCR.

7. HUMAN RESOURCE AND CAPACITY BUILDING

7.1 One health advocacy meeting on antimicrobial resistance

November 18th – 24th is observed as *World Antimicrobial Awareness week* (WAAW) globally. The goal of this week was to raise awareness on the antimicrobial resistance (AMR) to the public, health workers and the policy makers and to promote good practice in this area of concern, to limit the emergence and spread of resistant bacteria throughout the world. The slogan this year "Antimicrobials: handle with care".

Antimicrobials are the essential medicines used to treat infection with microorganism like bacteria, virus, fungi and parasites in human and animals. These agents have substantially reduced the burden of diseases in human and animals and also contributed to food security and safety**.** However, emergence and spread of AMR in several microorganisms is complicating the management of many infectious diseases. AMR occurs when these microorganisms change naturally and also triggered by inappropriate use, misuse or overuse in human, animals and plants whereby they don't respond to treatment with the antimicrobials which they used to be before. AMR endangers animal health and welfare, as well as food production and also adversely affects the functioning of human, animal and plant health systems and economies. It is also considered as one of the biggest global threat by the WHO. Hence, it requires inter-sector collaboration to reduce the emergence and spread of resistant microorganisms.

To mark the occasion and join the global move, **One Health Advocacy meeting on Antimicrobial Resistance** was conducted at Kuenphen Rabten Resort, Chang Jalu. The main objectives of the workshop were to create awareness to various one health stakeholders, on the AMR and to encourage the prudent usage of antimicrobials in respective areas. The meeting was organized by the Department of Livestock and funded by Ministry of Health.

The meeting was chaired by the Director, Department of Livestock and was attended by World Health Organization (WHO) representative to Bhutan and representation from the World Organisation for Animal Health (OIE). About 25 participants attended the advocacy program representing various agencies such as National Centre for Animal Health(NCAH), Thimphu Dzongkhag Livestock from Department of Livestock (DoL); AMR program, Department of medical Services and JDW National Referral Hospital from Ministry of Health (MoH); Drug Regulatory Authority (DRA); Khesar Gyalpo University of Medical Sciences of Bhutan (KGUMB); One Health Secretariat; National Plant Protection Centre (NPPC) from Department of Agriculture (DoA); Nature Conservation Division (NCD) from Department of Forests & Park Services (DoFPs); Media representatives from Information & Communication Division (ICTD), Ministry of Agriculture & Forests(MoAF), Bhutan Broad Casting Services(BBS) and Kuensel participated the meeting.

7.2 Training on Rabies diagnosis for the veterinary laboratory technicians

1. Introduction

Training on Rabies diagnosis was conducted from 12 to 16 of April 2021 at National Centre for Animal Health, Serbithang. About 17 participants attended representing from National Veterinary Laboratory, Serbithang; National Veterinary Hospital, Motithang; Regional Livestock Development centre, Tshimasham, Wangdue, Zhemgang, Kanglung; Dzongkhag Veterinary Laboratory, Dagana, Tsirang, Thimphu, Lhuntshe, Chukha, Wangdue.

The main objectives of the training were to enhance the diagnostic capacity of laboratories for Rabies in the country. It is also an approach towards the vision of eliminating dog mediated Rabies death in human by 2030.

The training was organized by the Department of Livestock and NCAH and was funded by FAO, TCP project.

2. Training Outline

The technical resource was provided by the National Centre for Animal Health (NCAH), Department of Livestock and Ministry of Health. The training comprised of technical presentations and hands on training on brain sample collection and Fluorescence antibody test (FAT).

