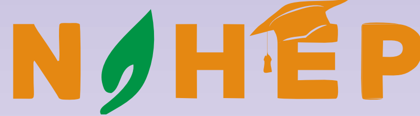

Compendium

Certificate Course

Under

IDP-NAHEP

on

Microbiological Diagnostic Techniques for Foodborne and Zoonotic Diseases

Organized by

College of Veterinary Science & Animal Husbandry

U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan (DUVASU), Mathura-281001 (U.P.), INDIA

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MOLECULAR TECHNIQUES FOR DETECTION OF VIRAL FOOD BORNE PATHOGENS

Sabapathi N. and Sameer Shrivastava

ICAR-Indian Veterinary Research Institute, Izatnagar- 243122

1. Introduction

Viral food borne pathogens now emerging threat in human and animal community. Food borne virus are majorly of RNA virus with icosahedral capsid and without envelope to sustain and invade in gastric environment as result majorly causes gastroenteritis. Common food borne virus are Human norovirus (HuNoV), Human rotavirus (HRV), Hepatitis A virus (HAV), Hepatitis E virus (HEV), Human astrovirus (HAstV), Aichi virus (AiV), Sapovirus (SaV), Human adenovirus (HAdV) and Enterovirus (EV). Among all norovirus is common and causes all three illness, hospitalization and death according to the Centers for Disease Control and Prevention. And these infections transmitted through the fecal–oral route by contaminated food and water, via person to-person, via contact with contaminated surfaces, and through aerosols. Early detection and screening of food sources play vital role to prevent the spread of food borne infections. Due to widely distinguished family of viral food pathogens, various modes of contaminated food as samples makes detection hard, time and cost consuming traditional method of diagnosis such as cultivate, isolate, enumerate the target microbe and electron microscopy. Over this traditional detection, recent molecular techniques which is based on nucleic acid identification make detection part quicker and more reliable (Gomara and Sarah, *et al.,* 2016).

2. Characteristics of Foodborne Viral Infections

Majority of food borne virus are RNA in nature, which leads to often mutation in nucleic acid sequence. Eventually leads to development of new strains and outbreaks. Viral family includes Human norovirus (HuNoV), Human rotavirus (HRV), Hepatitis A virus (HAV), Hepatitis E virus (HEV), Human astrovirus (HAstV), Aichi virus (AiV), Sapovirus (SaV), Human adenovirus (HAdV) and Enterovirus (EV). Food borne viruses are icosahedral structure and devoid of envelope these majorly make viruses extraordinarily resilient to environmental factors like heat and low pH (acidity). The foods that are most frequently linked

to foodborne viruses are undercooked meats, fruit and vegetables that are cultivated on soil that has been fertilized with animal excreta, and shellfish that is collected close to sewage outlets for humans. Foodborne viruses are excreted in large quantities through emesis or faeces and have their origins in the human intestine. The transmission of viral diseases is facilitated by their low infectious dosage and strong survival in food and other surroundings (D'Souza and Joshi, 2016)

Virus	Virus family	Genome	Host	Symptoms	Main route of transmission	Documented frequency of foodborne transmission	Source of food contamination
Norovirus	*Caliciviridae*	ssRNA	Human	Gastroenteritis	Faecal–oral Person-to-person Water and the environment	High	At source (faecal contamination) By infected food handler
Sapovirus	*Caliciviridae*	ssRNA	Human	Gastroenteritis	Faecal–oral Person-to-person	Rare	At source (faecal contamination) By infected food handler
Astrovirus	*Astroviridae*	ssRNA	Human	Gastroenteritis	Faecal–oral Person-to-person Water and the environment	Rare	At source (faecal contamination) By infected food handler
Aichi virus	*Picornaviridae*	ssRNA	Human	Gastroenteritis	Faecal–oral Water and the environment	Rare	At source (faecal contamination) By infected food handler
Rotavirus	*Reoviridae*	dsRNA	Human	Gastroenteritis	Faecal–oral Person-to-person	Rare	At source (faecal contamination) By infected food handler
Hepatitis A virus	*Picornaviridae*	ssRNA	Human	Hepatitis	Faecal–oral Person-to-person Water and the environment	Increasingly recognised	At source (faecal contamination) By infected food handler
Hepatitis E virus	*Hepeviridae*	ssRNA	Human	Hepatitis	Faecal–oral Water and the environment	Low/unknown	At source (faecal contamination)
			Zoonotic		Faecal–oral?	Unknown	Contaminated pork products

.

3. List of important food viral outbreaks

Brief list of some food viral outbreaks and samples collected during outbreaks

Virus type	Year	Sample type	Contamination source/location	Detection method
Norovirus	2013	Food	Unknown source (tourists), Yellowstone National Park	Epidemiology
	2012	Stool sample	Imported frozen raspberries	Epidemiology
	2011	Clinical, food, environmenta	Pasta, raw vegetables/French military unit	RT-PCR
	2009	Stool	Salad/German military base	RT-PCR
	2009	Clinical	Water resort	RT-qPCR, epidemiology
	2009	Water	Cruise ship	RT-qPCR
	2008	Food and clinical	Municipal drinking water/Sweden	Epidemiology
		Clinical	Deli meat	Nested RT-PCR; sequencing
	2008	Food and clinical	University dining hall	Epidemiology; RT-PCR
	2003		Oysters	Epidemiology; RT-PCR
Hepatitis A virus	2013	Food	Frozen berries in smoothies/Nordic countries	Epidemiology
	2013	Food	Antioxidant frozen berry and pomegranate mix	Epidemiology
	2011	Food	Sundried tomatoes	Epidemiology
	2011	Serum	Sushi bar, Japan	RT-PCR
	2009	Serum	Sun dried tomatoes	RT-PCR
	2009	Serum	Well water	Epidemiology, detection of anti-IgM
	2008	Serum/stool	Thai navy base	Detection of anti-IgM, RT-PCR
	2004	Clinical	Orange juice/Egyptian restaurant	RT-PCR
	2003	Serum	Green onions	Epidemiology, RT-PCR
Hepatitis E virus	2011	Serum	Italy	RT-PCR, detection of IgG and IgM by immunoassay
	2011	Food	Shellfish/England	RT-PCR
	2007	Blood	Uganda	RT-PCR
Rotavirus	2012	Clinical	Réunion, French island	RT-PCR
	2011	Clinical	Retirement communities	EIA, RT-PCR
	2011	Clinical	Intensive care unit/Brazil	RT-PCR
	2007	Clinical	Pediatric hospital/Mexico	ELISA
	1997	Stool, serum	Pediatric unit/toys	Epidemiology, EIA

4. Molecular techniques for detection of viral food borne pathogens

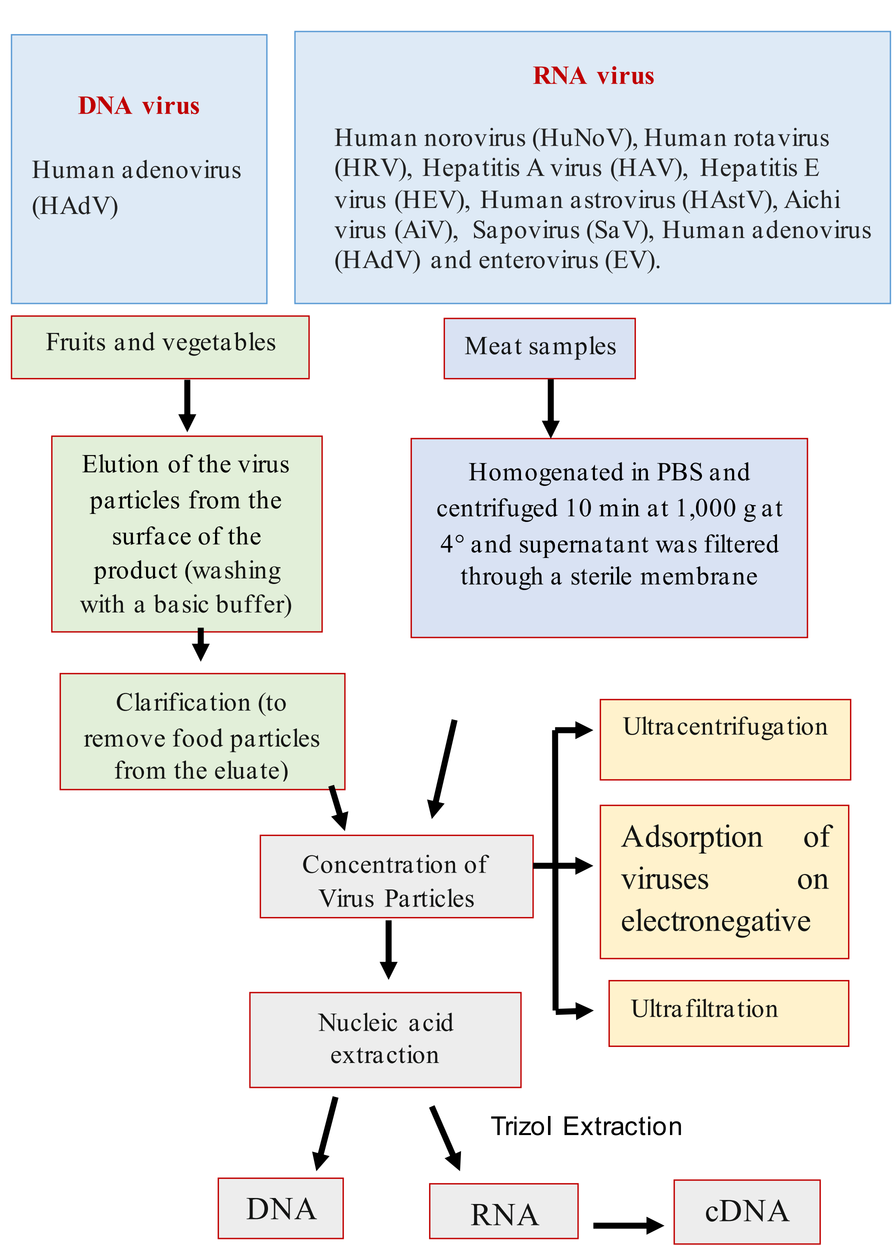
The target pathogen's specific DNA or RNA sequences are identified by nucleic acid-based approaches. To do this, a synthetic oligonucleotide (probes or primers) complementary to the target sequence is hybridised with the target nucleic acid sequence. Nucleic acid-based approaches identify the precise genes in the target pathogens, preventing unclear or incorrectly interpreted data. Molecular techniques are majorly targeting on nucleic acids of organism, such as

- Polymerase chain reaction (PCR)
- Real time polymerase chain reaction
- Nucleic acid sequence-based amplification (NASBA)
- Loop-Mediated Isothermal Amplification (LAMP)
- Transcription mediated amplification
- Microarrays
- Sequencing

5. Sample preparation

Recent advancement in molecular techniques made easy in viral detection from food and water sources. Critical part of detection is sample preparation and concentration of virus in sample. Constrains like large volume of sample, concentration of virus on surface will be less on samples washing from fruits and vegetable. In sewage contamination sample there will be presence of secondary viral DNA when we are going for nucleic acid isolation. It may be overcome by choosing proper method processing sample and followed by nucleic acid isolation.

Virus separation and concentration methods such as Elution techniques followed by concentrating step, Direct extraction of nucleic acid, Proteinase K treatment, Ultracentrifugation, Filtration – charged filters, Ultrafiltration and Flocculation. Further elution from sample contains less concentration of virus particles and use elution volume some concentration of virus methods that include Polyethylene glycol (PEG) precipitation, Two-phases separation, Evaporation, Ultracentrifugation, Ultrafiltration, Filtration based on charge, Flocculation, Cationic separation and anion exchange resin-based method, immunological and receptor binding methods and Celite concentration (Mattison and Bidawid, 2009).



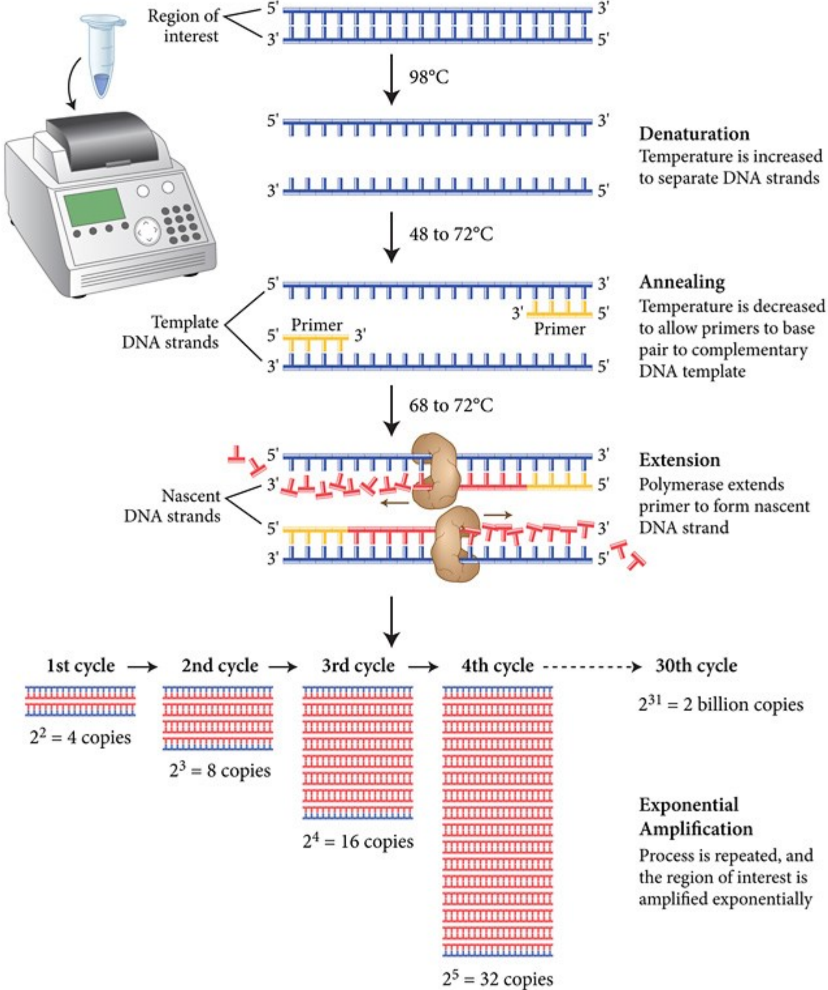
Schematic of sample preparation and detection

6. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) based assays are the most reliable methods for viral detection now a day. The components of PCR include template, two sets of primers, free nucleotide bases, and the DNA polymerase enzyme. The DNA template contains the specific region that you wish to amplify, such as the DNA extracted from viral contrate of fruit washing, food samples for example. Primers, or oligonucleotides, are short strands of DNA complementary to the 3' end of each target region. Both a forward and a reverse primer are required, one for each complementary strand of DNA. DNA polymerase is the enzyme that carries out DNA replication. Thermostable analogues of DNA polymerase I, such as Taq polymerase, which was originally found in a bacterium that grows in hot springs, is a common choice due to its resistance to the heating and cooling cycles necessary for PCR.

PCR takes advantage of the complementary base pairing, double-stranded nature, and melting temperature of DNA molecules. This process involves cycling through 3 sequential rounds of temperature dependent reactions: DNA melting (denaturation), annealing and enzyme-driven DNA replication (elongation). Denaturation begins by heating the reaction to about 95 C, disrupting the hydrogen bonds that hold the two strands of template DNA together. Next, the reaction is reduced to around 50 to 65 C, depending on the physicochemical variables of the primers, enabling annealing of complementary base pairs. The primers, which are added to the solution in excess, bind to the beginning of the 3' end of each template strand and prevent re-hybridization of the template strand with itself.

Lastly, enzyme-driven DNA replication begins by setting the reaction temperature to the amount which optimizes the activity of DNA polymerase, which is around 75 to 80 C. At this point, DNA polymerase, which needs double-stranded DNA to begin replication, synthesizes a new DNA strand by assembling free-nucleotides in solution in the 3' to 5' direction to produce 2 full sets of complementary strands. The newly synthesized DNA is now identical to the template strand and will be used as such in the progressive PCR cycles. Given that previously synthesized DNA strands serve as templates, the amplification of DNA using PCR increases at an exponential rate, where the copies of DNA double at the end of each replication step. The exponential replication of the target DNA eventually plateaus around 30 to 40 cycles mainly due to reagent limitation, but can also be due to inhibitors of the polymerase reaction found in the sample, self-annealing of the accumulating product, and accumulation of pyrophosphate molecules (Watzinger *et al.*, 2006).

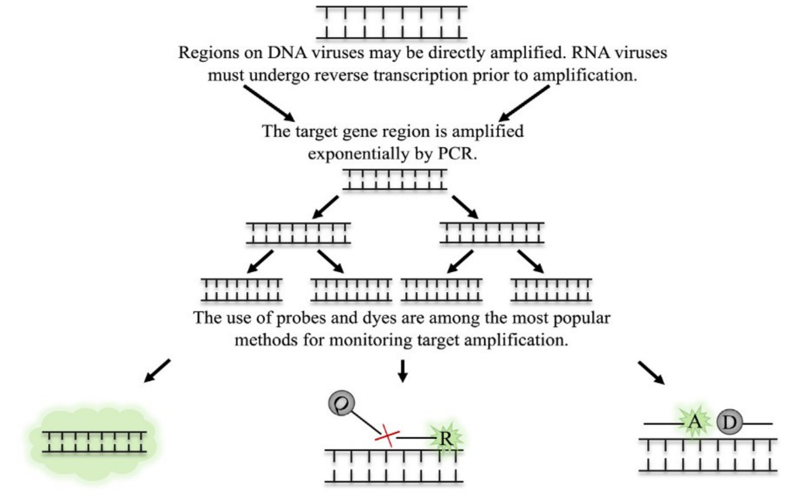


Schematic of the PCR. (Source URL: http://www.neb.com)

7. Real time polymerase chain reaction

Real time quantitative PCR (real time qPCR) measures the amplification of the target amplicon throughout the reaction. This is facilitated by DNA intercalating dyes, such as SYBR®green, or fluorescently labelled probes. RQ-PCR continuously measure the accumulation or reduction of fluorescence

signals during the amplification reaction. In contrast to quantitative PCR approaches based on data analysis at the end of PCR, the real-time PCR technology permits the detection of the number of amplicons generated during each amplification cycle in a real-time mode (=kinetic quantitative PCR). This technique has eliminated the need of post-amplification handling of the samples and has paved the way towards fully automated detection systems (Watzinger *et al.*, 2006).

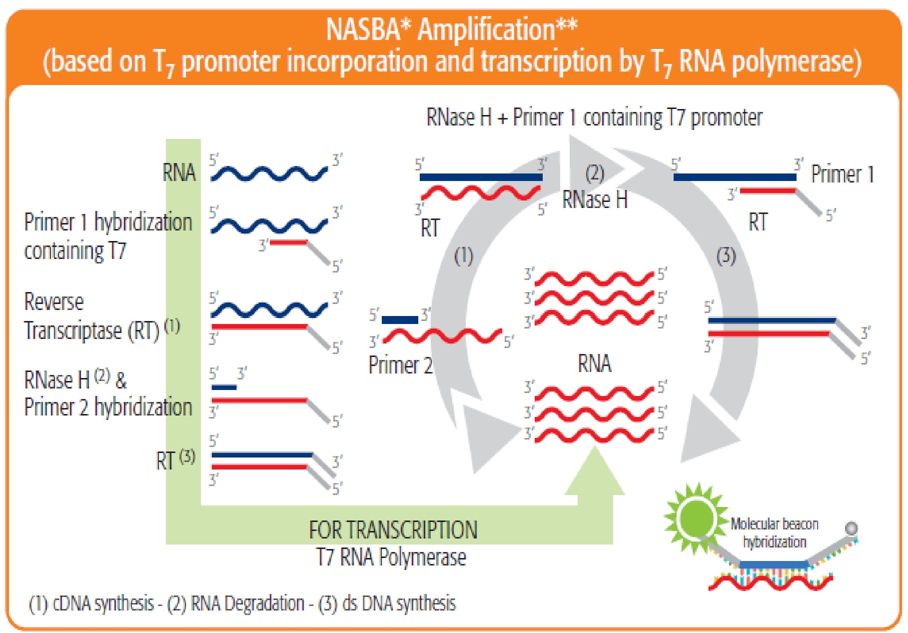


8. Nucleic acid sequence-based amplification (NASBA)

NASBA amplification system first employed in 1991 and optimized for the detection of HIV-1 sequences (detection level were 10 molecules of HIV-1 RNA). NASBA readily adaptable for detection of RNA viruses in both foods and clinical samples. Transcription-dependent amplification technique that uses promoter primers to recognize specific target sequences and synthesize RNA amplicons (Uyttendaele *et al.,* 1995).