The outline of the training was as follows:

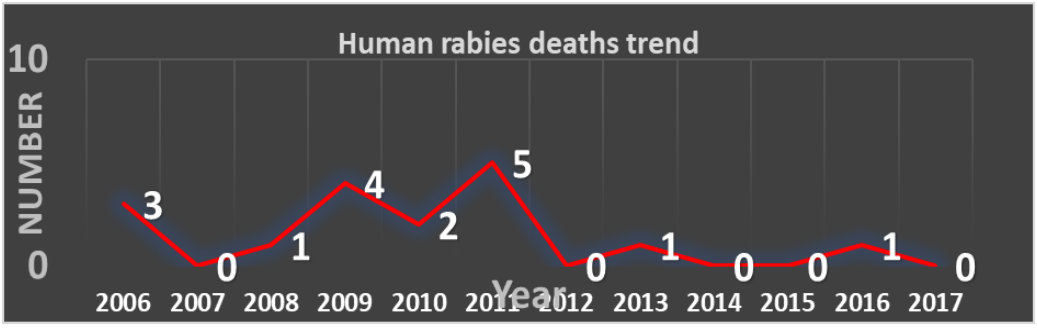
A. Theory Presentations:

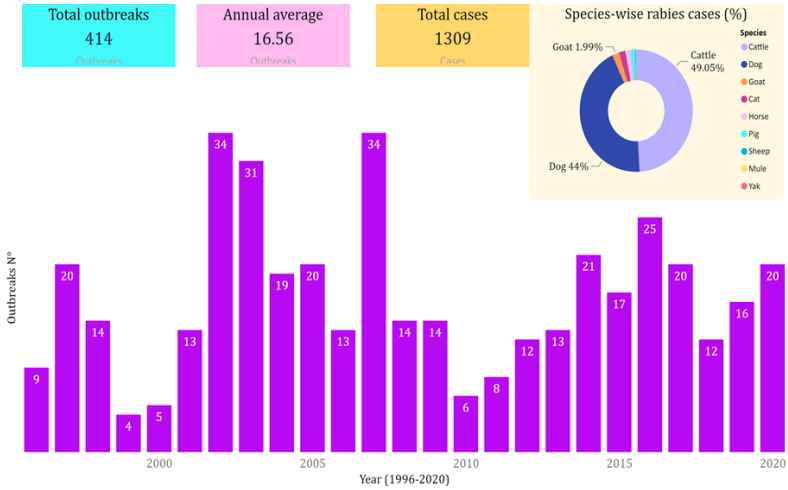
- Rabies Disease
- Rabies in Human & Prophylaxis
- Global status of Rabies
- Rabies situation in Bhutan and National Rabies Prevention Control Plan
- Laboratory Biosafety & Bio-security in relation to Rabies
- Sample collection, preservation, packaging, Transportation
- Laboratory Diagnosis- Rapid Test, Fluorescence Microscopy: Principles and applications: FAT, Molecular Test

B. Hands on Training

- Brain sample collection using drinking straw method
- Whole brain sample collection-opening the skull-(Dogs/ Pigs)
- Rapid test
- Fluorescence Antibody Test (FAT)

The training started with pre-test for all participants. Then by presentations on epidemiological scenario on Rabies distribution in different Dzongkhags from retrospective studies. Followed by presentation by health expertise on Rabies cases in human and its related prophylaxis recommended by the hospitals. The presentation went on with other introductory session to Rabies epidemiology, pathogenesis, symptoms and its preventive strategic methods indulged by NCAH, Serbithang.





Besides the Training on Rabies, the presentation on safety consideration while handling rabies samples, proper sample transportation, storage and lab testing were also presented by the Biosafety & Biosecurity section.

Theoretical presentations were followed by practical sessions. The hands on training were provided on brain sample collection using drinking straw method, Whole brain sample collection-opening the skull-(Dogs/ Pigs), Rapid test and Fluorescence Antibody Test (FAT).

The training concluded with the post-test and declaration of the results. Certificate were given to all participants by the Director of Department of Livestock (DoL). Finally, the training was ended with the closing remarks from the Director, DoL.