Three viral enzymes that work together to amplify RNA targets are

- Reverse transcriptase,
- Rnase H, and
- T7 RNA polymerase



Amplifying nucleic acids under isothermal conditions, unlike PCR which requires thermocycling system. Post amplification Analysis of NASBA products can be done by

- Denaturing agarose gel electrophoresis,
- Northern blotting and
- Molecular beacons

9. Difference between conventional PCR and NASBA

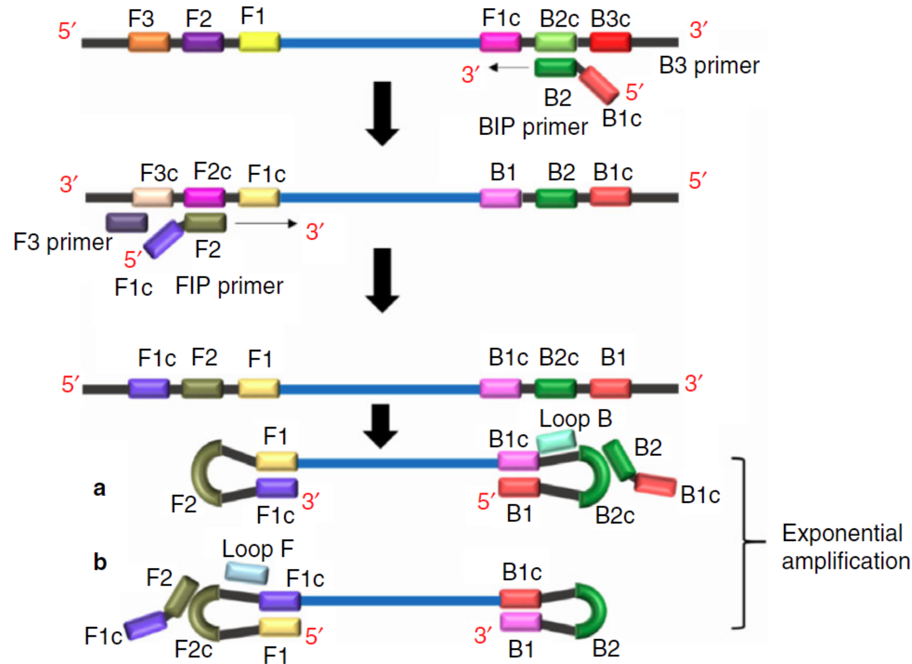
	PCR	NASBA
Target:	dsDNA	RNA, ssDNA
Initial process	RNA by reverse transcriptase step	dsDNA by prior heat denaturation
Phases	Three phases: • Denaturation (92–97°C, 1–2 min) • Hybridization (40–60°C, 1–2 min) • Extension (72°C, 1–2 min)	Two phases: • Conversion of RNA to dsDNA • Transcription
Reaction condition	Thermocycling required: 25–45 cycles	Isothermal reaction: 41°C, 90 min
Enzymes involved	One thermostable enzyme: • Taq DNA polymerase	Three enzymes: • AMV-reverse transcriptase • RNase H • T7-RNA polymerase
Primers	Two specific primers	Two specific primers with primer 1 (antisense, complementary) holding a T7-RNA polymerase promotor site
Amplification factor:	2^n n = number of cycles	x^n n = number of hypothetic cycles x = RNA copies generated during transcription (10–1000)
Amplification product:	ds DNA	antisense (3′–5′), complementary ssRNA

10. Loop-Mediated Isothermal Amplification (LAMP)

LAMP method was developed as an alternative method for viral detection. LAMP is a powerful and novel nucleic acid amplification method that amplifies a few copies of target DNA with high specificity, efficiency, and rapidity under isothermal conditions, using a set of four specially designed primers and a DNA polymerase with strand displacement activity. To detect RNA virus, LAMP has been merged with reverse transcription (RT) into RT-LAMP, which is completed in a single step, by incubating all the primers and enzymes (*Bst* polymerase and reverse transcriptase) with a constant temperature. Four different primers specially designed to recognize six distinct regions of the target DNA.

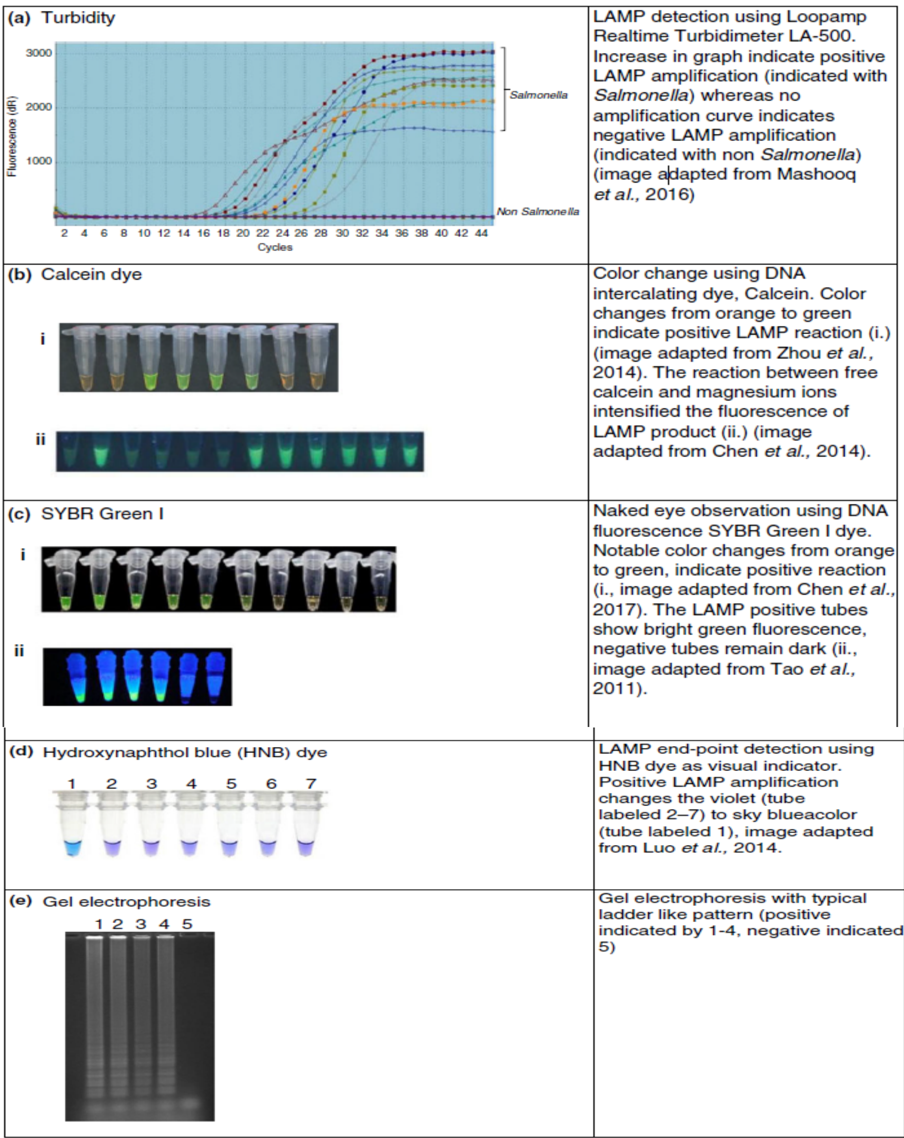
Primers

1. Forward Inner Primer (FIP) consists of a F2 region at the 3'-end and an F1c region at the 5'-end.
2. Forward Outer Primer (F3 Primer) consists of a F3 region which is complementary to the F3c region of the template sequence.
3. Backward Inner Primer (BIP) consists of a B2 region at the 3'-end and a B1c region at the 5'-end.
4. Backward Outer Primer (B3 Primer) consists of a B3 region which is complementary to the B3c region of the template sequence.



Stem-loop structure (refer a and b) serves as an initiator for LAMP cycling. Loop primers can be added as well for exponential amplification of LAMP. The final products obtained are a mixture of stem-loop DNA with various stem lengths and various cauliflower-like structures with multiple loops (Wong *et al*., 2017)

Various LAMP end-point detection method are showed in below table

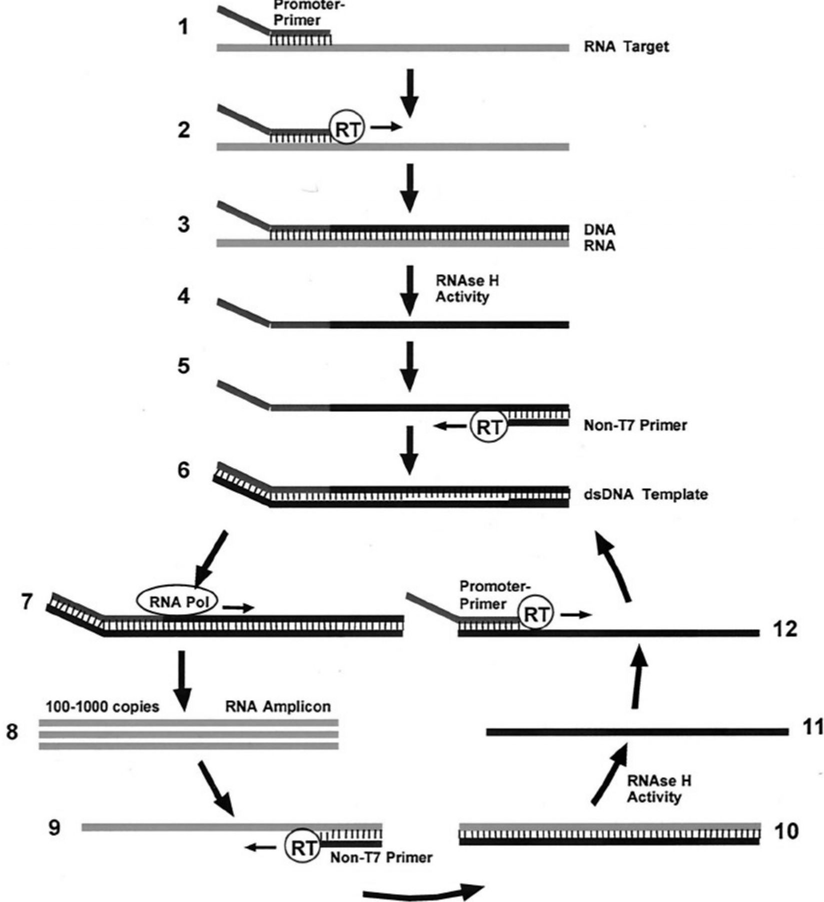


Method	Description
(a) Turbidity	LAMP detection using Loopamp Realtime Turbidimeter LA-500. Increase in graph indicate positive LAMP amplification (indicated with *Salmonella*) whereas no amplification curve indicates negative LAMP amplification (indicated with non *Salmonella*) (image adapted from Mashooq *et al.*, 2016)
(b) Calcein dye	Color change using DNA intercalating dye, Calcein. Color changes from orange to green indicate positive LAMP reaction (i.) (image adapted from Zhou *et al.*, 2014). The reaction between free calcein and magnesium ions intensified the fluorescence of LAMP product (ii.) (image adapted from Chen *et al.*, 2014).
(c) SYBR Green I	Naked eye observation using DNA fluorescence SYBR Green I dye. Notable color changes from orange to green, indicate positive reaction (i., image adapted from Chen *et al.*, 2017). The LAMP positive tubes show bright green fluorescence, negative tubes remain dark (ii., image adapted from Tao *et al.*, 2011).
(d) Hydroxynaphthol blue (HNB) dye	LAMP end-point detection using HNB dye as visual indicator. Positive LAMP amplification changes the violet (tube labeled 2–7) to sky bluecolor (tube labeled 1), image adapted from Luo *et al.*, 2014.
(e) Gel electrophoresis	Gel electrophoresis with typical ladder like pattern (positive indicated by 1-4, negative indicated 5)

11. Transcription mediated amplification (TMA)

TMA is an isothermal, autocatalytic target amplification method that has the potential to detect less than 50 RNA copies/mL. Promoter-primer hybridizes to the target DNA after heat denaturation, and the RNA polymerase creates a transcript of the target DNA. A second primer then binds to the

transcript, and the reverse transcriptase creates cDNA. The RNA in the resulting RNA-DNA duplex is degraded by the RNase H activity of the reverse transcriptase. The promoter-primer then binds to the cDNA and a new DNA is synthesized by reverse transcriptase, creating a double-stranded DNA molecule. The RNA polymerase recognizes the promoter sequence in the double-stranded DNA and synthesizes a number of RNA transcripts. Each of the newly synthesized RNAs reenters the TMA process and serves as a template for a new round of replication. RNA amplicons are detected by a hybridization protection assay with amplicon-specific acridinium ester labeled DNA probes, chemiluminescence is measured in a luminometer and signals are expressed numerically in relative light units (Meyer *et al.,* 2020)



Cycle of transcription-mediated amplification

12. Microarrays

Microarrays were originally used for the study of gene expression, but oligonucleotide DNA microarray has been widely used in the field of foodborne pathogen detection. DNA microarray capable of simultaneously detecting hundreds of viruses. Microarrays are made up of glass slides or chips coated with up to hundreds of specific oligonucleotide probes and these probes are chemically synthesized short sequences range from 25 to 80 bp. Each oligonucleotide probe is able to target a specific part of a gene sequence. Sample nucleic acid fragments (DNA, mRNA or cDNA) are labeled with fluorescent dye, and then they are denaturated to generate single-stranded fragments. These fragments will hybridize to the array through binding to their corresponding oligonucleotide probes. The results are obtained through the visualization of the fluorescence signal produced from the probe-sample complex. The fluorescence intensity is proportional to the concentration of each labeled nucleic acid fragment (Wang *et al.*2002).

13. Next-Generation Sequencing (NGS)

NGS is being introduced into the diagnosis of viral infections, using a metagenomics approach based on random PCR amplification to identify viral genomes present in samples. Most of food borne viruses are rapidly evolving RNA virus, and so NGS based detection more accuracy method WGS was used to analyze genomic variations among three linked patients. Characterization of infectious agents using NGS has been proven to be a powerful tool allowing for the development of fast PCR-based molecular assays.

PARTICULARS	SEQUENCING TECHNOLOGIES	
First - generation	**Basic method** • Chain termination • Chemical cleavage method	**Large scale sequencing and de nova sequencing** • Shotgun sequencing
Second - generation sequencing	**Sequensing by synthesis** • Pyrosequencing (Roche/454) • Reversible terminator (Illumina) • Ion proton semiconductor (Life Technologies) • Zero Mode Waveguide (Pacific Biosciences)	**Sequencing by Ligation** • Sequencing by hybridisation and ligation (SOLiD /Applied Biosystems) • DNA nanoballs
Third - generation sequencing	**Long Read sequencing method** • Single molecule real time (PacBio SMART) • Nanopore DNA sequencing (Oxford Nanopore)	**Short read sequencing method** • Helicos™ Single Molecule Sequencing (SMS) • GnuBIO platform
Fourth-generation sequencing	Nanopore-based sequencing (Silicon nanopores)	

Comparison of next-generation sequencing methods

Method	Single-molecule real-time sequencing (Pacific Bio)	Ion semiconductor (Ion Torrent sequencing)	Pyrosequencing (454)	Sequencing by synthesis (Illumina)	Sequencing by ligation (SOLiD sequencing)	Chain termination (Sanger sequencing)
Read length	2900 bp average[38]	200 bp	700 bp	50 to 250 bp	50+35 or 50+50 bp	400 to 900 bp
Accuracy	87% (read length mode), 99% (accuracy mode)	98%	99.90%	98%	99.90%	99.90%
Reads per run	35–75 thousand [39]	up to 5 million	1 million	up to 3 billion	1.2 to 1.4 billion	N/A
Time per run	30 minutes to 2 hours [40]	2 hours	24 hours	1 to 10 days, depending upon sequencer and specified read length[41]	1 to 2 weeks	20 minutes to 3 hours
Cost per 1 million bases (in US$)	$2	$1	$10	$0.05 to $0.15	$0.13	$2400
Advantages	Longest read length. Fast. Detects 4mC, 5mC, 6mA.[42]	Less expensive equipment. Fast.	Long read size. Fast.	Potential for high sequence yield, depending upon sequencer model and desired application.	Low cost per base.	Long individual reads. Useful for many applications.
Disadvantages	Low yield at high accuracy. Equipment can be very expensive.	Homopolymer errors.	Runs are expensive. Homopolymer errors.	Equipment can be very expensive.	Slower than other methods.	More expensive and impractical for larger sequencing projects.

RT-PCR products for sample were purified with silica-based membranes purification system and sequenced using suitable sequencer. Number of different NGS platforms using different sequencing technologies. NGS platforms perform sequencing of millions of small fragments of DNA in parallel. Bioinformatics analyses are used to piece together these fragments by

mapping the individual reads to the reference genome (Goodwin, McPherson and McCombie, 2016.).

Conclusion

In conclusions, most of food borne virus are RNA in nature. Molecular detections were more reliable and rapid techniques, which fundamentally involves in targeted amplification and detection of pathogen nucleic acid.

DIAGNOSTIC TECHNIQUES FOR ZOONOTIC PARASITES WITH SPECIAL REFERENCE TO NEUROCYSTICERCOSIS AND HYDATIDOSIS

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Introduction

Cysticercus cellulosae, the metacestode stage of the tape worm *Taenia solium* causes cysticercosis in pigs and neurocysticercosis (NCC) in human beings. The condition is endemic in pigs and humans in many developing countries of Latin America, Africa and Asia including India. Cysticercosis is classified as a List B disease by the Office International Epizootics (OIE) and about 50 million people are infected globally leading to about 50,000 deaths annually. Clinical neurocysticercosis in humans have been documented from various parts of India reflecting its high prevalence in the susceptible population. The *C. cellulosae* infection in pigs is widespread among swine in India and its occurrence is reported from different parts of the country with an overall prevalence ranging from 4.24% to 26%. The economic importance of the infection is never less so far as animal production is concerned, as swine cysticercosis affects the carcass quality resulting in heavy economic loss. *Taenia solium* cysticerci occur in the muscles and central nervous system of pigs (pork measles), bear, dogs and cats and in the muscles, subcutaneous tissues, eyes and central nervous system of humans. Cysts have a scolex bearing a rostellum and two rows of hooks (22-36) similar to the adult. Occasionally, in the brain of humans, they develop as racemose cysts up to 2 cm or more across that lack a scolex. Since pig is the intermediate host and important source of infection for humans, diagnosis of porcine cysticercosis is critical and serves the purpose of identification of endemic regions which will help in development of strategies to control cysticercosis (Gonzalez *et al.,* 1999). Further, an unequivocal detection of cysticercosis in humans is also critical for treatment of patients (Hancock *et al.*, 2006).