7.3 Training workshop on Laboratory Standard Operating Procedures (SOP) at NVH

The laboratory standard operating procedure (SOP) is a set of written instructions that describes, in detail, how to perform a laboratory process or experiment safely and effectively. Laboratory must have written SOPs when work involves the use of hazardous materials (biological or chemical) or physical hazards. It is equally important to have standardized and harmonized SOPs amongst the various veterinary laboratories to produce uniform results amongst the various laboratories. The national veterinary laboratory (NVL) at national centre for animal health (NCAH), is mandated to develop and standardizes the tests including SOPs for various veterinary laboratories in the country. The national veterinary hospital has recently developed laboratory diagnostic capacity for parasitology, biochemistry and hematology for clinical samples. However, NVH required familiarization/orientation on the laboratory techniques and also standard operating procedures. Hence, a two-day training workshop on various SOPs on sample collection, packaging, transportation, parasitology, hematology and biochemistry testing procedures was conducted at NVH. The main objectives of the workshop were: 1. To train and familiarize laboratory standard operating procedures on sample collection, packaging, storage and transportation 2. Standardize and harmonize laboratory SOPs on Parasitology, Hematology and Biochemistry 3. Asses the microbiology facility at NVH for future development of capacities.

The participants were trained and familiarized with following SOPS

1. SOP on sample collection for
    a. Faecal sample collection for parasitological tests
    b. Blood protozoa parasites collection and preparation
    c. Skin scrapping collection for parasitological tests
    d. Tissue aspirates collection
    e. Meat sample muscle biopsies collection.
    f. Blood sample collection for haematological tests
    g. Serum sample collection for biochemical tests and serological tests
    h. Urine sample collection for Biochemical test and bacteriological tests
2. SOP on faecal examination by Direct Method
3. SOP on faecal examination by Direct Method – Mucosal Impression Smear
4. SOP on flotation technique
5. SOP on Sedimentation technique
6. SOP on Quantitative Techniques – Stoll's Dilution Method
7. SOP on Blood Smear Preparation for Haematology
8. SOP on Blood smear staining techniques
9. SOP on Differential leucocytes count (DLC)
10. SOP on Sample Referral to National veterinary Laboratory

11. SOP on sample packaging, storage and packaging.

Assessment of Microbiology facility

The laboratory facility was assessed for microbiology facilities. Major equipment like Biosafety cabinets, laminar air flow, water bath, weighing balance, pH meter, granite table/working bench, hot air oven is accessible. However, to establish bacteriology laboratory below listed equipment and facility would be necessary. Once the equipment and facilities are available, basic bacteriology testing can be established.

Recommendations:

1. Equipment such as autoclave, at least two 2-8 °C fridges (one for clean media and reagents storage, other for samples and used plates storage), deep freezers (-20°C or -80°C) for storing samples, flame, consumables (glass /plastic apparatus- Petri plates, conical flask, beaker, measuring cylinders, bacteriological media) are essential.

2. Facility- a separate clean room is required for storing unprepared media, reagents and for media preparation.

3. Mycology laboratory establishment- Unlike bacteriology, fungal culture and identification is much easier and does not require various biochemical tests for identification and other facilities. Prior requirement for fungal culture is an autoclave, flame, media and consumables only. Hence, the fungal diagnostic facility can be started at NVH at the earliest.

8. FLEMING FUND ACTIVITIES

8.1 Training of animal health laboratory diagnosticians on use of Laboratory Information Management System (LIMS)

The training on Laboratory Information Management System (LIMS) for the laboratory diagnosticians for all the animal health laboratories of the country was organized. Three days training each were conducted in three regions v.i.z. at Monger on 5 to 7 of November 2020 for the eastern region, Punakha for western Region on 18 to 20 of November 2020 and Bumthang for central region on 26 to 28 of November 2020. In total of 52 participants were involved in the training and the resource persons were involved from National Centre for Animal Health and Namchoey consultancy. The participants represented, National Veterinary Laboratory, National Veterinary Hospital, Regional Livestock Development Centres and Dzongkhag Veterinary Laboratories. However, in compliance to COVID protocol, the participants from the red zone were not included in this training.
Data management on laboratory service operations is very challenging without proper record keeping. Further, due to the lack of electronic record (online system) it is tedious to compile the reports and perform analysis from hard copies. This affects the delivery of laboratory services as a whole in the country. The animal health services did not have such electronic recording system of the laboratory activities.

The same was recommended by the Technical Backstopping Mission of FAO conducted in 2017, and accordingly the National Centre for Animal Health, Serbithang has developed an online LIMS for storage of information on laboratory activities, report generation, report dissemination to clients and as a whole enhance animal health service delivery. The Database was tested several rounds in-house and was formally launched during July 2019.

It has the features for online entry of sample details, test result, diagnosis and recommendation. The system helps the veterinary laboratories to track samples from submission to testing and reporting. This database enables real time tracking of sample testing status through a paperless system. Besides data storage and test result dissemination, customized analysis can also be performed to provide decisions required in policy interventions. This database will immensely reduce turn-around-time for diagnostic service delivery as a whole. This system is intended for all the laboratory facilities under the Department of Livestock (DoL) viz. National Centre for Animal Health (NCAH), Regional Livestock Development Centres (RLDCs), Satellite Veterinary Laboratories (SVLs) and Dzongkhag Veterinary Laboratories (DVLs).

The Main objectives of the training was to support animal health laboratories in use of electronic recording system of all the laboratory activities and use paperless system of result dissemination, generation of accurate AST data for guiding the veterinary clinicians for prescribing right antibiotics and there by promoting their prudent use. In addition, the training of relevant officials will greatly support data generation and analysis for the forthcoming laboratory surveillance in chickens under the Fleming Fund Country grant.

The training was conducted by the National Veterinary Laboratory, Serbithang and is being funded by Fleming Fund Country Grant.

8.2 Animal Health Technical Working Group (TWG) meeting on Fleming Fund Country Grant activities

Under the Ministry of Agriculture & Forests, various organizations involved for AMR activities under Fleming Fund Project include National Veterinary Laboratory, two Regional Livestock Development Centres under Department of Livestock (DoL) and National Food Testing Laboratory, Bhutan Agriculture and Food Regulatory Authority (BAFRA). Since, two different organizations are involved, it is necessary to improve co-ordination of Fleming Fund activities. Hence, a two-day Animal Health TWG meeting was held w.e.f. 4th to 5th of May 2021 at Tashi Namgay Resort, Paro.

The main objectives of the meeting were to review the activities carried out, discuss on status and plan the way forward the activities. A total of 15 participants representing from Department of Livestock (Animal Health Division, National Centre for Animal Health, National Veterinary Hospital & Regional Livestock Development Centres); Bhutan Agriculture & Food Regulatory Authority (BAFRA), HQ, National Food Testing Laboratory, Yusipang and Fleming Fund Project Unit attended the meeting. During the meeting, the activities carried out by different agencies were reviewed and the issues resolved. In addition, the way forward was discussed including the prioritization of the activities for the Country grant 2. The meeting was funded by the Fleming Fund country grant.

8.3 Hands-on-training on microbiology techniques for culture, identification and antibiotic susceptibility testing of *Campylobacter, Enterococci, Salmonella* and *E. coli* from poultry

One of the main aims of Fleming Fund Project is to strengthen microbiology laboratory capacity for AMR diagnostics at the surveillance laboratories. The capacity of these laboratories needs to be enhanced and facilitated to identify, isolate and perform Antibiotic Sensitivity Test (AST) on WHO identified GLASS (Global AMR surveillance system) pathogens such as *Campylobacter spp, Enterococci spp, Salmonella spp* and *E. coli*. However, the surveillance laboratories identified do not have adequate capacity to carry out culture, identification and AST for the target pathogens. Therefore, it is important to train the laboratory technologist and technicians from all the surveillance sites on the sample processing, culture, identification and AST on the target pathogens.