Hydatidosis is an infection caused by tapeworms of the genus *Echinococcus*. Six species of *Echinococcus* have been recognized till date; among them four are of public health concern: *Echinococcus granulosus*

(which causes Hydatidosis), *Echinococcus vogeli* and *Echinococcus oligarthrus* (causing polycystic echinococcosis) and *Echinococcus multilocularis* (causing alveolar echinococcosis). The *E. granulosus* parasitizes Canids which are its definitive hosts inhabiting its small intestines. Domestic ungulates act as the intermediate hosts for the parasitic larval stage (metacestode). Based on molecular data, *E. granulosus* have been classified into several genotypes namely: a) G1, common sheep strain. b) G2: Tasmania sheep strain. c) G3: Buffalo strain. d) G4: Horse strain. e) G5: Cattle strain. f) G6: Camel strain. g) G7: Pig strain. h) G8: Cervid strain. i) G9: Human strain. (Poland) j) G10: Fennoscandian cervid strain. Cystic echinococcus (CE) occurs worldwide, but is endemic in Africa, South America, and Eurasia. *E. granulosus* is more prevalent in rural areas where sheep and goats are still slaughtered traditionally and carcass wastes are easily accessible to scavenging dogs and other wild carnivores. The liver is the most commonly affected organ; however, the lungs, spleen, kidney, brain, and breasts may be involved (Carmena and Cardona, 2013). Mortality from CE is usually due to the development of complications and is reported to be 2-4%. The disease course is typically slow and most CE patients remain asymptomatic for several years. In addition, due to non-specific symptoms, the diagnosis is often incidental. Hepatic alveolar echinococcus (AE) referring to the intrahepatic growth of the larvae of *E. multilocularis* is a rare yet serious disease. When the epidemiology of AE is analyzed, it is striking that the disease is encountered in the northern hemisphere only (Liu *et al.*, 2014). Complications of the echinococcal disease include allergic reactions to the dissemination of cyst contents due to spontaneous, traumatic or iatrogenic rupture, secondary infection, and cholangitis (Eckert *et al.,* 2001). While most CE patients have a single cyst, 20%-40% tend to harbor multiple cysts (Mihmanli *et al.*, 2016). During the last 10 years new sensitive and specific diagnostic methods and effective therapeutic approaches against Hydatidosis have been developed. Despite some recent progress in the control of Hydatidosis, it continues to be a major public health concern in several countries, and in several others, it constitutes an emerging or re-emerging disease.

Diagnostic approaches for *T. solium cysticercosis*

Currently, the ante-mortem diagnosis of porcine cysticercosis is based on lingual examination which can only detect moderate to heavy infections and requires technical skills. Moreover, the prevailing method of meat inspection for detection of the infection is poorly sensitive. Development of a specific and sensitive serodiagnostic tool against *T. solium* cysticercosis in pigs is, therefore,

important. An ante-mortem diagnosis of cysticercosis based on specific antibodies in humans and animals is essential for studying the epidemiology of *T. solium* and will aid immensely in the monitoring of infection rates during parasite control campaigns. Hence practical, rapid, and efficient ante-mortem diagnostic methods are of great significance.

Several serological techniques have been described to detect *T. solium* infection specific antibodies in humans and pigs, *viz*. radioimmunoassay, indirect hemagglutination test, double diffusion test, counter-immuno electrophoresis, complement fixation test, dipstick assay, latex agglutination, Dot-immunogold filtration assay, lymphocyte transformation test, flow through assay, ELISA and immunoblot using crude or partially purified antigens of *T. solium* prepared from cyst fluid or cyst tissue. However, these native antigen-based tests have moderate sensitivities and relatively poor specificities.

Currently among the antibody-based diagnostic assays western blot is considered as the gold standard which uses lentil lectin purified glycoproteins (LLGP) as antigen. This antigen consists of seven major diagnostic proteins of 13–50 kDa. It is the only assay recognized by the World Health Organization and the Pan American Health Organization for the serodiagnosis of cysticercosis (Pan American Health Organization, 1997). Since the LLGP antigen is a mixture of proteins and not all of them are diagnostic, it has not been successfully used in any format other than Western blot (Hancock *et al*., 2004). To overcome this limitation, Ito *et al*. (1998) developed a simple method to purify glycoproteins (GPs) using preparative isoelectric-focusing electrophoresis (IEFE) that is applicable for both immunoblot and ELISA for humans, pigs, and dogs. But ensuring the supply of the purified GPs in sufficient quantities is a major constraint because it depends on infected pigs for supplying the source material and, nowadays, strict regulations are in place for (international) transport of biological specimens. Furthermore, batch differences can exist between different antigen preparations. All these constraints could impede the application for specific diagnosis of cysticercosis (Deckers and Dorny, 2010).

Thus, the need for alternative, practical, reproducible, and economic supportive diagnostic methodologies is continuing that avoid the use of crude parasite material. The development of a single purified protein-based assays, such as recombinant antigenic proteins, would be an easy alternative for detection of infection in the susceptible host population (Hancock *et al*., 2004). In line with this, several recombinant proteins have been expressed in

different expression systems, viz. rGP50, rT24H, TsM10, Tsol-sHSP35.6, Ts14, Ag1v1/Ag2, Ts8B2, TsAg5, HP6, Ts18 etc., and have been used in ELISA with varying degrees of sensitivities and specificities.

Lee *et al.* (2011) developed a QuickELISA™ format for diagnosis of NCC. They analyzed a panel of 474 serum samples composed of 108 serum samples from donors with two or more viable cysts, 252 serum samples from persons with other parasitic infections and 114 serum samples from persons with no documented illnesses. The sensitivities and specificities of T24H QuickELISA™, GP50 QuickELISA™, and Ts18var1 QuickELISA™ were 96.3% and 99.2%, 93.5% and 98.6%, and 89.8% and 96.4%, respectively for detecting cases with multiple, viable cysts. They concluded that the T24H QuickELISA™ performed best among the three assays and had sensitivity and specificity values comparable to those of the LLGP-EITB.

However, detection of *T. solium* specific antibodies in serum only indicates exposure to the parasite and not necessarily established infection. Further, antibodies may persist long after the parasite has been eliminated by immune mechanisms and/or antiparasitic therapy. Furthermore, detection of anti-parasite antibodies in a population in an endemic village does not necessarily reflect the true prevalence, leading to misdiagnosis of a proportion of neurological cases. It can also lead to superfluous use of antiparasitic therapy in a patient where the parasites are not viable (Deckers and Dorny, 2010).

To overcome the above problems associated with the antibody detection in clinical conditions, antigen detection can provide a valuable alternative in that it reflects the presence of viable parasites (Deckers and Dorny, 2010). Further, antigen detection can also provide a tool for serological monitoring of antiparasitic therapy (antigen levels drop rapidly after successful anthelminthic treatment). Several assays have been developed to detect parasite antigens in serum, CSF or urine using either polyclonal or monoclonal antibodies (mAbs). However, so far only two mAb-based tests (B158/B60 Ag-ELISA and HP10 Ag-ELISA) have been validated and are used routinely for the detection of parasite antigens (Deckers and Dorny, 2010).

In humans, definitive diagnosis of NCC is made by direct demonstration of the parasite in tissues or radiological demonstration of scolex in cystic lesions using neuroimaging modalities *viz.* computerized tomography and magnetic resonance imaging (Prasad et al., 2008). Neuroimaging is the gold

standard for NCC diagnosis, but in many areas' endemic for NCC, this technology is either unavailable or prohibitively expensive. Magnetic resonance imaging (MRI) or computed tomography (CT) is used to visualize cysticerci in the CNS, providing evidence of the number of cysts, topography of lesions, stage of evolution of the cyst, and assessment of the level of the host's inflammatory reaction against parasites. Where available, CT scanning is the most common imaging tool used for diagnosis, especially in developing countries; however, CT is less effective than MRI at identifying intraventricular cysts, which comprise up to 22% of all NCC cases (Zhao *et al.,* 2015).In 2017, Del Brutto et al. revised the diagnostic criteria for NCC to include neuroimaging with a view to eliminating false-positive diagnoses in endemic areas (from serological examinations) and increase diagnosis in non-endemic areas where NCC is often overlooked. The revised diagnostic criteria determine that NCC cannot be definitively diagnosed without neuroimaging, and that for a definitive NCC diagnosis, the tapeworm scolex (head) should be visible on the scan. However, neuroimaging is unavailable in many endemic areas, training of radiologists for correct interpretations of the scans can be problematic in developing countries, and the high cost of imaging precludes initial and sequential scans.

Diagnostic approaches for hydatidosis

Cystic Echinococcosis**:** Most cases of CE at the asymptomatic early stage are diagnosed incidentally. Diagnosis relies on imaging and immunological tests. Ultrasonography is a convenient tool for diagnosis that indicates the location, number, and size of the cysts with relative ease. However, small-sized cysts may not be detected by ultrasonography. The criteria for classification of liver cysts on ultrasonography, developed by World Health Organization (WHO) in 2001, is followed. There are three categories of cysts: Active, transitional, and inactive. Types 1 and 2 cysts are considered "active" while Type 3 cysts are considered "transitional". Types 4 and 5 cysts are categorized as "inactive" (Grisolia *et al.,* 2009). Type 3 cysts, which are considered transitional, are further divided into two subgroups: CE3a (separated endocysts) and CE3b (solid type containing daughter vesicle). CE3a cysts are inactive while CE3b cysts are active. Magnetic resonance imaging (MRI) and computer tomography (CT) may be required in some cases, where ultrasonography fails to provide a definitive diagnosis. These include cases with subdiaphragmatic cyst or secondary infection of cysts, complicated cases such as biliary fistula or cases with extra-abdominal spread etc. Use of MRI for

diagnosis and follow-up examination is known to be superior to CT (Stojkovic *et al.*, 2012).

Serologic diagnostic methods are used to support the radiological diagnosis and for follow-up assessment. The indirect hemagglutination (IHA) is usually non-specific and is of value in tandem with other investigations such as enzyme-linked immunosorbent assay (ELISA) and immunoblotting (Craig *et al.,* 2003). Concomitant use of IHA and ELISA is associated with diagnostic sensitivity rates up to 85%-96%. Immunoblotting is generally used to confirm the diagnosis in cases where IHA and ELISA findings are not definitive. *E. granulosus* antigen B and antigen 5 (Ag5) are the most specific antigens used for immunological diagnosis (Ito, 2002). However, the sensitivity of the serological tests tends to vary with the location, stage, and size of the cyst. Rate of seronegativity is relatively higher in patients with CE1, CE4, and CE5 cyst types as compared to those with CE2 and CE3 types. Moreover, seropositive patients may continue to remain so for more than 10 years despite treatment. This may lead to unnecessary treatment and an increase in costs. A novel 32 kDa EpC1 antigen, which is located in the germinal layer of the hydatid cyst and the early protoscolex of *E. granulosus*, demonstrated antibodies in 92.2% of preoperative human cases of CE compared with 84.5% cases detected using native antigen B.

Percutaneous fine needle aspiration (FNA) biopsy under ultrasound guidance is used in suspected cases with equivocal radiological and serological test results. Observing the protoscolexes and cyst membranes, or Echinococcal antigen or DNA in aspirated fluid confirms the diagnosis.

Hepatic alveolar echinococcosis: The radiological imaging methods are the main methods of diagnosis of AE and the serologic examinations are used to support the diagnosis. Ultrasonography is the diagnostic method of choice. On ultrasonography, a pseudotumoral mass with hypo and hyperechoic areas together that contain irregular, limited, and dispersed calcifications is diagnostic (Bresson-Hadni *et al.,* 2006). Doppler ultrasonography may be useful for imaging of biliary tracts and vascular infiltrations. Although CT renders the anatomical details in a better manner, MRI is considered the best method to determine invasion of the contiguous structures. Percutaneous cholangiography is an important method for diagnosis in order to view the relation between the alveolar lesions and the biliary tracts. In addition, cranial and thoracic imaging should be required to rule out extra-hepatic involvement in AE patients (Bresson-Hadni *et al.,* 2006).

The immunological diagnostic methods are helpful for diagnosis as well as for monitoring the effectiveness of the treatment. The serological investigations for AE (ELISA or IHA test) are more specific than the ones used for the diagnosis of CE (antigens Em2 and Em II/3-10 are highly specific to AE) (Gottstein e*t al.,* 1993). However, EM2-ELISA may remain positive for many years even in the treated cases as the EM2 antigen is present in inactive lesions. The most active component of AE is the protoscolex that has EM16 and EM18 antigens. The activity of the lesion can be obtained by using these antigens in immunoblot tests. In addition, EM18 is helpful for distinction between AE and CE (Zhang and McManus, 2006).

MICROBIOLOGICAL DIAGNOSTIC TOOLS FOR DETECTION OF IMPORTANT BACTERIAL FOOD BORNE PATHOGENS

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The food safety is a major cause of concern at present because a large number of food products are commercialized and available for the consumption of general public. At the same time, people are more aware about food contamination and food safety. Many different agents like microorganisms, chemicals, biological and other substances can contaminate foods and cause foodborne illness.

The microorganisms viz. Pathogenic bacteria and viruses are very important food contaminants and are responsible for the highest number of foodborne illness outbreaks worldwide. Salmonella spp., Campylobacter spp., Listeria monocytogenes, Staphylococcus aureus, pathogenic Escherichia coli, Norovirus and hepatitis E virus, are the main pathogens that cause the highest number of outbreaks linked to food sources. Bacterial contamination of food products presents a challenge for the food industry and poses a high risk for the consumer. Despite increasing awareness and improved hygiene measures, foodborne pathogens remain a threat for public health, and novel methods for detection of these organisms are needed.

Since the food industry is growing very fast and monitoring of food safety is of prime concern, the rapid testing methodologies are essentially required for monitoring of food borne pathogenic bacteria. Although, traditionally used culture-based methods are still considered the gold standard as they are inexpensive and simple to use but they require longer time to provide results. Generally further confirmation is required by the use of other tests like biochemical tests, molecular tests (typically PCR), or mass spectrometry. Delayed culture become useless in many cases due to the perishable nature and limited shelf-life of many foods. In order to overcome the limitations of culture-based tests, various more rapid and culture-independent methods are developed time to time to detect viable foodborne pathogens.

Detection of foodborne pathogens

The methods available to detect viable food borne bacteria in food can be broadly categorized into:

1. Culture-based Methods.
2. Culture independent methods.

1. Culture-based methods

The culture-based methods are regarded as the 'gold standard' for microbiological analysis of food. Traditional culture relies on the ability of bacteria to grow and multiply on laboratory media and form visible colonies. These methods still represent the first choice for many food testing laboratories as they are sensitive, inexpensive, easy to use, and give either qualitative or quantitative information on the number and type of viable microorganisms present in the food samples (Doyle, 2001). However, culture-based methods are generally time taking as a series of steps are required before a definitive identification, which may include pre-enrichment, selective enrichment, plating on selective media, and then biochemical or serological confirmatory tests. The entire culture process typically requires 2–3 days for preliminary isolation and up to a week for final confirmation of the species isolated. Furthermore, the non-uniform distribution and often low abundance of pathogens in a food sample, the heterogeneity of food matrices, and the presence of indigenous bacteria which might interfere with isolation of specific pathogens can influence the accuracy of culture results. Culture-based methods might also have limited detection capability if microorganisms in an injured state or a VBNC state are present in the food being tested.

2. Culture-independent methods

The following culture-independent approaches are being used for detection of viable foodborne pathogens:

a) Immunoassay
b) Chemical Methods
c) Physical Methods-Biosensors, Impedance
d) Nucleic acid–based methods
e) Microarray
f) Bacteriophage-based detection methods.

a. Immunoassay

Immunological methods have had a major impact on the development of rapid methods for foodborne pathogens and constitute the largest proportion of commercially available rapid tests for food pathogens.

Agglutination methods: Agglutination methods are employed for particulate antigens which are found in many foodborne pathogens. Serotyping is most widely applied to Gram-negative enteric bacterial pathogens such as Salmonella and Escherichia. Among Gram positives, serotyping is important for the genus Listeria. Latex agglutination is one of the simplest examples of agglutination which is observed when a sample containing the specific antigen (or antibody) is mixed with an antibody (or antigen) which is coated on the surface of latex particles. It has been used successfully in case of Campylobacter, Escherichia coli O157:H7, Listeria monocytogenes, Salmonella and Shigella, Staphylococcus aureus.

Gel diffusion methods: Gel diffusion methods have also been widely used for the detection and quantitation of bacterial toxins and enterotoxins e.g., in Staphylococci and C. perfringens; and C. botulinum toxins.

Enzyme-linked immunosorbent assay (ELISA): A typical ELISA is performed with a solid-phase (polystyrene) coated with antigen and incubated with antiserum. Following incubation and washing, an enzyme-labelled preparation of anti-immunoglobulin is added. After gentle washing, the enzyme remaining in the tube or microtiter well is assayed to determine the amount of specific antibodies in the initial serum. A commonly used enzyme is horseradish peroxidase and its presence is measured by the addition of peroxidase substrate. Sandwich technique ELISA technique is the most common form of immunoassay. In sandwich technique, the Antigen on foodborne pathogen is sandwiched between antibody. It has high sample throughput but the individual steps of the ELISA procedure, are fairly labour intensive. The entire procedure takes between 45 min to 2 h to complete after addition of an overnight enrichment broth. Because of interference from the food matrix or debris, ELISA for foodborne pathogens generally require the food sample to undergo an overnight incubation step. This has led to numerous immunoassays being used in combination with separation and concentration methods to eliminate or shorten the preincubation time.

Immunoseparation: Immunoseparation is used to concentrate specific target bacteria, then coupled with a specific ELISA to identify the pathogen

from a short enrichment incubation. In immunomagnetic separation method, paramagnetic beads are employed which are surface activated and can be coated with antibody by incubating in the refrigerator (up to 24 hours). The unabsorbed antibody is removed by washing. The coated beads are added to a food slurry that contains the homologous antigen, thoroughly mixed, and allowed to incubate (few minutes to several Hrs) and the complex is collected by a magnet followed by elution of antigen. The concentrated antigen is then assayed by other methods like FAT, Flow cytometry, ELISA etc.

Fluorescent Antibody: An antibody to a given antigen is made fluorescent by coupling it to a fluorescent compound and when the antibody reacts with its antigen, the antigen–antibody complex emits fluorescence. The fluorescent markers used are rhodamine B, fluorescein isocyanate, and fluorescein isothiocyanate. The use of the indirect method eliminates the need to prepare FA for each organism of interest.

Solid phase RIA: This technique consists of adding a radioactive label to an antigen, allowing the labelled antigen to react with its specific antibody, and measuring the amount of antigen that combined with the antibody by the use of a counter to measure radioactivity. It has been used successfully to detect staphylococcal enterotoxins. Solid-phase RIA can be used to detect as little as 1 ng of toxin per gram by iodination of enterotoxins. Because of its requirement for an isotope and lack of portability, the RIA method is rarely used now in food microbiology.

b. Chemical Methods:

Thermostable Nuclease Test: The thermonuclease enzymes persist even if the bacterial cells are destroyed by heat, chemicals, or bacteriophage. Most strains of *S. aureus* and S. intermedius produce thermonuclease. The organism is heated to destroy heat labile thermonuclease. It is then inoculated on medium containing DNA and toluidine blue. The DNA is broken down by heat stable nucleases resulting into luidine blue changing to red or pink. The heat-stable nuclease can be detected faster than enterotoxin (about 3 hours versus several days) as it appears to be produced by enterotoxigenic cells before enterotoxins appear and the nuclease is detectable in unconcentrated cultures of food specimen, whereas enterotoxin detection requires concentrated samples.

Use of Fluorogenic and Chromogenic Substrates

Some of the fluorogenic and chromogenic substrates employed in culture media in food microbiology are 4-methylumbelliferyl-β-d-glucuronide (MUG), 4-methylumbelliferyl-β-d-galactoside (MUGal), o-nitrophenyl-β-d-galacto-pyranoside (ONPG). These substrates are employed in various ways in plating media, MPN broths, and for membrane filtration methods. MUG is the most widely used fluorogenic substrates. It is hydrolysed by β-d-glucuronidase to release fluorescent 4-methylumbelliferyl moiety, which is detected with long-wave ultraviolet light. e.g. EHEC –ve

Adenosine Triphosphate Measurement

Since ATP is a primary biological source of energy found in all the living cells, the ATP bioluminescence test represents the presence of a living microbe. The ATP disappears within 2 hours after cell death. One of the simplest ways to measure ATP is by use of the firefly luciferin–luciferase system. In the presence of ATP, luciferase emits light, which is measured with a luminometer. The amount of light produced by firefly luciferase is directly proportional to the amount of ATP added. It has been used as a rapid method for estimating microbial numbers in clinical microbiology.

$$\text{D-Luciferin} + ATP + O_2 \xrightarrow[Mg^{2+}]{\text{Firefly Luciferase}} \text{Oxyluciferin} + AMP + PPi + CO_2 + \text{Light}$$

Limulus amoebocyte lysate (LAL) test: LAL test employs a lysate protein obtained from the blood (actually hemolymph) cells (amoebocytes) of the horseshoe crab (Limulus polyphemous). The lysate protein is the most sensitive substance known for endotoxins. For LAL, small quantities of a lysate preparation is added to aliquots of food suspensions, followed by incubation at 37◦C for 1 hour. The presence of endotoxins causes gel formation of the lysate material. LAL reagent can detect 1.0 pg of LPS.

C. Physical Methods

Luminescence: Luminescence in marine bacteria such as *Vibrio fischeri and V. harveyi* is controlled by genes. The primary genes (designated lux) for luminescence are lux A and lux B. The specific gene is carried from one bacterium to another via a bacteriophage. If Y. enterocolitica is the bacterium of interest, one selects a phage that will infect the widest range of strains. To

this phage, the lux genes are inserted by recombination methods. these transduced phages are not luminous but when added to their specific host bacteria, the lux gene-bearing phages enter and multiply, thus host cells (bacterium) produce luminesce by the increased production of more lux genes. The emitted light can be measured by luminometry.

Biosensor based methods: Biosensors are considered a novel approach for the rapid detection of foodborne pathogens. The word biosensor refers to an effective and creative analytical device that has a biological sensing function. A biosensor is an analytical device, which converts a biological response into an electrical signal. It is widely used for the detection of microbial contamination. Biosensors typically deliver fast, on-site tracking and thus provide real-time details. Their function depends on the interaction between biologically active agents, the transducer, and a signal conversion unit. Biosensors typically contain two main components: a target recognition component such as receptors, nucleic acids, or antibodies anda signal transducer that transforms target recognition into physically detectable signals.

Impedance: It is the apparent resistance in an electric circuit to the flow of alternating current, corresponding to the actual electrical resistance to a direct current. When microorganisms grow in cultured media, they metabolize substrates of low conductivity into products of higher conductivity andthere by decrease the impedance of the media. When the impedance of broth cultures is measured, the curves are reproducible for species and strains.

D. Nucleic acid–based methods

Nucleic acid–based methods are based on detection of specific DNA or RNA sequences. The commonly advocated methods are:

Ribotyping: It is a rapid automated method for microbial diagnostics. DNA is extracted from cells and digested with an endonuclease such as EcoRI. The fragments are separated by agarose gel electrophoresis. Separated fragments are transferred to a nylon membrane and hybridized with an appropriately labelled cDNA probe which is derived from ribosomal RNA (rRNA) by reverse transcriptase. The automated device creates ribo prints that are matched or compared to those of known strains stored on computer software.

Nucleic Acid Probes: The probe contains sequences that code for a specific product. DNA fragments of unknown organisms are prepared by the use of restriction endonucleases. After separating fragment strands by

electrophoresis, they are transferred to cellulose nitrate filters and hybridized to the labelled probe. The hybridized product is assessed by autoradiography

Polymerase chain reaction (PCR): it is the most commonly used nucleic acid amplification method for detecting pathogenic microorganisms, and over the last two decades, many different modifications of original PCR protocol have been developed (Priyanka et al. 2016). Although the standard PCR is rapid, specific and sensitive but this method does not ascertain the viability of detected cells. To ascertain the viability of detected cells, the use of cell viability dyes in combination with DNA amplification methods called “viability PCR”, has been developed (Pan and Breidt, 2007). In Viability PCR tests, the cells are stained with ethidium monoazide (EMA) or propidium monoazide (PMA) dyes before DNA amplification. These dyes can enter only the perforated cell membranes and bind to the DNA. Subsequent exposure of cells having dye bound DNA to light causes irreversible damage to DNA resulting into a strong inhibition of PCR amplification of DNA and hence only DNA from cells with an intact membrane will be amplified (Emerson et al., 2017).Use of viability PCR tests for rapid detection of foodborne pathogens has been extensively explored.

The detection of messenger RNA (mRNA) is considered a better indicator of cell viability than DNA, since this molecule is only present in metabolically active cells. Reverse transcription-PCR (RT-PCR) is one of the RNA-based molecular techniques most commonly used that uses the reverse transcriptase enzyme to convert originally extracted mRNA into complementary DNA (cDNA). The newly synthesized cDNA is then used as a template for exponential amplification using conventional PCR (RT-PCR) or quantification using quantitative PCR (RTqPCR). However, the application of RT-qPCR is less common in bacterial foodborne pathogens, probably because the method is too laborious, or there is rapid degradation of RNA in samples, which may lead to false negative results.

Microarrays: DNA microarrays consist of multiple-specific oligonucleotides or PCR probes spotted mechanically on to a microchip (nylon membrane, glass slide, or silicon chips) in a lattice-type configuration. Target nucleic acid, which may be either PCR products, genomic DNA, or RNA is then applied to the microarray and hybridization detected by a fluorescent label. The fluorescence pattern is then recorded and analyzed using a scanner. It has high specificity and provide detailed genetic characterization of specific foodborne pathogens.

Bacteriophage-based detection methods: The bacteriophages have high specificity and natural affinity for their host cells and they can only replicate inside living cells, meaning that phage-based methods can be tests to demonstrate cell viability. Most phage-based tests employ lytic phages as lysing agents, and detection of the new progeny phages or intracellular material released from target bacterial cells provides the indication of cell viability. The phage amplification assay or the plaque assay has been explored for different foodborne pathogens such as Salmonella Typhimurium and Staphylococcus aureus, Salmonella Enteritidis and Escherichia coli 0157:H7, Listeria monocytogenes and Mycobacterium avium subsp. paratuberculosis because it is simple and the rapid. Faster phage-based detection can be achieved by combining the lytic part of the plaque assay and an alternative endpoint detection method, such as immunological or molecular tests to detect either progeny phages or phage DNA, respectively.

CURRENT TRENDS IN DIAGNOSIS OF FUNGAL ZOONOTIC DISEASES AND MANAGEMENT OF FUNGAL INFECTIONS IN ANIMALS

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Fungi are ubiquitous. They thrive on soil, on plants, indoor surfaces and on skin and inside the body. Of these, a few hundred of them are known to be zoonotic. Although, recent years have observed an enormous increase in the frequency and severity of fungal infections. Environmental/climatic changes owing to urban expansion and intensive farming practices can also affect the growth and spread of fungi. A One Health approach thus is significant for the prevention and control of fungi that can spread through the environment and between animals and people.

Early diagnosis of fungal infection is crucial for effective treatment. Very few clinical mycologists, cost, time taken to get the result, sensitivity and specificity of the tests demand newer diagnostics which meet the contrasting needs presented by the increasing diversity of fungi in association with the use of immunosuppressive agents. Diagnosis of fungal infection has relied primarily on methods such as direct microscopic examination of clinical samples, histopathology, and culture. Such approaches require personnel having expertise in diagnostic mycology. Consequently, increased emphasis on the use of molecular methods and antigen detection is being driven especially for invasive fungal infections.

Fungal zoonotic infections, categorized on the basis of involvement of tissues/organs, are of four types: (1) Superficial (2) Cutaneous (3) Subcutaneous and (4) Systemic. Of these, cutaneous and systemic mycoses include some of the better known zoonoses. Successful diagnosis primarily requires proper collection and transport of appropriate specimens, and comprehensive procedures in the laboratory. For this, all efforts should be made to collect specimens free from bacterial contamination as possible.

Specimen collection and transport

Selection, collection and transport of samples to the laboratory forms the basis of diagnostics, particularly in the recovery of fungi. Penicillin (20 U/mL), streptomycin (1,00,000µg/mL) or chloramphenicol (0.2 mg/mL) may be added to the specimen to prevent the overgrowth of other commensals. In cases of delay, the specimen should be stored under refrigeration at 4°C for no longer than 24 hrs as it preserves the viability of pathogens and also reduces the growth of contaminants. Blood and Cerebrospinal fluid, on the other hand, should not be refrigerated. Blood should be kept at room temperature or in an incubator at 35°C while CSF, should be transported at room temperature. All specimens are transported in sterile containers and processed as early as possible.

Diagnostic methods

1. Direct Microscopic Count

This procedure can often provide the first microbiologic proof of the cause of disease in patients with fungal infection. Preparations for direct examination of clinical specimen include KOH, India ink, and calcofluor white. In addition, a few staining techniques such as Giemsa and periodic acid Schiff (PAS) are effective.

Microscopy can identify a dermatophyte by the presence of fungal hyphae (branched filaments) making up a mycelium, Arthrospores (broken-off spores), Arthroconidia (specialised external spores), Spores inside a hair (endothrix) or outside a hair (ectothrix). A yeast infection can be identified by the presence of: Yeast cells, which may be dividing by budding, Pseudohyphae (branched filaments similar to those of a dermatophyte) forming a pseudomycelium.

2. Culture

Culture from a clinical sample is the gold standard for diagnosis of fungal infection. Culture has the advantage of yielding the specific etiological agent if positive. Moreover, culture allows for susceptibility testing. Common media for primary fungal isolation include Sabouraud dextrose agar and Brain-Heart Infusion agar (BHI). Culturing is performed either in petri dishes or screw top tubes. The media may be enriched with 5% to 10% sheep blood to support the growth of certain fungi. Specimens that may be contaminated with other microorganisms, such as urine or sputum, are set up on agar media containing antimicrobials. Chloramphenicol, streptomycin, or penicillin is incorporated into the agar to inhibit the

growth of bacteria. The plates are incubated at 25-30ºC and the growth may take several weeks. The most common procedure for microscopic examination of fungal cultures is a direct mount of the fungal isolate. This is achieved by preparing tease mount or cellophane tape mount. The procedure has certain limitations particularly in invasive disease, such as it

a. may take many days to a result with several of the filamentous fungi.

b. may only become positive late in infection

c. identification of less common fungi that may cause opportunistic infections requires a high level of expertise on the part of laboratory personnel.

3. Serology

Serologic tests for patient antibodies have been useful for non-culture-based diagnosis of fungal infection. Latex particle agglutination, immunodiffusion, counter current immune electrophoresis, immune-fluorescent antibody, ELISA, and western blotting/immunoblotting are the various serological techniques used. Ex. Complement Fixation and immunodiffusion are the most common serologic tests for diagnosis of histoplasmosis. The immunodiffusion test detects precipitating antibodies to *Histoplasma* H and M antigens.

Advantages

a. results may be positive when culture results are negative or samples are difficult to obtain.

b. if positive, serological results may reduce the need for culture of potential hazardous fungi, for example, *Coccidioides* spp.

c. serology is a minimally invasive sample, which lowers barriers to testing.

Disadvantages

a. sometimes low levels of sensitivity and specificity

b. Some tests, particularly CF, are time consuming and require trained personnel

c. Immunocompromised patients may show a reduced antibody response, which would dramatically reduce the value of serologic assays

d. Interpretation of serological results may be confounded by the inability of serology that measures IgG to distinguish between current or previous infection.

e. Finally, sensitivity is dependent on the type of disease and the timing of testing relative to the disease process, for example, early versus late.

4. Molecular Diagnostics

Availability of protein and DNA sequence data, has led to the progression of this area. The area accelerates with each new advance in technology, and the growing need to identify a broader range of fungi.

Polymerase chain reaction (PCR)

PCR is a central component for many molecular methods, either as the main diagnostic

strategy or as one of the preliminary steps in the diagnostic assay. Consequently, diagnostic PCR encompasses a number of different approaches. The simplest consists of conventional PCR in which species-specific primers that have been designed based on existing sequence or data, are used to amplify fungal DNA from clinical specimens. The readout generally consists of the presence or absence of a band, with the size of the band often being a secondary factor in identification.

Advantages

- simple and inexpensive

Disadvantages

- wide intra- and interlaboratory variation
- Requires some prior suspicion about the identity of the isolate

Variations of PCR include Real time PCR or quantitative real-time PCR; qPCR) . Quantitative PCR instruments display the output graphically in real-time as amplification cycles proceed. Output is typically detected by

fluorescence, which can be as simple as measuring the amount of double stranded product, or adding a probe to the reaction that is specific for a target sequence within the amplicon.

Isothermal PCR techniques, Loop Mediated Isothermal Amplification (LAMP) is carried out at 60–65 °C and utilizes a polymerase that displaces one of the DNA strands as a new strand is synthesized. Another isothermal amplification method used for fungal identification is called Nucleic Acid Sequence-Based Amplification (NASBA), which uses RNA as the template. This reaction typically is done at 41 °C. A technique called Rolling Circle Amplification (RCA) uses a viral polymerase to amplify the target sequence in the form of a circular template. RCA has been successfully used with the Mucorales and *Cryptococcus*, species.

Fluorescence in situ hybridization

Fluorescent in situ Hybridization (FISH) can locate the exact position of particular DNA or RNA sequences in the biological materials. FISH probes are used to target sequences of ribosomal RNA or mitochondrial genes due to presence of sequence databases and result in multiple copies in each cell. Limitations of the FISH method can include fungal and substrate inherent autofluorescence, insufficient permeability. Variations of the assay include Peptide Nucleic Acid (PNA) FISH, which utilizes a peptide nucleic acid as the probe. PNA-FISH has also been used to identify several fungal species, including Candida, *Aspergillus*, *Fusarium*, and *Scedosporium* species.

DNA array Hybridization

DNA array hybridization or Reverse Dot Blot Hybridization (RDBH) or macroarray, technique is based on hybridization of amplified and labelled genome regions of interest to immobilized oligonucleotides spotted on a solid support platform. It is now considered a powerful and practical technique for the detection and identification of fungi and other microbes, such as bacteria, from complex environmental samples without the need for isolation in culture.

DNA sequencing

Sequence-based identification of fungi is an extremely useful diagnostic tool due to availability of databases, such as GenBank. GenBank contains an extensive number of ribosomal RNA gene (or rDNA) sequences, with more than 500,000 records for ITS1 and 28S deposits. The latter region, also called the D1/D2 region, is not as sensitive as the ITS region and in many cases can only provide genus-level discrimination.

Whole-genome sequencing

WGS can provide epidemiological information about each strain, and the potential for drug resistance, all in the same run and without a prior suspicion of fungal species. However, this technology is still too expensive, complicated, and too slow for routine use in most clinical microbiology laboratories.

Mass spectrometry

Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) uses a laser to ionize biomolecules, which are then detected and measured based on their mass-to-charge ratio. The time-of-flight (TOF) component generates a peptide mass fingerprint that is used to interrogate a database. It is fast, providing an identification in minutes, and also requires minimal sample preparation. However, MALDI-TOF MS requires analytes from pure cultures, which can add days to the turnaround time. Fingerprint variation depending on culture conditions, makes difficult to standardize fungi. MALDI-TOF MS has successfully identified several major human fungal pathogens, including *Aspergillus, Candida, Cryptococcus*, and Fusarium species, the Mucorales, dimorphic fungi (*Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Coccidioides* species), and some dermatophytes.

Management of fungal infections

1. Superficial and Cutaneous fungal infections are typically treated with antifungal drugs, applied directly to the affected area (called topical drugs). Topical drugs may include creams, gels, lotions, solutions, or shampoos.
2. Measures should be taken to keep the affected areas dry, such as applying powders.
3. Isolation of the affected animal should be followed.
4. Maintain clean environment.
5. Animal feed to be kept in hygienic conditions, clean, dry and consumed in a short time, and changed with new fresh feedstuffs.
6. Equipment and other materials in contact with infected animals should be disinfected.
7. Balanced nutrition to animals should be provided in order to maintain immunity.
8. Education and awareness to livestock owners.

CONVENTIONAL TO MOLECULAR TECHNIQUES FOR DIAGNOSIS OF BRUCELLOSIS AND TUBERCULOSIS IN ANIMALS

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Abstract

Brucellosis and bovine tuberculosis aresome of the major zoonotic diseases affecting man and animals. Brucellosis primarily causing heavy economic and production losses to animals especially to ruminants is basically an occupational hazards disease in human and to those who drinks raw milk. Similarly, bovine tuberculosis is primarily a concern for animals especially cattle but can cause zoonotic disease in human, to those who take raw milk and meat. Both the disease needs a one health approach for implementing the control strategy in human and animals.

Introduction

Mankind had since historical days have been impacted with diseases caused by pathogenic microorganism and in that bovine tuberculosis and brucellosis is one of the major diseases which had impacted both animals and human beings. Emergence of zoonotic infectious diseases is a prevailing trend of 21stcentury and there are plethora of evidences to show that these diseases are existing in the developing world but they are given attention when there is a major outbreak and attracts news attraction. These are often called as neglected zoonoses which are afflicting humans since time immemorial and acquired notoriety as chronic disabling diseases particularly in populations relying on livestock or wildlife for financial and social capital. Brucellosis and bovine tuberculosis are some of the most widespread infectious zoonoses included in the category of the neglected zoonotic disease, by World Health Organization. Both the disease had a world- wide distribution, particularly affecting low income and developing countries. We will be discussing the overview of both the disease with particular emphasis on laboratory diagnosis.

Brucellosis

Brucellosis is one of the most infectious, widespread zoonotic disease showing great impact on human and animal health, livestock products,

agriculture trade in endemic countries It has been identified as one of significant neglected zoonotic disease by WHO, FAO, OIE especially in low income countries where there are no proper disease monitoring systems and effective control measures 1897 Brucellosis is recognized as world's most laboratory acquired infection and also exhibits strong occupational character due to different ways of transmission. Brucella is a facultative, intracellular, Gram negative cocobacilli organism which is non-motile. There are around 30 identified species of brucella so far but the most pathogenic species responsible for leading causes of animal and human brucellosis are *B.melitensis, B.abortus, B.suis*and *B.canis*. Even though most of the countries have shown progress in eradicating brucellosis in livestock, expansion of urbanization, international travel, increased taste for exotic goods and food products contaminated with Brucellosis, importation of such goods is an increasing concern over years. Due to this, the geographical distribution of disease is changing causing its re-emergence. Despite the pathogen's ability to adopt to modern world, diagnosis are challenges in humans due to similar clinical presentations. Lack of proper diagnosis, poor human intervention, are of special concern in brucella endemic and low income countries. Moreover, neglecting the animal reservoir of disease is a hindrance to stop its spread and re-emergence. Therefore, a combined one health approach is needed involving measures at human, animal and environmental interfaces by veterinary and medical professionals to curb the disease transmission.

Brucellae transmission in animals is mainly due to ingestion of feed and water contaminated with aborted materials, vaginal secretions and placental fluids. It is also transmitted through contact, oronasal mucosa, venerally and through abraded skin. Transmission in humans is mainly due to drinking of infected raw milk or unpasteurized milk and ingestion of cheese contaminated with brucellae of infected animals. Transmission by oral route always depends upon pathogenicity of strain, health condition of consumer and infectious dose of organism. Occupational exposure is another major way of transmission which may be direct or indirect. The occupational groups which are at risk are livestock farm workers, abattoir workers, veterinary assistants, veterinarians, laboratory personnel etc. Infection is transmitted mainly by handling of aborted material like foetus, fluids and foetal membranes, contact with vaginal secretions through skin abrasions and conjunctiva.