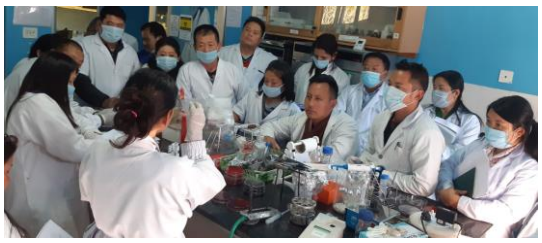
Hence, five days long hands-on-training on microbiology techniques for culture, identification and antibiotic susceptibility testing of *Campylobacter, Enterococci, Salmonella* and *E. coli* from poultry was conducted at (NCAH), Serbithang from 28th October -1 st November 2020. The participants were laboratory technologist/technicians from Department of Livestock (National Veterinary Laboratory, Regional Laboratories of Regional Livestock Development Centre, National Veterinary Hospital) and BAFRA (National Food Testing Laboratory). The training was conducted by Puspa M Sharma Sr. Laboratory Officer from NCAH and Mr. Dorji Tshering, Sr. Laboratory Technician from Royal Centre for Disease Control (RCDC).

The main objectives of the workshop were:

1. Skilled microbiology laboratory technologist/technicians on handling and culture, identification of *Campylobacter, Enterococci, Salmonella* and *E. coli*
2. Skilled microbiology laboratory technologist/technicians on performing antimicrobial susceptibility testing (AST) for *Campylobacter, Enterococci, Salmonella* and *E. coli*
3. Laboratory technologist/technicians well versed with SOPS
4. Laboratory technologist/technicians well versed with surveillance plans and processing of samples
5. Laboratory technologist/technicians trained on report of the results.

mCCDA plates, XLD plates, MaC plates

Processing of whole meat sample



Demonstration on cecal samples processing and direct plating

8.4 Hands-on training on internal quality control and maintenance of ATCC reference cultures

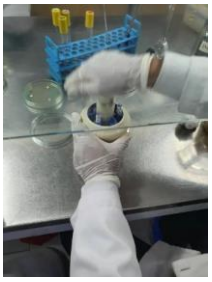
One of the main objectives of Fleming Fund Project is to strengthen microbiology laboratory capacity for AMR diagnostics at the surveillance laboratories. The capacity of these laboratory needs to be enhanced and facilitated to identify, isolate and perform Antibiotic Sensitivity Test (AST) on WHO identified GLASS (Global AMR surveillance system) pathogens such as *Campylobacter spp, Enterococci spp, Salmonella spp and E. coli*. In performing the diagnostic tests

the control strains has to be used and ran parallel to the tests. The American Type Cell Culture control strains are used as controls for identification and antibiotic susceptibility testing by most of the international reference laboratories. These ATCC controls strains are available for purchase and can be cultured and maintained for use over the years. Hence, training on culture and maintenance of ATCC organisms is extremely important and focused on culture, usage, maintenance and storage of ATCC organism. In addition, performance of antibiotic susceptibility testing, interpretation and recording of the Quality control ranges. Hence, a three days hands-on-training on culture and maintenance of ATCC reference strains was conducted at NFTL, Yusipang from 24 to 26 of June 2021. The training focused on culture, usage, maintenance and storage of ATCC organism. In addition, performance of antibiotic susceptibility testing, interpretation and recording of the Quality control ranges. The participants were laboratory technologist and technicians from BAFRA (National Food Testing Laboratory). The training was conducted by Ms. Puspa Maya Sharma, microbiologist/Sr. Laboratory Officer from NCAH.

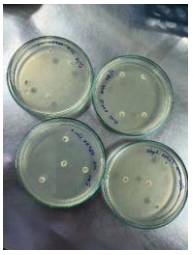
The main objectives of the workshop were:

1. To review and finalize the standard operating procedure for internal quality control and maintenance of ATCC reference cultures.
2. Skilled microbiology laboratory technologist/technicians on culture and maintenance of ATCC control strains
3. Laboratory technologist/technicians well versed with SOP and record maintenance.

a. Preservation in 20% glycerol



b. Dispersion of antibiotic disks



c. MHA plates with antibiotic discs

8.5 Hands on training to laboratory and field staffs on sample collection, packaging and transportation" on ceaca samples from poultry for AMR surveillance

Puspa Maya Sharma, Sr. Laboratory Officer,

Hands on training on sample collection, packaging and transportation of caeca samples for AMR surveillance was conducted on18-22nd October 2020 for the laboratory staff at Paro.