The disease in cattle is characterized by placentitis resulting in abortions during last trimester, retained placenta and still births in pregnant females. The disease is usually asymptomatic in young and non-pregnant females. The

infection in adult male cattle causes poor semen quality, arthritis, epididymitis, orchitis, hygromas and sometimes infertility in both sexes. In humans the incubation period of the disease is 1-3 weeks and manifests as acute febrile illness with undulant fever which may persists or prolong into chronic incapacitating diseases with severe implications asorchitis in males, arthritis, endocarditis, spondylitis and recurrent fever which may persist long for years.

Diagnosis

Laboratory Protocols for Diagnosis of Brucellosisrevolves around serological test, isolation and molecular test. The basic screening test for bovine brucellosis is herd screening by milk ring test which is basically done for pooled milk. Rose Bengal Plate Test (RBPT) is one of the most widely used screening tests for brucellosis using serum and for confirmation complement fixation test or ELISA is used. Isolation of brucellae is cumbersome and require biosafety level 2 facility and the bacterium is also fastidious and require around 7 days to grow in laboratory culture with requirement of 5% Carbon di-oxide. The molecular test revolves around genus specific PCR of bcsp31 followed by bruce ladder PCR which require a cocktail of 8 pars of primer for differentiating the species involved. Now we will discuss the test in brief

Rose Bengal Plate Test (RBPT): It require the test to be performed at room temperature with 25-30µl of each serum sample on a microscopic slide and an equal volume of Shaked antigen followed by mixing and observing within 3 min for the agglutinin as positive reaction.

Milk Ring Test (MRT): 2-3ml pooled fresh raw milk samples and 30-75µl antigen (room temperature) is kept in a test tube for incubation at 37ºC for 1 hour or overnight at 4°C and the result is seen as a colored red/blue ring formation near meniscus.

Indirect Enzyme Linked Imunosorbent Assay (i-ELISA) for sera samples as per the manufacturer protocol.

Isolation and identification of *Brucella*spp. *Brucella* suspected material presented for culture should be handled either in biosafety containment level III or level II. Proper gloves, N95 masks and protective clothing should be worn during culture procedures. The choice of clinical samples are aborted foetuses (stomach content, spleen, lung), placenta, vaginal secretion/swab (especially after abortion) and Milk, semen, arthritic/hygroma fluid. Direct isolation on solid media (Tryptose Soy Agar, Brucella agar with supplement and Blood Agar) is used. Incubate the plates at 37ºC with 5%CO_2. Observe the

plates for growth every 24 hours. *Brucellaabortus* is slow growing and requires 3-7 days for colonies to appear. Plates should not be discarded until 10 days. *Brucellasuis* shows growth in comparatively lesser time, 2-4 days. Gram negative cocco-bacilli can be seen under microscope (100X). *Brucella* spp. are catalase, oxidase, urease tests positive; produces H_2S and shows agglutination with convalescent sera.

The primer used for brucella identification are shown below

Genus specific Polymerase Chain Reaction (PCR) for BCSP31 gene

Primers: 223 bp product length (Bailey *et al*. 1992)	
BCSP-B4 (F)	tggctcggttgccaa tat caa
BCSP-B5 (R)	cgcgcttgcctttcaggtctg

Species specific Polymerase Chain Reaction (PCR) and Bruce Ladder Multiplex PCR: 8 primer cocktails (Add 1µl of each forward and reverse primer, resulting in a primer cocktail of 16µl) (Lopez-Goni *et al*., 2008)

Name of Primer Sequence	**5'-3'**	**Product Length(bp)**
BMEI0998 (F)	atcctattgccccgataagg	1,682
BMEI0997 (R)	gcttcgcattttcactgtagc	
BMEI0535 (F)	gcgcattcttcggttatgaa	450
BMEI0536 (R)	cgcaggcgaaaacagctataa	
BMEII0843 (F)	tttacacaggcaatccagca	1,071
BMEII0844 (R)	gcgtccagttgttgttgatg	
BMEI1436 (F)	acgcagacgaccttcggtat	794
BMEI1435 (R)	tttatccatcgccctgtcac	
BMEII0428 (F)	gccgctattatgtggactgg	587
BMEII0428 (R)	aatgacttcacggtcgttcg	
BR0953 (F)	ggaacactacgccaccttgt	272
BR0953 (R)	gatggagcaaacgctgaag	
BMEI0752 (F)	caggcaaaccctcagaagc	218
BMEI0752 (R)	gatgtggtaacgcacaccaa	
BMEII0987 (F)	cgcagacagtgaccatcaaa	152
BMEII0987 (R)	gtattcagccccgttacct	

Species	Fragments amplified
Brucellaabortus	1682bp, 794bp, 587bp, 450bp, 152bp
Brucellamelitensis	1682bp, 1071bp, 794bp, 587bp, 450bp, 152bp
Brucellaovis	1071bp, 794bp, 587bp, 450bp, 152bp
Brucellasuis/canis/neotomae	1682bp, 1071bp, 794bp, 587bp, 450bp, 272bp, 152bp

The MLST and whole genome sequencing can also be performed for epidemiological and phylogenetic analysis. Basically brucellosis which is a neglected zoonosis and endemic disease in most of middle and low income countries causes severe economic devastation in livestock industry and significant human burden reducing the countries potential to reduce or eradicate poverty. The disease has become a challenge in developing countries due to underreporting, inadequate funding for conducting vaccination programmes at national level. Even though, it is most wide spread zoonosis and laboratory acquired infection there are wider gaps in knowledge about disease diagnostics and vaccines which need to be filled to control and eradicate brucellosis. Focussing on natural reservoirs of disease to reduce the incidence in human population, developing vaccines to provide protection and strategies to differentiate between infected and vaccinated animals play a role in controlling the disease. Measures to control or mitigate the disease burden especially in low resource communities will improve food security, house hold income, human and animal health. In present scenario, there is a desperate need for a public policy to diminish the socio-economic impacts of brucellosis in human and animal populations, allocating resources for vaccine development, campaigns, surveillance and research. Ultimately to end this challenging disease, a multinetwork approach measure like ‘One Health ‘, collaboration between nations, states, public and private enterprise is needed.

Bovine tuberculosis

Bovine tuberculosis is a chronic bacterial disease of animals caused by members of the *Mycobacterium tuberculosis* complex(MTBC), primarily by *M. bovis* and other members of the MTBC such as *M. africanum, M. canettii, M. microti M. tuberculosis*, *M. caprae*, etc. Bovine tuberculosis is a major zoonotic disease, cattle serve as the main source of infection for humans. It also affects other domesticated and pet animals such as sheep, goats, equines, pigs, dogs, cats and wildlife species such as wild boars, deer and primates causing a general state of illness like loss of appetite, fluctuating fever, diarrhea,

dyspnoea, swelling of lymph nodes etc. Intermittent hacking cough, pneumonia, weight loss and eventual death are the major signs. Although the infection in cattle herds has been controlled in most of the developed countries but bovine tuberculosis remains a serious problem for animal and human health in many developing countries. Cattle are considered to be the major reservoir *M. bovis*, and are the main source of infection for humans. Nevertheless, the disease has been reported in many other domesticated and non-domesticated animals.

A total of 21.8 million (7.19%) cattle are estimated to be infected with bovine tuberculosis in India.Bovine tuberculosis remains endemic in developing countries including India where cattle and human coexist creating a huge economic impact on agricultural industries and animal productivity threatening the livelihoods of farmers and ranchers.

Diagnosis: The diagnosis of *Mycobacteria bovis* revolves around detection of pathogen by culturing and biochemical characteristics. Even though these methods are highly specific, sensitive and are in the first line of standards for detection of disease, it is time consuming. Screening by single, double tuberculin tests using PPD and interferon gamma assay for *M. bovis* in live animals are the options with their own limitation of specificity and cost effectiveness respectively. During post-mortem examination, the post- mortem lesions with visible tubercle and acid- fast staining gives a fair idea of tuberculosis infected animal. Molecular-based techniques includes polymerase chain reaction for *Mycobacterium* genus specific PCR like hsp65 (Telenti *et al.*, 1993), *Mycobacterium tuberculosis* bacillus complex PCR like IS6110 (Eisenach *et al.*, 1990), IS1081(Collins and Stephens, 1991) and finally a *M. bovis* specific PCR based on any of the region like 500bp Fragment PCR (Rodriguez *et al.*, 1995), Mb 400 primers which are intended to flank Region of Difference RD4 (Sales *et al.*, 2014), RD9 (Parsons *et al.*, 2002).

Tuberculin test

The standard method for detection of bovine tuberculosis is the tuberculin test, which involves the intradermal injection of bovine tuberculin purified protein derivative (PPD) and the subsequent detection of swelling (delayed hypersensitivity) at the site of injection 72 hours later. This may be performed using bovine tuberculin alone or as a comparative test using avian and bovine tuberculins. The comparative intradermal tuberculin test is used to differentiate between animals infected with *M. bovis* and those responding to bovine tuberculin as a result of exposure to other mycobacteria. This

sensitisation can be attributed to the antigenic cross-reactivity among mycobacterial species and related genera. The test involves the intradermal injection of bovine tuberculin and avian tuberculin into different sites, usually on the same side of the neck, and measuring the response 3 days later.

Acid Fastness/Ziehl-Neelsenstaining is carried out on the fresh tissue which use to show basic carbolfuchsin stained small rods of *M. bovis* with backgroundof malachite green. The isolationof *M.bovis*require prior digestion and decomtamination of the samples followed by inoculating them the tissues in Lowensten Jensen pyruvate media or agar-based medium such as Middlebrook 7H10 followed by incubation at 37 degree Celsius for 2 to 4 months under 5% carbon di-oxide. *M. bovis* can be differentiated by usual few important bio-chemical test like niacin test, nitrate test, pyrazinamide test and Thiophene-2-carboxylic acid hydrazide(T2CH) test.

DNA can be extracted by kit-based method or conventional phenol-chloroform method. The hot-cold lysis method is applicable only for pure colony ofMycobacteriabut doesnot give result for tissue samples. The mycolic acids resist the proper lysis of the *Mycobacterium*, hence lysis step should be more robust than other bacteria. The PCR based diagnosis revolve around Mycobacterium genus specific hsp65, followed by complex specific IS6110 or IS1081 followed by species specific 500bp, Rd4 or RD9 based PCR.

Sl. No	Region or gene targeted	Sequence 5'-3'	Product Size(bp)	Reference
1.	hsp 65	ACCAACGATGGTGTGTCCAT	441	Telenti *et al.* (1993)
		CTTGTCGAACCGCATACCCT		
2.	IS6110	CCTGCGAGCGTAGGCGTCGG	123	Eisenach *et al.* (1990)
		CTCGTCCAGCGCCGCTTCGG		
3.	IS1081	ACAGGCGAGCCCGGATCTGCTG	248	Wards *et al.*, 1995
		GTTCAGCTCGCTTGCGGCGCTG		
4.	RD4	AACGCGACGACCTCATATTC	400	Sales *et al.* (2014)
		AAGGCGAACAGATTCAGCAT		
5.	500bp	TCGTCCGCTGATGCAAGTGC	500	Rodriguez *et al.*,1995
		CGTCCGCTGACCTCAAGAAG		

The whole genome sequencing for mycobactyerial samples require DNA to be extracted in the laboratory in bulk amount and later can be sent to the WGS sequencing provider. Most of the WGS sequencer doesn't accept the live TB bacterium as they require additional BSL-3 setup to handle.

Conclusion

Both bovine tuberculosis and brucellosis is a serious age-old problem in spite for so much attention these pathogen are getting from academician and government trying to give proper importance to these pathogen, the disease is widely present in developing and under-developed countries. The reasons could be many some of the reason in Indian condition is requirement of biosafety level 2 and 3 facility requirement which is a costly affair to do research on these pathogen at least in veterinary sector. The inability of application of test and culling procedure due to socio- economic reasons is another one. The pathogen is looming large in animal farms and is posing serious threat to human. The government had to give special focus with specialized funding for both the pathogen in animal sector like it included the free vaccination for brucellosis. Similarly, bovine tuberculosis also needs specialised focused funding. Awareness among general public, livestock owners, medical fraternity is also needed for prevention and control of both the disease. One health approach invoicing multiple stake holder is needed to prevent and control bovine brucellosis and bovine tuberculosis.

RECENT ADVANCES IN MOLECULAR TECHNIQUES FOR ENSURING FOOD SAFETY IN PRODUCTS OF ANIMAL ORIGIN AND PUBLIC HEALTH RISKS RELATED TO FOOD SAFETY ISSUES

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Introduction

Foodborne illness, commonly referred to as food poisoning, result from consumption of food or drink contaminated with pathogens including bacteria, viruses, parasites or toxins. It leads to a considerable amount of loss in terms of man hours and treatment costs. Foodborne pathogens pose a constant threat to public health with implications on socioeconomic development and international trade worldwide. The assessment of microbiological quality of foods is the first step in ensuring safe and wholesome food. The World Health Organization (WHO) estimates that 600 million – almost 1 in 10 people in the world – fall ill after eating contaminated food and 420,000 die every year, resulting in the loss of 33 million healthy life years (DALYs). Further, children under 5 years of age carry 40% of the foodborne disease burden, with 125,000 deaths every year (WHO, 2017). Although various pathogens have been identified to cause foodborne illness; majority of the outbreaks have been caused by *Campylobacter* spp.*, Salmonella, L. monocytogenes* and *Escherichia coli* O157:H7. Parasites lik*e T. spiralis, Cryptosporidium* spp. and *A. cantonensis* have also been implicated to cause food poisoning outbreaks due to consumption of animal origin foods. In recent years, viruses such as Norovirus, Rotavirus, Hepatitis A virus and Astrovirus have been found associated with cases of food poisoning from foods of animal origin. Despite advances in hygiene, consumer knowledge, food treatment and processing techniques, foodborne agents continue to plague mankind. Changes in food production and distribution systems, microbial adaptation, and lack of support for public health resources and infrastructure have led to the emergence and re-emergence of many foodborne diseases. Application of microbiological quality control programs throughout the production chain is essential in order to

minimize the risk of infection to the consumer. These programs need to be ably supported by reliable, rapid and accepted test systems to detect the presence or absence, or even the degree of contamination of pathogens. The ability to detect, identify, and quantify unwanted microorganisms in food is important to the food industry as well as to the consumer. Further, these identification techniques are crucial in epidemiologic investigations of foodborne disease outbreaks and hence to formulate suitable preventive and control measures. Conventional methods for detection of bacteria in foods are generally the classical cultural methods which rely on enrichment and isolation of organisms, followed by biochemical and/or serological identification. These methods of detection, though reliable and cost effective, require procedures of days to weeks before final results are obtained. Another disadvantage of conventional methods is that cells which are viable but otherwise non-culturable cannot be detected. Introduction of the molecular techniques in microbial diagnostics has been established as a valuable alternative to conventional diagnostics. There is an improvement in traditional methods and laboratories have begun to adopt nucleic acid-based tests to identify pathogens rapidly. The advantages of nucleic acid-based tests include rapid results in a short period of time, even though low in number (theoretically a single cell) and detecting specific organism of interest.

During the last decades, molecular methods developed extensively which operate by detecting specific DNA or RNA sequences of the target pathogenic organism. This is done by hybridizing the target nucleic acid sequence to a synthetic oligonucleotide (probes or primers) which is complementary to the target sequence (Zhao *et al.,* 2014). Nucleic acid based methods detect the specific genes in the target pathogens, therefore preventing ambiguous or wrongly interpreted results. The recent nucleic acid-based methods described are simple polymerase chain reaction (PCR), multiplex polymerase chain reaction (mPCR), real-time/quantitative polymerase chain reaction (qPCR), nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP) and microarray technology. Polymerase chain reaction, or PCR, is the most commonly used nucleic acid amplification method for detecting pathogenic microorganisms, and over the last two decades, many different advances on the original PCR protocol have been described (Priyanka *et al.,* 2016). However, despite being rapid, specific and sensitive, standard PCR–based detection methods used alone do not provide any indication about the viability of detected cells, as they are not able to discriminate between DNA derived from live as opposed to dead cells. To overcome this limitation, the use of cell viability dyes in combination with

DNA amplification methods, sometimes termed viability PCR, has been investigated (Pan and Breidt, 2007). Viability PCR tests are commonly performed using ethidium monoazide (EMA) or propidium monoazide (PMA) dyes. Before any DNA amplification is applied, cells are stained with EMA or PMA, which can only enter perforated cell membranes binding to the DNA. Subsequent exposure of cells to light leads to irreversible damage of nucleic acid resulting in a strong inhibition of PCR amplification. The end result is that only DNA from cells with an intact membrane will be amplified (Emerson *et al.,* 2017). Use of viability PCR tests for rapid detection of foodborne pathogens has been extensively explored, and different endpoint detection approaches, but particularly qPCR and Loop-mediated isothermal amplification (LAMP), have been successfully applied (Law *et al.,* 2015; Priyanka *et al.,* 2016). A limitation of the viability PCR approach is that integrity of the cell membrane is not always a reliable indicator of the viability of cells. Evidence suggests that some cells might remain intact even if they do not show any metabolic activity, leading to false positive results (Ayrapetyan and Oliver, 2016). Moreover, bacterial cells may have perforated cell walls at some point during their growth, or during cell wall synthesis, so that inhibited DNA amplification in that case might also generate false negative results (Stiefel *et al.*, 2015). The detection of messenger RNA (mRNA) is considered a better indicator of cell viability than DNA, since this molecule is only present in metabolically active cells (Sheridan *et al.* 1998). Reverse transcription-PCR (RT-PCR) is one of the RNA-based molecular techniques most commonly used (Lleò *et al.*, 2000). RT-PCR uses the reverse transcriptase enzyme to convert originally extracted mRNA into complementary DNA (cDNA). The newly synthesized cDNA is then used as a template for exponential amplification using conventional PCR (RT-PCR) or quantification using quantitative PCR (RT-qPCR). RT-qPCR appears to be the first choice for the rapid detection of viral foodborne pathogens in food (Terio *et al.,* 2017). However, it seems that application for detection of bacterial foodborne pathogens is less common and currently appears to be limited to inactivation studies or challenge tests (Baskaran *et al.,* 2016). This is probably because the method is too laborious, or due to the rapid degradation of RNA in tested samples, which might also lead to false negative results (Xiao *et al.,* 2012). Various molecular methods for assessing microbiological quality of foods are:

1. Polymerase chain reaction (PCR)

The PCR is an *in vitro* enzymatic method which allows several million-fold amplification of a specific DNA sequence within few hours. This technique

was invented by Mullis *et al.* (1986) and is not only useful for molecular biologist and geneticist but also useful for food microbiologists. This is a sensitive, cost effective, precise, authentic and potentially applicable technique for microbiological quality control of food products due to its lesser complexity and fast reliable nature. The ingredients required for successful PCR amplification are template DNA, pair of forward and reverse oligonucleotide primers, all four deoxynucleotide triphosphates, a thermostable DNA polymerase enzyme and reaction buffer. The PCR technique involves denaturation, primer annealing and elongation steps for a set number of times depending on the degree of amplification required. This generates billions of copies of desired DNA segment from picograms quantities of starting DNA in matter of few hours (Chikuni *et al.,* 1994). However, the other parameters are also important for successful amplification of desired PCR products, such as DNA quality, primer concentration, different thermo cyclers, brand of DNA polymerase, Mg++concentration, annealing temperature and final extension periods (Meunier and Grimont, 1993; Macpherson *et al.,* 1993). Theoretically, PCR can amplify a single copy of DNA a million-fold in less than 2 hr, hence, it has the potential to disseminate or greatly reduce the dependence on cultural enrichment. In a PCR system, assuming a sensitivity of 1 cell/reaction tube, approximately 103 bacteria/ml sample required to ensure a reliable and repeatable amplification (Wang *et al.,* 1997). Although PCR is a powerful technology, the reactions can be dramatically affected by the presence of inhibitory compounds in foods and selective microbiological media like bile salts and acriflavin. A problem to routine use of PCR in food testing laboratory is that the procedures are rather complicated and very clean environment is needed to perform the tests. Further, PCR cannot distinguish between live and dead cells and hence providing more false negative results (Biswas *et al.,* 2008).