The main objectives of the workshop were:

1. To improve skills of veterinary officers and laboratory staffs on sample collection, packaging and transportation.
2. Sensitization on SOPS
3. Sensitization on surveillance plans and processing of samples

Sample collection:

Why caecum sample is preferred?

Collecting whole caeca is preferred: Representative of on-farm antimicrobial use as there is less opportunity for environmental contamination of samples. Collecting whole caeca is preferable to taking a swab of the caecal content as the higher volume of material in the whole caecum is likely to increase the chance of detecting the bacteria of interest if they are present.

Sample collection method:

Collect whole intact-full caeca from healthy animals within 10 min of slaughter.

1. Identify the caecum
2. Tight the intestinal area at two points above the caecum. Cut the intestinal area with scissors in between the tighten points to prevent spread of faecal contents.
3. Hold the caecum with forceps and pour the whole caecum into sterile plastic bag by cutting the tip of the caecum.

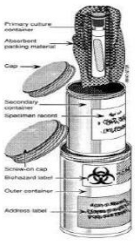
Put the caecum into sterile plastic bag and place the sample at 4°C immediately

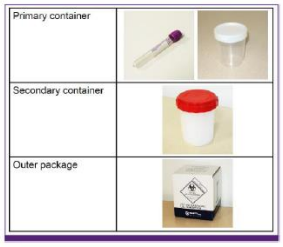
Steps involved in caeca sample collection:

Sample Packaging

1. Samples must be packed in a primary and secondary container/leak proof zipper bag.
2. Double layer of protection is required to ensure that the biological contents in the container do not contaminate other samples or environment in case of leakage or spillage. The recommended procedure for packing caeca is as follows:
3. Both the caeca are put in a primary container (zip-lock plastic bag) and placed in a well-labelled secondary container (zip-lock plastic bag)/ any other leakproof container
4. The secondary packaging is placed in an outer container; this will be cool boxes (foam boxes) / UN boxes with ice gel packs.

Ensure that there is no direct **contact between the ice and the sample**.





Sample Transportation

1. The samples should be sent safely to the laboratory as soon as possible by the fastest available means.
2. The samples should ideally be transported immediately or within 24 hours under refrigerated condition. If not, they must be stored in a refrigerator at 4-8° C and transported to the laboratory the next day.
3. The laboratory analysis should begin immediately after the sample reaches the laboratory.
4. All the samples sent to the laboratory should strictly comply with packaging instruction mentioned above.

9. PUBLICATIONS

9.1 Analysis of pigs mortality at National Nucleus Pig Breeding Centre, Yusipang

NK Thapa[1], Puspa Maya Sharma[1], Menuka Rai[2] and RB Gurung[1]

[1]National Centre for Animal Health, Serbithang, Thimphu [2] National Nucleus Pig Breeding Centre, Yusipang, Thimphu,

ABSTRACT: A retrospective study was conducted to understand the extent and causes of mortality in pigs at the National Nucleus Pig Breeding Centre, Yusipang. This study used