2. Multiplex PCR

Multiplex PCR is a technique in which more than one pair of primer is used to amplify the different fragment of target gene simultaneously to save time and minimize the expense on detection of food borne pathogens (Slavik *et al.,* 2003; Bottero *et al.,* 2004). However, great care must be taken to ensure that primers have same melting temperature and they must not interact with each other. Kawasaski *et al.* (2009) used a multiplex PCR system for simultaneous detection of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157:H7 in foods. The multiplex PCR assay was able to detect all three pathogens when found in food at less than or equal to 5 bacteria per 25 gm of food. Fukushima *et al.* (2010) reported simultaneous screening of 24

target genes of food borne pathogens in 35 food borne outbreaks using multiplex PCR assays. Zhou *et al.* (2011) used multiplex PCR method for identification of *Campylobacter* isolates in retail broiler meat and typed using pulsed-field gel electrophoresis (PFGE).

3. Real-time PCR

Real-time PCR has become a very attractive method for the detection of food borne pathogens since it offers a continuous monitoring technique for PCR product formation throughout the reaction, which eliminates post-PCR analysis process, shortens the detection time compared to standard PCR, and reduces the risk of amplicons contamination by laboratory environments (Heid *et al.,* 1996; Klein and Juneja, 1997). In addition, Real-time PCR is a quantitative method and is often used to determine the number of pathogens in various samples (Heid *et al.,* 1996; Long *et al.,* 2008). Real time PCR method allows built in product detection (both quantitative and qualitative) during the entire reaction period. RT-PCR is so named as one can continuously monitor the development of amplicons in a fluorimeter. SYBR-Green (a cyanine dye, binding preferably to dsDNA) or other fluorescent labelled probes that emit lights during amplification are widely used in RT-PCR. The emitted light signals corresponding to DNA amplification recorded at frequent intervals generating a curve showing product generation. The more targets DNA amplifies in the sample, the earlier amplicons can be detected and the peak curve is generated. Baggi *et al.* (2005) have used RT-PCR for rapid detection of diarrheagenic *E. coli* using SYBR Green dye and best sensitivity and specificity was observed. For the detection of food samples 5' nuclease multiplex PCR can also be employed. The method uses the 5' nuclease activity of T*aq* polymerase (Holland *et al.*, 1991) and FRET (Fluorescence Resonance Energy Transfer) technology in same experimental setup with multiple primers and probes. The method can be optimized by the amount of each primer pair to achieve the maximum amplification in separate reaction condition and the target loci must be checked separately for amplification with same reaction conditions and same PCR program. Real-time PCR using RNA as template is more authentic since the RNA is present only in viable microbes. RNA is first reversely transcribed to cDNA and in second step used for amplification. The kits are developed for the molecular detection of pathogenic bacteria in foods and environmental samples, such as *Salmonella, Listeria monocytogenes, E. coli* O157:H7, *Campylobacter*. Huang *et al.* (2007) identified 8 food borne pathogens by multicolour combinational probe coding technology (MCPC) in a single real time PCR.

4. DNA fingerprinting

DNA fingerprinting techniques have also been used for detection and identification of pathogens. Generally, the genomic fingerprinting methods require isolation of DNA from cells of pure isolated colonies, restriction enzyme digestion, and amplification or hybridization of fragments with appropriate primers or probes, which may take at least five to seven days to complete. An automated RiboPrinter system, a compact robotic instrument, can perform all these tasks within eight hours. The DNA fingerprint pattern is then compared with the built-in database, and the precise identification is made for each bacterium. Other commonly used fingerprinting techniques include pulsed-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD) analysis, amplified fragment-length polymorphism (AFLP), restriction fragment-length polymorphism (RFLP), rep-PCR, and multilocus sequence typing (MLST).

A. Random Amplified Polymorphic DNA Analysis (RAPD): RAPD, a PCR based technique, generates pattern of DNA bands on gel electrophoresis using amplification of random DNA segments with primers of arbitrary nucleotide sequence. Short length primers bind randomly along the prokaryotic genome resulting number of different DNA fragments. The array of fragments is examined for similarity of genotypes based upon the number and size of the amplicons. RAPD has been used in detection of *Listeria* species in poultry processing environment and vegetable processing plants to identify the source of contamination and dissemination.

B. Amplified Fragment Length Polymorphism: Amplified Fragment Length Polymorphism (AFLP) represents another genotyping technique based on selective amplification of restriction fragments of DNA molecule (Vos *et al.*, 1995). The method involves restriction endonucleases digestion of total purified genomic DNA followed by ligation of the resulting fragments by a double-stranded oligonucleotide adapter complementary to the base sequence of the restriction site. The adapters are designed such that the original restriction site is not restored after ligation, thus preventing further restriction digestion. Selective amplification of sets of these fragments in PCR is achieved with primers corresponding to the contiguous base sequences in the adapter, the restriction site plus one or more nucleotides in the original target DNA. The resulting PCR-amplified DNA fragments are then analyzed by gel electrophoresis. AFLP can be applied for the determination of sources of contamination, especially in cases such as livestocks (Siemer *et al.,* 2005).

AFLP combined with automated laser fluorescence analyzer been used for rapid and reliable identification at the strain level (Aarts *et al*., 1999).

C. Restriction Fragment Length Polymorphism: Restriction Fragment Length Polymorphism (RFLP) is a very simple method that uses particular restriction enzyme digestion of the genomic DNA. It is used for the comparison of the number and size (mass) of the fragments produced by restriction endonucleases cutting at a specific recognition site of the target DNA. The resulting DNA fragments are examined by electrophoretic separation. Presence, absence, or changes in the mass of the resulting DNA fragments are evidence of changing DNA sequences. This method requires pure culture for the discrimination of bacteria at the species level. RFLP in combination with PCR has been used for the accurate detection of *Staphylococcus* and *Listeria* spp. (Paillard *et al*., 2003).

5.Loop Mediated Isothermal Amplification (LAMP)

Loop mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method that relies on an auto-cycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment (Notomi *et al.,* 2000). *Bst* DNA polymerase is the largest fragment of *Bacillus stearothermophilus* DNA polymerase protein that contains the 5'-3' polymerase activity, but lacks the 5'- 3' exonuclease domain which performs 5'-3'exonuclease activity. *Bst* DNA polymerase has strand displacement activity which synthesizes DNA with loop forming primers to yield long stem loop products under isothermal condition. LAMP is different from PCR in that four to six primers perform the amplification of the targeted gene, the amplification uses a single temperature step at 60-65^{0}C for about 60 min, and the amplification products have many types of structures in large amounts. Thus, LAMP is faster and easier to perform than PCR, as well as being more specific. Furthermore, gel electrophoresis is not required, because the LAMP products can be detected indirectly by the turbidity that arises due to a large amount of by-products, pyrophosphate ion being produces, yielding an insoluble white precipitate of magnesium pyrophosphate in the reaction mixture. Haro-Kudo *et al.* (2005) used LAMP assay for the rapid detection of pathogens within 60 min. The 220 strains of 39 serotypes of *Salmonella Enterica* subsp. *Arizonae* were amplified, but not 62 strains of 23 bacterial species other than *Salmonella*. Tang *et al.* (2011) used LAMP for rapid and sensitive detection of *Listeria monocytogenes* in food.

6. Nucleic acid sequence-based amplification (NASBA)

NASBA is developed by Compton (1991) in the early 90s and it operates by amplifying nucleic acids under isothermal conditions, unlike PCR which requires thermocycling system. NASBA is normally used for the amplification of RNA whereby the single-stranded RNA template is converted into complementary DNA (cDNA) by the reverse transcriptase during the reaction. NASBA reaction occurs at around 41°C, involving two specific primers and three enzymes: avian myeloblastosis virus (AMV) reverse transcriptase, T7 RNA polymerase and RNase H. The NASBA amplicons can be detected by agarose gel electrophoresis (Leone *et al.*, 1998; Zhao *et al.*, 2014). The post-NASBA product detection methods such as agarose gel electrophoresis or enzyme-linked gel assay is considered labour-intensive and not cost-effective. This leads to the development of a novel real-time NASBA which uses fluorescently labelled probes which are molecular beacons to detect the single-stranded RNA amplicons, thus, producing a homogenous NASBA assay (Leone *et al.*, 1998). Real-time NASBA has been used for the detection of various foodborne pathogens such as *Salmonella enterica, Vibrio cholerae, Staphylococcus aureus, Campylobacter jejuni, and Campylobacter coli* (Fykse *et al.*, 2007; O'Grady *et al.*, 2009). In addition, real-time NASBA is able to detect viable microorganisms that present in food samples through mRNA amplification and the detection of RNA targets will indicate the presence of viable cells (Simpkins *et al.*, 2000). Real-time NASBA has been used to distinguish viable from non-viable bacterial cells and RNase treatment is usually required to degrade target mRNA from dead cells before nucleic acid extraction or treating the samples with RNase-free DNase is required prior to performing the NASBA assay (Dwivedi and Jaykus, 2011). NASBA offers high-throughput analysis and it has been commercialized as kits. There are several commercial NASBA kits manufactured by Life Sciences, KIT Biomedical Research, Gen-Probe and bioMérieux (Gracias and McKillip, 2007). However, the commercial NASBA kit that is used for the detection of foodborne bacterial pathogens is mainly the Nuclisens EasyQ® Basic Kit (bioMérieux). Nuclisens EasyQ® Basic Kit can be used for the detection and identification of Listeria monocytogenes, Salmonella enterica and Vibrio cholerae (Fykse *et al.,* 2007).

7. DNA Microarray

Microarrays are made up of glass slides or chips coated with up to hundreds of specific oligonucleotide probes and these probes are chemically synthesized short sequences range from 25 to 80 bp (Severgnini *et al.,* 2011).

In DNA microarray, DNA probes targeting specific genes are first printed on the microarray chip and then exposed to test samples containing DNA. Complementary DNA present in the sample will hybridize with the probe in a spot, and the unbound or weakly bound DNA is removed by washing. The hybridized products are then detected and quantified by fluorescence. An array usually contains thousands of probes, and a micro array experiment is able to achieve goals from many pathogen detection tests in parallel. Therefore, DNA microarrays have tremendously promoted many types of investigations and offered a useful tool for microbiological quality control of food products. Identification of various food pathogens simultaneously was carried out using the microarray technique by Jiang *et al.* (2004). Oligonucleotide array is gaining popularity in investigating the clinical samples as it does not require costly reagents and machines. Zhang *et al.* (2006) used an electronic microarray technique for detection and differentiation of viable *Campylobacter* species, *C. jejuni, C. coli* and *C. lari*. This is achieved by using mRNA of the 60 kDa heat shock protein as the viability marker. This technique was able to detect as few as two viable Campylobacter cells. Liu *et al.* (2006) used same microarray method for detection of viable pathogenic *Escherichia coli, Vibrio cholera* and *Salmonella typhi*. Four unique genes, the *E. coli* O157:H7 LPS gene (*rfbE*) and H7 flagelling gene (*fliC*), the *V. cholera* O1 LPS gene (*rfbE*), and the *S. typhi* LPS gene (*tyv*) were chosen as the targets for detection. The technique was able to detect as few as two to 150 cells of *E. coli* O157:H7.

The advantages and limitations of various methods have been enlisted below:

Method	Advantages	Limitations	References
1. Simple PCR	• High sensitivity	• Affected by PCR inhibitors, Requires DNA purification	Mandal *et al.,* 2011; Zhang, 2013; Park *et al.,* 2014
	• High specificity	• Difficult to distinguish between viable and non-viable cells	
	• Automated		
	• Reliable results		
2.Multiplex PCR	• High sensitivity	• Affected by PCR inhibitors	Mandal *et al.,* 2011; Zhang, 2013;
	• High specificity	• Difficult to distinguish between	

		viable and non-viable cells	Park *et al.,* 2014
	• Detection of multiple pathogens	• Primer design is crucial	
	• Automated		
	• Reliable results		
3.Real-time PCR	• High sensitivity	• High cost.	Mandal *et al.,* 2011; Zhang, 2013; Park *et al.,* 2014
	• High specificity	• Difficult for multiplex real-time PCR assay	
	• Rapid cycling	• Affected by PCR inhibitors.	
	• Reproducible	• Difficult to distinguish between viable and non-viable cells	
	• Does not require post-amplification products processing	• Requires trained personnel.	
	• Real-time monitoring PCR amplification products	• Cross contamination may occur	
4.NASBA	• Sensitive	• Requires viable microorganisms	Lauri and Mariani, 2009; Zhao *et al.,* 2014
	• Specific	• Difficulties in handling RNA	
	• Low cost		
	• Does not require thermal cycling system		

	• Able to detect viable microorganisms		
5.LAMP	• High sensitive	• Primer design is complicated	Zhao *et al.,* 2014
	• High specificity	• Insufficient to detect unknown or unsequenced targets	
	• Low cost		
	• Easy to operate		
	• Does not require thermal cycling system		
6.Oligonucleotide DNA microarray	• High sensitivity	• High cost	Lauri and Mariani, 2009; Mandal *et al.,* 2011; Park *et al.,* 2014
	• High specificity	• Difficult to distinguish between viable High specificity and non-viable cells	
	• High throughput	• Requires trained personnel	
	• Enables detection of multiple pathogens	• Requires oligonucleotide probes and labelling of target genes	
	• Allows detection of specific serotype		
	• Labor-saving		

Conclusion

Pathogen testing is a key component and critical success factor in the formulation and implementation of effective food safety and process control programs. The essential steps for reducing or eliminating pathogenic microorganisms from food begins with their detection and/or quantification. The entire food processing chain beginning from the farm till consumer is to be

taken into account for keeping food safe. Needless to say, there is no single procedure or technology that can detect all microbes in food. However, the recent advances in molecular methods have ensured that microbiological quality assessment is more accurate with a short turnaround time along with higher sensitivity and specificity. The complex food matrix must be taken into account while choosing the appropriate tests. A variety of specific and rapid techniques for detection of foodborne pathogens have been on the anvil in recent years thus resulting in the availability of a wide range of diagnostics. The applicability of a diagnostic method depends on attributes like specificity, sensitivity, rapidity, portability, cost effectiveness and on-site testing ability, with each technique having its own advantage and disadvantages. Techniques like real-time PCR and MALDI-TOF MS cater to the need of service labs while biosensors and DNA microarrays have the capability for on-site testing of multiple pathogens in foods. Though rapid tests have been made available in the form of commercial kits, the complexity of foods continues to present challenging problems with most of the tests requiring pre-enrichment of food before subjecting it to final testing. Further research is needed to check cost effectiveness and efficacy of emerging techniques for in situ diagnosis of foodborne infections. The need of hour is to standardize, validate and harmonize protocols between laboratories at local, national and international levels to harness the maximum benefits.

CONVENTIONAL TO MOLECULAR TECHNIQUES FOR DIAGNOSIS OF RICKETTSIAL AND CHLAMYDIAL ZOONOTIC DISEASES IN ANIMALS AND ITS MANAGEMENT

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The *Rickettsiaceae* is a diverse collection of obligately intracellular gram-negative bacteria comprising the genera *Rickettsia, Ehrlichia,* and *Orientia*. The genus *Rickettsia* is non motile, non-spore-forming, highly pleomorphic bacteria that may occur in the forms of cocci (0.1 μm in diameter), bacilli (1–4 μm long), or threads (up to about 10 μm long). Bacterial genus *Rickettsia* was named after Howard Taylor Ricketts, in honor of his pioneering work on tick-borne spotted fever. This genus consists of two antigenically defined groups: spotted fever group and typhus group, which are related. The scrub typhus rickettsiae differ in lacking lipopolysaccharide, peptidoglycan, and a slime layer, and belong in the separate, although related, genus *Orientia*. *Rickettsia* species cause rickettsial pox, epidemic typhus, murine typhus, Rocky Mountain spotted fever, and other spotted fevers. The Rickettsia are primarily parasites of arthropods such as lice, fleas, ticks and mites, in which they are found in the alimentary canal and transmit to people by the bite of these arthropod vectors. In vertebrates, including humans, they infect the vascular endothelium and reticulo-endothelial cells. From the portal of entry in the skin, *Rickettsiae* spread *via* the bloodstream to infect the endothelium and sometimes the vascular smooth muscle cells. These organisms cannot grow in artificial nutrients. Most *Rickettsia* grows in the cytoplasm and nucleus of the cell. They can be cultured in HeLa, Hep2, Detriot-6, mouse fibroblasts, and other continuous cell lines. *Rickettsia* grows in the yolk sac of 5-6 days old chick embryo. The rickettsial diseases commonly found in India include Scrub typhus, Murine flea borne typhus, Indian tick typhus and Q fever. Q fever and trench fever have been excluded because the former is not arthropod borne and the latter is not an obligate intracellular parasite.

Chlamydiae are obligate intracellular bacteria. They lack several metabolic and biosynthetic pathways and depend on the host cell for

intermediates, including ATP. *Chlamydiae* exist in two forms; infectious particles called elementary bodies and intracytoplasmic, reproductive forms called reticulate bodies. The chlamydiae consist of three species, *C. trachomatis, C. psittaci*, and *C. pneumoniae*. The first two have many serovars based on differences in cell wall and outer membrane proteins. *Chlamydia pneumoniae* comprises one serovar, the TWAR organism.

Scrub typhus (Bush typhus)

Scrub typhus is the commonest and most widespread zoonotic disease among the diseases caused by rickettsial organisms both in India and globally. Scrub typhus is an acute, febrile, infectious illness that is caused by *Orientia* (*Rickettsia*) *tsutsugamushi*. It is also known as tsutsugamushi disease (from tsutsuga meaning dangerous and mushi meaning insect or mite). Scrub typhus was first described from Japan in 1899, where it was found to be transmitted by mites. Scrub typhus differs from other members in its genetic make-up and therefore there are considerable differences in virulence among individual strains of *Orientia tsutsugamushi*. The Rickettsial diseases remain grossly under diagnosed as routine laboratory tests are unlikely to be diagnostic and presentation non-specific. Scrub typhus is transmitted by the mite *Leptotrombidium deliense*. The vector mites inhabit sharply demarcated areas in the soil where the microecosystem is favourable (mite islands). Human beings are infected when they trespass into these mite islands and are bitten by the mite larvae (chiggers). The mite feeds on the serum of warm-blooded animals only once during its cycle of development, and adult mites do not feed on man. The microbes are transmitted transovarially in mites. Chigger index (average number of chiggers infesting a single host) of > 0.69 (critical value) is an indicator for implementation of vector control measures. Most cases of scrub typhus occur in rural areas of Southeast Asia, Indonesia, China, Japan, India, and northern Australia. Anyone living in or traveling to areas where scrub typhus is found could get infected.

Table 1. Difference between Rickettsia and Chlamydia

Features	*Rickettsia*	*Chlamydia*
Cytochrome	+	-
Metabolism	Aerobic	Anaerobic
DNA & RNA	+	+
Size of cell	500 nm	300 nm
Intracellular parasite	+	+

Growth in living cell	+	+
Growth on media	-	-
Inclusion bodies	+	+
Antibiotic sensitive	+	+
Transmission by vector	+	-

Table 2. Difference between *Rickettsia* and *Coxiella*

Character	*Rickettsia*	*Coxiella burnetii*
Taxonomic Position	F: *Rickettisiasae*	F: *Coxiellacae*
Growth	Freely in cytoplasm	Inside cell in Phagosome
Phase variation	No	Yes
		Phase-I-in host
		Phase-II-After passage in lab
Transmission	Essentially vector borne	Through all routes including by tick vector but Mainly Aerosol
Disease	Spotted fever-RMSF	Q fever
Weil-felix reaction	+ve	-ve

Table 3a. Human infections caused by *Rickettsia*

Bacteria	Diseases
Rickettsia prowazekii	Epidemic or louse-borne typhus; relapsing louse-borne typhus or Brill-Zinsser disease
Rickettsia typhi	Endemic or flea-borne murine typhus
Rickettsia rickettsiae	Tick borne Rocky Mountain spotted fever
Rickettsia akari	Rickettsial pox
Rickettsia conori	Boutonneuse fever, Indian Tick Typhus

Table 3b. Diseases caused by *Rickettsia* (Typhous group)

Typhus	Scrub typhus	*Orientia tsutsugamushi*	Larval mite (chigger)	Rodents	Asia-Pacific region from maritime Russia and China to Indonesia and North Australia to Afghanistan; recently recognized in Chile as well as some countries of Africa
		Orientia chuto	Unknown	Unknown	United Arab Emirates
Typhus fever	Epidemic typhus, sylvatic typhus	*R. prowazekii*	Human body louse, flying squirrel ecto-parasites	Humans, flying squirrels	Central Africa; Asia; North, Central and South America
	Murine typhus	*R. typhi*	Flea	Rodents	Temperate, tropical and subtropical areas worldwide

(Source:https://wwwnc.cdc.gov/travel/yellowbook/2020/travel-related-infectious-diseases/rickettsial-including-spotted-fever-and-typhus-fever-rickettsioses-scrub-typhus-anaplasmosis-and-ehr)

Flea-borne (murine) typhus

Flea-borne (murine) typhus is a disease caused by bacteria called *Rickettsia typhi*. It is also known as endemic typhus. Flea-borne typhus is spread to people through contact with infected fleas (*Xenopsylla cheopis*). Fleas become infected when they bite infected animals, such as rats, cats, or opossums. When an infected flea bites a person or animal, the bite breaks the skin, causing a wound. Fleas poop when they feed. The poop (also called flea dirt) can then be rubbed into the bite wound or other wounds causing infection. People can also breathe in infected flea dirt or rub it into their eyes. No person-to-person transmission. Flea-borne typhus occurs in tropical and subtropical climates around the world including United States (California, Hawaii, and Texas), Africa (Tunisia, Morocco, Ivory Coast, Central African Republic, Madagascar) Southeast Asia (India, Indonesia, Philippines, Thailand, Cambodia, Vietnam, Myanmar, or Laos). Symptoms of flea-borne typhus begin within 2 weeks after contact with infected fleas or flea dirt. Signs and symptoms may include fever, chills, body aches and muscle pain, loss of appetite, nausea,

vomiting, stomach pain, cough and rash (typically occurs around day 5 of illness). Severe illness is rare and most people recover completely, sometimes without treatment. Untreated disease can cause severe illness and damage to one or more organs, including the liver, kidneys, heart, lungs, and brain. Flea-borne typhus is treated with the antibiotic doxycycline which can be used in persons of any age.

Table 3c. Diseases caused by *Rickettsia* (Spotted fever group)

Rickettsiosis	*Rickettsia aeschlimannii*	Tick	South Africa, Morocco, Mediterranean littoral
African tick-bite fever	*R. africae*	Tick	Sub-Saharan Africa, West Indies
Rickettsial pox	*R. akari*	Mite	Countries of the former Soviet Union, South Africa, Korea, Turkey, Balkan countries, North and South America
Queensland tick typhus	*R. australis*	Tick	Australia, Tasmania
Mediterranean spotted fever or Boutonneuse fever	*R. conorii*[1]	Tick	Southern Europe, southern and western Asia, Africa, India
Cat flea rickettsiosis	*R. felis*	Flea	Europe, North and South America, Africa, Asia
Far Eastern spotted fever	*R. heilong-* *jiangensis*	Tick	Far East of Russia, Northern China, eastern Asia
Aneruptive fever	*R. helvetica*	Tick	Central and northern Europe, Asia
Flinders Island spotted fever, Thai tick typhus	*R. honei*, including strain "marmionii"	Tick	Australia, Thailand

Japanese spotted fever	*R. japonica*	Tick	Japan
Mediterranean spotted fever-like disease	*R. massiliae*	Tick	France, Greece, Spain, Portugal, Switzerland, Siciliy, central Africa, Mali, United States
Mediterranean spotted fever-like illness	*R. monacensis*	Tick	Europe, North Africa
Maculatum infection	*R. parkeri*	Tick	North and South America
Tickborne lymphadenopathy (TIBOLA), *Dermcentor*-borne necrosis and lymphadenopathy (DEBONEL)	*R. raoultii*	Tick	Europe, Asia
Rocky Mountain spotted fever; Brazilian spotted fever, febre maculosa, São Paulo exanthematic typhus, Minas Gerais exanthematic typhus	*R. rickettsia*	Tick	North, Central, and South America
North Asian tick typhus, Siberian tick typhus	*R. sibirica*	Tick	Russia, China, Mongolia
Lymphangitis-associated rickettsiosis	*R. sibirica mongolotimonae*	Tick	Southern France, Portugal, China, Africa
TIBOLA, DEBONEL	*R. slovaca*	Tick	Southern and eastern Europe, Asia; recently in US tick colony (unknown origin)

(Source:https://wwwnc.cdc.gov/travel/yellowbook/2020/travel-related-infectious-diseases/rickettsial-including-spotted-fever-and-typhus-fever-rickettsioses-scrub-typhus-anaplasmosis-and-ehr)

Indian Tick Typhus

Indian tick typhus is caused by the bacteria *R. conori* and transmitted by the dog tick *Rhipicephalus sanguineus*. Some species of *Haemaphysalis* and *Hyalomma* ticks may also transmit the infection. This disease is also known as Boutonneuse fever, Marseilles fever, Mediterranean spotted fever, Mediterranean tick fever, African tick typhus, and Kenya tick typhus. First described in Tunisia in 1910 by Conor and Bruch and was named boutonneuse (French for "spotty") due to its characteristic papular skin-rash. The disease occurs in much of Africa, Southeast Asia, India, and areas of Europe and the Middle East adjacent to the Caspian, Mediterranean, and Black Seas. The disease first observed in India in the foothills of the Himalayas. Subsequently, reported from many parts of the country including Allahabad, Narsapatnam, Ratlam, Secunderabad, Trichinapally, Bangalore, Jhansi, Darjeeling, Pune and Lucknow. Man is an accidental host. The infection is maintained in nature by wild rodents and their ticks. Dogs play a very important role by introducing infected ticks into the human environment.

Spotted fever group

All spotted fever group (SFG) diseases transmitted by ticks, except *R. akari*, which is mite borne. *Rickettsiae* of this group possess a common soluble antigen and multiply in the nucleus as well as in the cytoplasm of host cells. Many species have been recognized in this group.

Organism	Disease
R. rickettsi	Rocky mountain spotted fever
R. siberica	Siberian tick typhus
R. conori	Indian, Mediterranean, Kenyan and South African tick typhus
R. australis	Queensland tick typhus
R. japonica	Oriental spotted fever

Ticks serve as both reservoir and vector for rickettsial diseases as they are transmitted transovarially in ticks. Ticks are not harmed by the rickettsiae and remain infected for life.

Q fever

Q fever is caused by the gram-negative intracellular bacteria, *Coxiella burnetii*. The agent differs from other rickettsiae in its filterability and high degree of resistance to physical and chemical agents (it is more resistant than most nonsporogenic microorganisms). It does not produce agglutinins in the

Weil-Felix test, nor does it cause a cutaneous rash in man, and it can be transmitted without the intervention of a vector. The disease is also known as Pneumorickettsiosis, Balkan influenza, coxiellosis, abattoir fever, Australian Q fever, hiberno-vernal bronchopneumonia, nine-mile fever, quadrilateral fever, Burnet's rickettsiosis, Balkan grippe, Goat flu and Query fever. First identified in Australia in 1935, Q fever has since been found throughout the world with the exception of New Zealand. Domestic ruminants are considered the main reservoirs of C. burnetii, but cats, dogs, rabbits, birds, etc., have also been reported to be implicated in human disease/infection. There is clear epidemiological and experimental evidence that the infection is principally transmitted by inhalation of desiccated aerosol particles, and through exposure in the vicinity of infected animals, their reproductive tissues or other animal products, like wool. Ingestion has been often suggested, particularly through the consumption of dairy products derived from contaminated raw milk, but no good evidence has shown significant transmission to humans by food. Q fever also seems very rarely transmissible from person to person, although exposure during childbirth, through sexual transmission or blood transfusion is possible. The incubation period ranges from two weeks to 39 days, with an average of 20 days. The disease has a sudden onset, with fever, chills, profuse sweating, malaise, anorexia, myalgia, and sometimes nausea and vomiting. The fever is remittent and usually lasts from 9 to 14 days. The main clinical manifestations of chronic Q fever are endocarditis, valvular, vascular or aneurismal infections, hepatitis, pneumonia or chronic fatigue syndrome. The acute form resolves quite quickly after appropriate antibiotic therapy, but the chronic form requires prolonged antibiotic therapy (for 2 years or more), coupled with serological monitoring.

Diagnosis

Diagnosis of rickettsial infections is often difficult. The clinical signs and symptoms (e.g., fever, headache, nausea, vomiting, and muscle aches) resemble many other diseases during the early stages when antibiotic treatment is most effective. The diagnostic tools available include serologic assays, molecular testing, cultures, immunochemistry, and Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF). Serology has been the mainstay for diagnosis of rickettsial infections since 1916, with the development of the Weil-Felix test. Advancements in serological assays have provided greater sensitivity and specificity; however, they remain an imperfect diagnostic tool. Currently, the indirect immunofluorescence assay (IFA) is the

reference-standard serological assay, however, due to cost, laboratory equipment, and technical expertise required, other assays remain in use.

Serological tests

The Weil-Felix test (WFT) is a non-specific heterophile agglutination reaction, utilising cross-reactivity between rickettsiae and various *Proteus* serotypes (*P. vulgaris* OX-19 antigen (Ag)-typhus group antibody (Ab); *P. vulgaris* OX-2 and OX-19 Ag- spotted fever group Ab) for detection of anti-rickettsial antibodies. Agglutinating antibodies, mainly IgM, are detectable 5–10 days after symptom onset. Poor sensitivity and specificity of the WFT have been demonstrated for all rickettsial groups and the test is now rarely used; however, in some resource-limited settings, this remains the only test available.

The indirect immunofluorescence assay is performed using fluorescein-labelled conjugate to detect serum antibodies to rickettsial antigens fixed to a slide. The majority of laboratories test perform IgG IFA, as IgM antibodies do not appear significantly earlier and are less specific. IFA IgG assays demonstrate high sensitivity (83–100%) and specificity (91–100%) from the second week of illness onwards, for both SFG and TG infections. IFAT is the WHO reference test for detection of Phase I and II antibodies to discriminate acute and chronic *C. burnetii* infection.

Microimmunofluorescence (MIF) allows for simultaneous detection of multiple rickettsial antigens in a single well. This can assist in differentiation of species; if a species demonstrates a fourfold higher dilution compared to others, this may be suggestive of a causative organism. However, this is not definitive, and cross-reactions can hinder this technique. Western blotting and cross-adsorption assays may overcome this issue. Western blot analysis allows detection of both non-specific lipopolysaccharide (LPS) and species-specific surface protein antigens (SPA), facilitating species-level diagnosis.

The enzyme-linked immunosorbent assay (ELISA) detects the binding of specific antibodies to antigens in a serum sample. When secondary anti-human antibodies conjugated with an enzyme are bound to antibodies from a serum sample and subjected to a substrate, an enzymatic reaction will be measurable in a positive specimen. The inhibition ELISA has been used only for the diagnosis of scrub typhus and seems to be more sensitive than IFA in the early phase of the disease

Molecular diagnostic methods

The Nucleic Acid Amplification Tests (NAATS) has been used to identify numerous *Rickettsia* species directly from clinical specimens. Many different molecular probes and primers have been used to identify *Rickettsia* DNA, including 16S rRNA gene, *gltA* (citrate synthase), *ompA* (outer membrane protein A), *sca0, sca4* and *sca5* (outer membrane proteins), HSP60, and genes encoding lipoproteins (17-kDa antigen). Patient specimens have included blood, buffy coats, plasma, tissue (fresh, frozen, and paraffin-embedded), and swabs from the base of ulcers. The conventional PCR format, due to a large number of PCR products, is more prone to contamination. For this reason, a single-use primer PCR has been introduced.

The most recent diagnostic tool is the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF). This technique has been using with promise application for the Tick-borne infections inside the arthropods. The future role of this new method could be applied to help the clinical decision.

Isolation techniques

Shell vial technique, a method originally employed for the isolation of cytomegalo virus, has been the gold standard for *Rickettsia* isolation and culture due to its versatility and efficiency. In shell vial technique, only a small volume of cell and inocula are required, which are put in close contact through centrifugation, a critical step to enhance the penetration of the bacteria into the cells. Several successful attempts have been made for the isolation *of Rickettsia* species from yolk sacs of embryonated chicken eggs, however experimental results have shown that tissue culture cells provide more bacteria and are easier to purify. Different cell lines have been used for initial isolation tests, including Vero ATCC-CCL81 *(Cercopithecus aethiops* kidney), L929 *(Mus musculus* fibroblasts), HEL (Human embryonic lung). Particularly, Vero CCL-81 and L929 allows a fast isolation for highly infected samples; whereas the other mentioned cell types can sustain a prolonged incubation, with poorly infected samples. Tick cell lines have also been used to study differences in the proteomic and genomic expression among the vector and host.

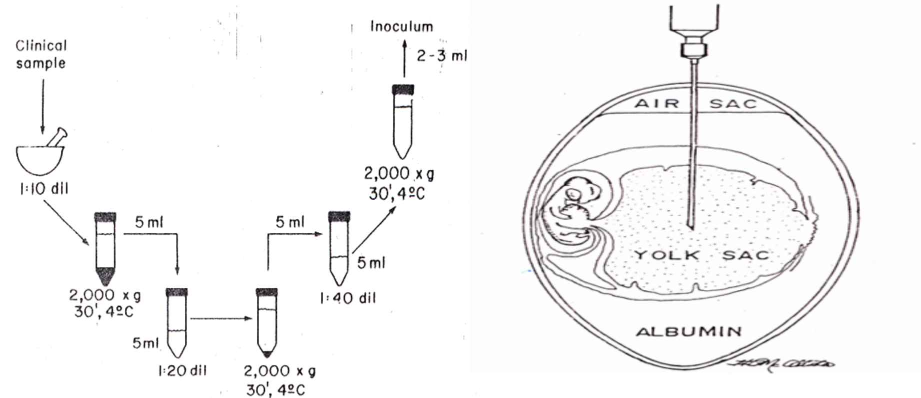

Fig: Inoculation in yolk sac of embryonated chicken egg

Prevention & Control

- No vaccine is available for preventing rickettsial infections except Q fever vaccine –Q vax (Australia)
- Antibiotics are not recommended for prophylaxis of rickettsial diseases and should not be given to asymptomatic people.
- Travelers should be instructed to minimize exposure to infectious arthropods during travel (including lice, fleas, ticks, mites) and to animal reservoirs (particularly dogs) when traveling in endemic areas.
- The proper use of insect or tick repellents on skin or clothing, self-examination after visits to vector-infested areas, and wearing protective clothing are ways to reduce risk.
- Raw milk should be pasteurized at 72°C for 15 sec to destroy *C. burnetii*

Table 4. Tests used routinely for detection of *Rickettsia*

Type of test	Methods	Advantages	Disadvantages
Indirect immunofluorescence antibody assay (IFA)	Serologic	High sensitivity and specificity for IgG.	Low sensibility for IgM; Operator-dependent
The enzyme-linked immunosorbent assay (ELISA)	Serologic	More sensitive than IFA for the detection of low antibodies level; Absorbance of the enzyme reaction is measured with a spectrophotometer	Could be negative during the early phase
Nucleic acid amplification tests (NAATs)	molecular methods	Quick response; could be used during acute disease. Provides the differentiation between different species	High costs; low sensitivity if used peripheral blood and serum. antibiotics reduce the sensitivity

Control programmes: Initiation of national level control programmes across the globe such as the 'National Q fever management programme' introduced by Commonwealth Government in October 2000. A 'Task Force on Rickettsioses' in India at ICMR -to streamline, strengthen and coordinate the research work on diagnosis, prevention & control

EMERGENCE AND DETECTION OF ANTIMICROBIAL RESISTANCE IN FOOD BORNE PATHOGENS AND MAJOR CHALLENGES OF AMR WITH CURRENT SCENARIO

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Antimicrobial resistance (AMR) is a critical public health problem, which can shake the foundation of modern healthcare. The misuse and or overuse of antibiotics in livestock is known to have contributed to the emergence of AMR. Global Research on Antimicrobial Resistance study show that drug-resistant bacterial infections contributed to almost five million human deaths in 2019, making AMR a leading cause of death globally. Infections caused by drug resistant organisms could lead to increased mortality and prolonged duration of hospitalization, causing a huge financial burden to the affected persons, healthcare systems, and hinder the goals of sustainable development. With rising AMR, antibiotics are increasingly becoming ineffective for treating diseases in humans. If there is no timely containment, AMR is likely to cause nearly 10 million deaths by 2050 and result in significant global economic losses. It would also impact nutrition security, livelihood and hinder the attainment of the Sustainable Development Goals.

Foodborne diseases have become a major public health problem worldwide due to the significantly increased incidence. Although it is difficult to estimate the global incidence of foodborne diseases as some of the cases are under-reported especially in developing countries, but the increased incidence of foodborne diseases was reported in many parts of the world. For instance, the outbreak of foodborne disease in Taiwan increased rapidly from 121 in 1995 to 177 in 1996 and since then the incidence keep rising. According to report from Centers for Diseases Control and Prevention (CDC), approximately 48 million people in the United States get ill, 128000 people are hospitalized and 3000 people die annually due to food-borne diseases despite United States has the safest food supplies in the world (Centers for Disease Control and Prevention, 2011). In addition, about a quarter of the population is at a higher risk for foodborne diseases nowadays.

Generally, foodborne diseases are caused by the consumption of food or water contaminated with pathogens or their toxins. Pathogens that caused foodborne diseases are often referred as foodborne pathogens and they include bacteria, viruses, fungi and parasites (Zhao *et al.,* 2014). There are 31 identified foodborne pathogens in the United State and it is estimated that viruses are the primary causes of illnesses whereas bacteria are the primary causes of hospitalizations and deaths. The common foodborne pathogens which are responsible for most of the foodborne disease out-breaks are *Listeria monocytogenes, Escherichia coli* O157:H7, *Staphylococcus aureus, Salmonella enterica, Bacillus cereus, Vibrio spp., Campylobacter jejuni, Clostridium perfringens* and Shiga toxin-producing *Escherichia coli* (STEC).

The increasing amounts of street foods and the increasing demand for minimally processed ready-to-eat products have begun to concern public health agencies on food safety. Foodborne pathogens are present in various foods such as fruits, vegetables and ready-to-eat products which are consumed without any further treatment. This may lead to foodborne diseases if food safety issues are not taken into consideration. Also, foodborne diseases are often associated with the consumption of raw or undercooked foods such as seafood, meat and poultry; It is essential to analyze the food for the presence of foodborne pathogens in order to ensure a safe food supply and to minimize the occurrence of foodborne diseases.

Understanding the gravity of the situation and the threat posed by AMR on healthcare, the World Health Assembly (WHA) adopted the Global Action Plan (GAP) on AMR in 2015. Following this, many countries have formulated their own National action plans (NAPs). The Indian Ministry of Health and Family Welfare published the National Action Plan for containing AMR in April 2017. It was submitted at the 70th WHA in Geneva in May 2017.This 5-year NAP on AMR (2017–2021) outlines the priorities and implementation strategies for curbing AMR in India.