secondary data on pig deaths, observed clinical signs recorded and molecular laboratory reports to assess the extent and causes of mortality. The data were analysed descriptively using Microsoft excel. The farm average mortality rate was recorded at 6.78 in 2018-19. Highest mortality rate was recorded in adult pigs (13.3%), followed by sucklers (5.9%), weaners (5%) and growers (2.9%). Seasonally, the highest mortality was recorded between October to January and December to February in the sucklers and weaners, respectively. Majority of the mortality in sucklers (97.6%) and weaners (67.7%) are recorded as sudden death. Similarly, a large number of growers (31%) are also recorded to have died suddenly, followed by lameness (24.1%), Blue ear (20.7%). In the adult group, highest case reported was associated with Chronic illness and weakness (27.3%) followed by digestive related illness (21.2%), high respiration (15.2%) and reproductive problems (12.1%). As per the necropsy findings, cause of highest death was due to hepatic disorder (28.9%) followed by cardiopathy (15.6%), and respiratory infection and septicaemia (12.5%). The main isolates in the bacterial infection and septicaemia were *Escherichia coli, Erysipelothrix rhusiopathiae, Klebsiella, Streptococcus and Staphylococcus. Salmonella* was isolated from diarrhoeal cases in piglets in the month of May 2019. Similarly, *Staphylococcus hyicus, Streptococcus, Corynebacterium, Actinomyces, Actinobacillus and Escherichia coli* were also isolated from animals with reproductive disorder. Molecular analysis confirmed negative against ASF, CSF, PRRS and Brucellosis. The three main steps in biosecurity measures - segregation, cleaning and disinfection needs to be strictly instituted. NNPBC should have adequate space for isolation of the sick animals, proper water supply for cleaning and adequate stock of disinfectants for routine disinfection. Additionally, proper health monitoring of the animals also needs attention. Keywords: Hepatic; pig; molecular analysis; mortality; Suckler; weaner.

(Publications accepted in BJAS and submitted online)

Bhutan Journal of Animal Science (BJAS) Volume 5, Issue 1, Page 108-112, 2021 108

9.2 Case report cerebral cysticercosis in a wild bengal tiger *(panthera tigris tigris)* in Bhutan

Yoenten Phuentshok[a,*], Kinley Choden[b,*], Cristian Alvarez Rojas[c], Peter Deplazes[c], Sonam Wangdi[b], Kuenzang Gyeltshen[b], Nirmal Kumar Thapa[a], Karma Rinzin[d], Dechen Dorjee[e], Marc Valitutto[f], Martin Gilbert[g], Boripat Siriaroonrat[h], Waleemas Jairakh[h], Chutchai Piewbang[i,j], Kannika Phongroop[i,j], Somporn Techangamsuwan[i,j], Puspa Sharma[a], Tenzinla Tenzinla[a], Tshewang Dema[a], and Ratna Bahadur Gurung[a]

[a]*National Centre for Animal Health, Department of Livestock, Ministry of Agriculture and Forests, Serbithang, Bhutan*
[b]*Nature Conservation Division, Department of Forests and Park Services, Ministry of Agriculture and Forests, Taba, Bhutan*
[c]*Institute of Parasitology, Veterinary and Medical Faculty, University of Zurich, Zurich, Switzerland*
[d]*Animal Health Division, Department of Livestock, Ministry of Agriculture and Forests, Thimphu, Bhutan*

[e]*World Wildlife Fund country office, Kawajangsa, Thimphu, Bhutan*
[f]*Global Health Program, Smithsonian Conservation Biology Institute, Washington, DC 20008, United States of America*
[g]*Department of Population Medicine and Diagnostic Sciences, Cornell University, College of Veterinary Medicine, Ithaca, New York 14853, United States of America*
[h]*Bureau of Conservation and Research, Zoological Parks Organization, Bangkok, Thailand*
[i]*Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand*
[j]*Animal Virome and Diagnostic Development Research Group, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand*
* *Joint first authors*

Abstract

Infectious causes of mortality have received little attention in free-ranging wild tigers. We present a case of neurocysticercosis in a wild Bengal tiger (*Panthera tigris tigris*) that presented with advanced neurological disease outside the city of Thimphu in Bhutan during March 2018. Despite supportive care in captivity, the tiger subsequently died, and two intracerebral cysts were observed during post mortem examination. Samples were analysed for the presence or exposure to pathogens or potential pathogens of felids, particularly
those associated with neurological disease or immunosuppression including canine distemper virus, Nipah virus, rabies virus, Japanese encephalitis virus, feline morbillivirus, feline calicivirus, feline herpesvirus, feline leukemia virus, feline immunodeficiency virus and *Toxoplasma gondii*. All results were negative with the exception of feline herpesvirus confirmed by PCR and antibody titers to *Toxoplasma gondii* (titer 1:256). DNA extracted from the cysts were amplified using primers for COX1 and NADH and sequenced amplicons confirmed the presence of a Taeniid cestode. Further sequencing of the complete COX1 and CYTB genomes of the parasite revealed 98.7% and 98.31% homology with the corresponding sections of the complete genome of a *Taenia solium* sequence from the Asian group (AB086256). Eggs harvested by fecal floatation were identified using a published multiplex PCR and amplification of the partial NADH gene and identified as *T. hydatigena* and *T. regis*. The confirmation of encysted *T. solium* together with the neurological presentation supports the diagnosis of neurocysticercosis, an infection previously unrecorded in tigers or other non-domestic felids.
Published in International Journal of parasitology: Parasites and Wildlife 14(2021) 150-156

9.3 Occurrence of *Echinococcus granulosus sensu lato* and other taeniids in Bhutan

Puspa M. Sharma, Nirmal K. Thapa, Pema Tshomo, Tshewang Dema, Cristian A. Alvarez Rojas, Tenzin Tenzin, Ratna B. Gurung, Tshering Norbu, Lhatru Lhatru, Phurpa Namgyel, Chimi Jamtsho, Kinzang Drukpa, Yoenten Phuentshok, Krishna P. Sharma, Sonam Pelden and Peter Deplazes.

Abstract: The present research shows the results of a national study documenting the occurrence and genetic diversity of Echinococcus and Taenia species across Bhutan. Environmental dog faecal samples (n = 953) were collected from 2016 to 2018 in all 20 Bhutanese districts, mainly in urbanised areas. Cystic echinococcosis cysts were isolated from 13 humans and one mithun (*Bos frontalis*). Isolation of taeniid eggs from faeces was performed

by sieving/flotation technique, followed by DNA isolation, PCR and sequence analyses for species identification (gene target: small subunit of ribo- somal RNA, rrnS). Haplotype diversity of *E. granulosus s.s.* was based on the sequence (1,609 bp) of the cox1 gene. A total of 67 out of 953 (7%) dog faecal samples were positive for at least one taeniid species. From the 670 free-roaming dog faecal samples, 40 (5.9%) were positive for taeniid DNA, 22 (3.2%) of them were identified as *E. granulosus s.s.* and four (0.5%) as *E. ortleppi* (G5). From the 283 faecal samples originating from yak-grazing areas, 27 (9.5%) were taeniid positive, including eight (2.8%) infected with *E. granulosus s.s.* and four (1.4%) with *E. ortleppi*. *E. granulosus s.s.* was identified in all isolates from human and the cyst from mithun. A haplotype network (cox1 gene) from *E. granulosus s.s*, including isolates from 12 dogs, two human and one mithun, revealed eight different haplotypes. The most common cox1 haplotype was the globally distributed Eg01, followed by Eg40 and Eg37 (previously described in China). Five new cox1 haplotypes (EgBhu1–5) originated from human, dogs, and a mithun were identified. The study indicated the contamination of urban areas and pastures with Echinococcus eggs in seven districts in Bhutan. The molecular characterisation of *E. granulosus s.l.* revealed different *E. granulosus s.s.* haplotypes as well as *E. ortleppi*. The transmission of *T. multiceps* was documented only in the western part of the country. Considering the zoonotic feature of *E. granulosus s.s.* and *E. ortleppi* and the economic impact of coenurosis caused by *T. multiceps* (also known as gid) in Bhutan, the findings of this study represent a significant contribution towards an epidemiological baseline for the establishment of a national control program.

Pathogens 2021,10,330 https://doi.org/10.3390/pathogens 10030330