AMR in food animals

According to the statistics of 2015, India was the largest producer of milk and the second largest producer of fish in the world. Further, the poultry consumption in India is expected to rise by 577 per cent between year 2000 and 2030. With such a huge potential of food animal industry, antimicrobial agents are being used in abundance to increase the productivity. India produced $137{,}685.8 \times 10^3$ tonnes of milk in 2013-2014 with major contributions from States of Uttar Pradesh (17.6%), Rajasthan (10.6%) and Andhra Pradesh

(9.4%). On analyzing milk samples for the estimation of AMR in livestock, 48 per cent of Gram-negative bacilli detected in cow and buffalo milk were extended-spectrum β-lactamases (ESBL) producers (West Bengal) and 47.5 per cent were resistant to oxytetracycline (Gujarat). Among the Gram-positive organisms isolated from these milk samples, 2.4 per cent of *S. aureus* were vancomycin resistant (West Bengal). The rate of methicillin resistance was 21.4 and 14.5 per cent for *S. aureus* from Karnataka and Jabalpur, Madhya Pradesh.

In the poultry industry of India, $1{,}916 \times 10^3$ tonnes of broiler meat are produced each year with maximum production by States of Haryana (18.4%), West Bengal (17.1%) and Uttar Pradesh (14.1%). Three studies on ESBL producing *Enterobacteriaceae* have documented the rate of ESBL producers to vary from 9.4 per cent in Odisha to 33.5 per cent in Madhya Pradesh to 87 per cent in Punjab. Other four studies reported the presence of *Salmonella* species in broilers to vary from 3.3 per cent in Uttar Pradesh to 23.7 per cent in Bihar with 100 per cent isolates being resistant to ciprofloxacin, gentamicin and tetracycline in Bihar and West Bengal. While, from M.P. in broiler, layer and breeder presence of *Salmonella* species were 11.26% They showed resistance to norfloxacin (81.48%), ciprofloxacin (92.59%) and ampicillin / sulbactum (80%), respectively (Bordoloi *et al.*, 2018).

India, where 9579×10^3 tonnes of fish is produced in a year, is becoming an important hub of aquaculture industry. In the common Tilapia fish found in the lakes of Maharashtra, 48 per cent *Enterobacteriaceae* isolated from the gut were ESBL producers. *Vibrio cholera* and *V. parahaemolyticus*, isolated from the retail markets of shrimps, shellfish and crabs in Kerala were 100 per cent resistant to ampicillin, 100 per cent susceptible to chloramphenicol while resistance to ceftazidime ranged from 67 to 96 per cent.

AMR in environment

Antimicrobial-resistant bacteria and their genes have been reported from different water sources of India. The major sources are the pharmaceutical waste waters and hospital effluents that are released into the nearby water bodies without adequate treatment. The rate of isolation of *E. coli* resistant to third generation cephalosporin was 25, 70 and 95 per cent when the inlet to the treatment plant was domestic water alone, domestic waste along with hospital effluent and hospital effluent alone, respectively. The two largest rivers of India, Ganges and Yamuna, span across a massive area of land and receives multiple inlets with varying concentration of drug-resistant bacteria. The rate of ESBL producers was 17.4 per cent among Gram-negative bacteria isolated

from these north Indian rivers with detection of resistance genes like *bla*NDM-1 and *bla*OXA48 23. Of the 283 *E. coli* isolates from the south Indian river Cauvery in Karnataka, 100 per cent were resistant to third generation cephalosporin. The groundwater and surface water that are used for drinking and recreational purposes have been reported with 17 per cent rate of *E. coli*, resistant to third generation cephalosporin, in central India, seven per cent in north India (Kashmir), 50 per cent in east India (Sikkim) and 100 per cent in south India (Hyderabad). The samples in these studies were collected from water sources like rivers, ponds, lakes, springs, hand pumps and tube-wells.

Conventional methods

The conventional methods for detecting the foodborne bacterial pathogens present in food are based on culturing the microorganisms on agar plates followed by standard biochemical identifications. Conventional methods are usually inexpensive and simple but these methods can be time consuming as they depend on the ability of the microorganisms to grow in different culture media such as pre-enrichment media, selective enrichment media and selective plating media. Usually conventional methods require 2 to 3 days for preliminary identification and more than a week for confirmation of the species of the pathogens. Conventional methods are laborious as they require the preparation of culture media, inoculation of plates and colony counting (Mandal *et al.,* 2011). Furthermore, conventional methods may be limited by their low sensitivity. False negative results may occur due to viable but non-culturable (VBNC) pathogens. The failure to detect foodborne pathogens would increase the transmission risk of pathogens.

Antibiotic Susceptibility Testing: As the Broth dilution method are time-consuming, many laboratories in the United States adopted disc diffusion tests in the early 1950s. Kirby Bauer tests also known as the Disc diffusion test is used for antibiotic susceptibility testing. Lack of standardization creates a problem in the 1960s and later Kirby and Bauer reviewed the description. In 1961, WHO standardized the procedure. Currently, CLSI updates and modifies the original procedure which ensures uniformity worldwide. This test is mainly performed to determine the sensitivity or resistivity of aerobes or facultatively anaerobes against different classes of antibiotics.

Immunological-based methods

The detection of foodborne pathogens by immunological-based methods is based on antibody-antigen interactions, whereby a particular antibody will bind to its specific antigen. The binding strength of a particular antibody to its

antigen determines the sensitivity and specificity of immunological-based methods. Immunological-based methods involve the use of polyclonal and monoclonal antibodies (Zhao *et al.,* 2014).

ELISA offers a sensitive and accurate detection of foodborne pathogens. However, the operation of ELISA requires specialized equipment and trained personnel.). Hence, other immunological detection methods which are rapid, cheap, simple and reliable are required. Lateral flow immunoassays such as dipstick and immunochromatographic strips have been developed for rapid on-site detection of foodborne pathogens. Lateral flow immunoassay device is made up of four sections which are arranged orderly on a plastic backing, with sample pad starting at the bottom, followed by conjugate pad, nitrocellulose membrane and then absorbent pad. The sample fluid will migrate along the four sections of lateral flow immunoassay via capillary action. The sample fluid encounters and mixes with the conjugate, which can be antibody or antigen labelled by a colour particle, at the conjugate pad and then pass through the lines in the nitrocellulose membrane that immobilized with antibody or antigen.

The colour particle can bind to the antibody or antigen immobilized at test line depending on the analytes present in the sample. The colour can be visualized approximately two to 10 min after the addition of sample. There are two basic formats of lateral flow immunoassays: competitive assay which used to test analytes with the detection of foodborne pathogens by lateral flow immunoassay employs labels such as monodisperse latex, colloidal gold, carbon and fluorescent tags. Immunochromatographic strip to detect *Escherichia coli* O157 in enriched samples had used colloidal gold particles as label. This study had showed that the detection limit for *Escherichia coli* O157 without enrichment was 1.8×10^5 CFU/mL and after enrichment was 1.8 CFU/mL. Niu *et al.* (2014) employed an immune-chromatographic test strip based on sandwich format with colloidal gold as label for the detection of *Staphylococcus aureus*. The detection limit for *Staphylococcus aureus* the study was 10^3 CFU/mL. Moreover, Xu *et al.* (2013) developed a novel immunochromatographic strip test which based on sandwich format with fluorescent microspheres as label for the detection of *Campylobacter jejuni.* The detection limit of this test was 10^6 CFU/mL. Lateral flow immunoassay is also used for detection of other foodborne bacterial pathogens such as Listeria spp. and Salmonella. It can also be used to detect toxins which may cause food-borne diseases such as brevetoxins and staphylococcal enterotoxin B.

ELISA is one of the most commonly used immunological methods for the detection of foodborne pathogens. Sandwich ELISA is the most effective

form of ELISA whereby it involves two antibodies (Zhao *et al.*, 2014). The primary antibody is usually immobilized onto the walls of the microtiter plate wells. The target antigen like bacterial cells or bacterial toxins from the food sample binds to the immobilized primary antibody and the remaining unbound antigens are removed. After that, an enzyme-conjugated secondary antibody is added and it will bind to the antigen and the remaining unbound antibodies are removed. The complex consisting antigen sandwiched between two antibodies is formed and it can be detected by adding a colourless substrate which will be converted into a coloured form in the presence of the enzyme. There are different types of enzymes can be used in ELISA, some of the most commonly used enzymes include horseradish peroxidase (HRP), alkaline phosphatase and beta-galactosidase.

Many studies have been performed using the sandwich ELISA for rapid detection of foodborne pathogens. For example, Kumar *et al.* (2011) performed the detection of pathogenic *Vibrio parahaemolyticus* in seafood with sandwich ELISA, using monoclonal antibodies against the TDH-related hemolysin (TRH)of pathogenic Vibrio parahaemolyticus. The detection limit of this assay was 103 cells of pathogenic *Vibrio parahaemolyticus*. Commercial ELISA test kit such as BIOLINE Salmonella ELISA Test is also available for the detection of Salmonella in food products. The detection limit of this test kit was 1 CFU/25 g sample with minimum four of the 20 food matrixes tested (Bolton *et al.,*2000). ELISA is also commonly used for the detection of toxins present in foods such as *Clostridium perfringens* α, β, and ε toxin, *Staphylococcal enteroxins* A, B, C and E, botulinum toxins and *Escherichia coli* enterotoxins.

Recently, high-throughput and automated ELISA systems such as BD Phoenix M50or VIDAS (BioMerieux) and Assurance EIA (BioControl) are available for the detection of foodborne pathogens. VITEK immunodiagnostic assay system (VIDAS) is system that performs entire ELISA procedure automatically. It utilizes enzyme-linked fluorescent immunoassay (ELFA) which is similar to ELISA, but it is a more sensitive fluorescent immunoassay for reporting the results. Generally, this system can complete an assay in 45 min to 2 h which also depends on the test kit. The VIDAS system involves the use of reagent strip and a plastic tube known as solid phase receptacle (SPR). A liquid sample of an enriched sample is placed in the reagent strip that contains all the required reagents in a ready-to-use format. The SPR serves as the pipette and the solid phase for the assay. The instrument will perform ELFA by automatically transferring the sample to the SPR that coated with antibodies in its interior wall in order to capture the target pathogen or toxin. The SPR is then

automatically transferred to a series of wells that contain enzyme-conjugated secondary antibodies and enzymes in a sequence manner. Once the assay is completed, the result will be automatically analyzed by the instrument and interpreted as positive or negative.

Nucleic acid-based methods such as PCR, mPCR, qPCR, and DNA microarray have high sensitivity and they are widely used for the detection of foodborne pathogens, but these methods require trained personnel and specialized instruments. Alternative nucleic acid-based methods such as NASBA and LAMP are available for the detection of foodborne pathogens and their toxins. NASBA and LAMP are relatively sensitive, specific and cost efficient. They do not require thermocycling system therefore they are useful especially in low resource settings. Furthermore, numerous biosensors-based methods have recently emerged and employed in the field of foodborne pathogen detection due to their rapidness and cost effectiveness.

Biosensors based methods are easy to operate and they do not require trained personnel, furthermore the techniques can be used for the detection of foodborne pathogens without sample pre-enrichment. Combination of several rapid methods for the detection of a particular foodborne pathogen is also possible as the use of only one detection method may not be sufficient to confirm the detected pathogen. Further studies on the effect of different combinations of rapid methods for foodborne pathogen detection are required in order to develop the most effective and accurate detection method.

Challenges of AMR in India

India has been referred to as ‘the AMR capital of the world’. While on one hand, emergence of newer multi-drug resistant (MDR) organisms poses newer diagnostic and therapeutic challenges, on the other hand India is still striving to combat old enemies such as tuberculosis, malaria and cholera pathogens, which are becoming more and more drug resistant. Factors such as poverty, illiteracy, overcrowding and malnutrition further compound the situation. Lack of awareness about infectious diseases in the general masses and inaccessibility to healthcare often preclude them from seeking medical advice. This, more often than not, leads to self-prescription of antimicrobial agents without any professional knowledge regarding the dose and duration of treatment. Among those who seek medical advice, many end up receiving broad-spectrum high-end antimicrobials owing to lack of proper diagnostic modalities for identifying the pathogen and its drug susceptibility. Low doctor to patient and nurse to patient ratios along with lack of infection prevention and

control (IPC) guidelines favour the spread of MDR organisms in the hospital settings. Easy availability of over-the-counter (OTC) drugs, further contributes to AMR.

The rise in the pharmaceutical sector has caused parallel rise in the amount of waste generated from these companies. With the lack of strict supervisory and legal actions, this waste reaches the water bodies and serves as a continuous source of AMR in the environment. Another important challenge could be the use of antimicrobial agents as pesticides and insecticides in the agriculture industry, although the evidence for the same is currently lacking. India has vast agricultural lands and farmers already face a lot of adversities at the hands of harsh weather, difficult terrain and natural calamities. They fall an easy prey to the lure of protecting their hard-earned field from pests and rodents by using antimicrobial agents without considering the future consequences. This large reservoir of antimicrobial agents forms a favourable niche for the emergence of MDR pathogens who then drift into the water bodies with rains and floods. The paucity of data on the extent of AMR, especially in animals and environment, presents hurdles to framing and implementation of policies on the control of AMR.

HANDLING, PRESERVATION AND TRANSPORT OF SAMPLES FOR DIAGNOSIS OF FOOD BORNE PATHOGENS

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Food is one of the three basic necessities of man and is required for sustenance of life on earth. The Government and Non-Governmental agencies the world over is striving to meet the rising demand for food, i.e., food security for all. In our haste for achieving this goal it is vital that we do not miss another important aspect, food safety. We may produce food in abundance in the near future, but until and unless it is safe and wholesome for human consumption, we are nowhere close to achieving food security. Human health concerns due to contaminated, adulterated, low quality food products containing microbial, chemical, or physical hazards are a common occurrence. This problem of unsafe food is humongous, with the World Health Organization (WHO) attributing around 33 million Disability Adjusted Life Years (DALYs) to food bore illnesses in 2010. The impact of such agents is much more as the above estimates are based on just 31 of the major food borne concerns out of the nearly 250 different foodborne hazards recognized globally. Together, these 31 global hazards caused approximately 600 million foodborne illnesses and 420,000 deaths in 2010. The burden and type of food borne illness varies from region to region with higher cases been reported in low-income countries especially in children below 5 years of age. Some important foodborne pathogens are *Salmonella, E. coli, Campylobacter, Listeria, Brucella, Shigella, Vibrio cholera, Mycobacterium,* Norovirus, Hepatitis A virus, *Cryptosporidium, Entamoeba, Giardia, Echinococcus, Taenia, Toxoplasma* and *Trichinella*.

The myriad potential hazards may contaminate the food commodity anywhere in the production chain right from production at farm to processing, packaging, transport, retail and final consumption at home. Therefore, it is important that the agents be recognized and prevented from entering the food commodity to limit the occurrence of such food borne outbreaks. The Food Safety and Standards Authority of India defines a food borne outbreak as "the occurrence of two or more cases of a similar foodborne illness resulting from the ingestion of a common food and epidemiologic analysis implicates the food

as the source of the illness OR the observed number of cases of a particular illness that may be foodborne exceeds the expected number. The WHO Foodborne Disease Epidemiology Reference Group (FERG) burden study attributed nearly 100 million cases of FBD in India per year in 2010. This corresponds to around one in 12 people falling ill, 120,000 deaths and a burden of over 8 million Disability Adjusted Life Years (DALYs). Therefore, to prevent entry of hazards in the food chain regular monitoring of food samples be done. Also, in cases of food borne outbreaks proper testing is required to implicate and control the hazard. But for testing to be meaningful the food sample should be collected, handled, packaged, and transported as per the standard guidelines to prevent any erroneous results.

Why to go for food sampling:

1. To verify the compliance with the criteria laid down in Food Safety and Standards Regulations.
2. To verify food safety management system/ process control.
3. To check the compliance of individual batches.
4. To obtain general information on the microbiological status of certain products placed on the market.
5. Monitoring and surveillance.
6. To investigate suspected food-borne outbreaks, customer complaints.

General points to be considered before collection of samples for detection of food borne pathogens

The reliability of the analysis and interpretation of the results exclusively depend on the process of collection, storage and transport of the sample. The condition of the sample received for examination influences the outcome of the analysis. If samples are improperly collected and mishandled or are not representative of the sampled lot, the laboratory results will be meaningless. It is very important that the sample should be uniform, homogenous and a true representative of the entire lot/ batch. A representative sample is essential when microbes are unevenly and sparsely distributed within the food, for this an established sampling procedure and plan must be applied uniformly. Extraneous organisms from hands, clothing, sampling equipment, or the processing environment may contaminate samples and may lead to erroneous results. Aseptic sampling techniques should therefore be adopted. A definite sampling plan may be followed as per the guidelines of FSSAI. Further,

improper transportation and storage especially at high temperatures may also affect the test results.

Collection of samples for detection of food borne pathogens

Samples should be collected following aseptic technique as far as possible. Basic tools like spoons, scoops, spatula, trier, knife, scissors, forceps, tongs, hand/ electric drills, chisel, hammer, swabs, syringes, template, etc should be made sterile before venturing out for sampling. Sterilization is generally preferred by exposure to dry heat (170 °C for 30 min/ 160 °C for 60 min/ 150 °C for 150 min) in hot air oven or steam (121 °C, 15 lb pressure for 20 min) in an autoclave. Other methods like exposure to a flame, immersion in 70% ethanol, ignition with 96% ethanol or exposure to a gamma radiation may also be done just before collection of samples in case of unavailability of the above two methods.

The sampler should keep their hands away from the mouth, nose, eyes and face. During sampling, hands should be sanitized with 70% isopropyl alcohol/ 70% ethanol and sterilized gloves be used during the collection of the sample. Care should be taken to prevent contamination of the external surface of the gloves prior to or during the sample collection process. Sampler should never touch the inside of the sterile container or lid and open lid not be allowed to become contaminated.

Samples should be collected on clean, dry, leak-proof, wide-mouthed and sterile containers of a size suitable for sample to be collected. The containers should be made of plastic, metal, but never glass to prevent spillage in case of breakage. For solids or semi-solids, clean, dry, wide-mouth, cylindrical receptacles of suitable waterproof, material should be used. Sample must be submitted in original and in sealed condition. Dry or canned foods that are not perishable and are collected at ambient temperatures should not be refrigerated. Frozen samples should be collected in pre-chilled containers. The sample collected should be adequate for laboratory analysis. Sample size for analysis has been given under the provision of Rule No. 13 (FSSA, 2011) and varies according to class of food item.

In case of outbreaks leftover foods from a suspect meal, ingredients used to prepare suspected foods, and foods known to be associated with the pathogen are collected. Unopened packages of food from the same batch may also be collected. This can help establish whether the food was contaminated before it arrived at the site of preparation. Any ingredients and raw items used in the preparation of the suspect meal that are still available should be sampled. The

conditions under which samples were collected, the names of the suppliers and distributors, and coding information on packaged foods should be recorded, so that the distribution channels of the product can be determined. It is useful to take photographs of any samples taken, as well as any labels and coding information on packaged food products. Environmental samples may be taken from work surfaces, food contact surfaces of equipment, containers, and other surfaces, such as refrigerators and door handles. Environmental samples may also include water used for food processing. Hand swabs and stool samples of food handlers and from clinical cases may also be taken.

Packaging and Labelling

In order to maintain integrity, containers should be secured or sealed. The stopper should be securely fastened to prevent leakage of the contents in transit. The bottle, jar or other container should be completely wrapped in strong thick paper and put in a zip pouch to prevent leak. It should be secured with shock-absorbing materials to protect damage during transit. Samples of frozen foods may be packed in insulated cartons containing dry ice. Proper care should be taken to provide information related to the sample by labelling. Label information should match that put on the test requisition form and include code number of the sample, name of sender, date, time and site of collection, food type, batch/ lot number, storage temperature, and type of testing requested.

Storage and Transportation

The samples collected should be transported to the laboratory as soon as possible (within 24 hrs) maintaining storage and transportation temperatures as close to collection conditions as possible (frozen stays frozen, cold stays cold). Chilled samples shall be transported at 2-8 °C and never frozen. Frozen or refrigerated products shall be transported in insulated containers of rigid construction under frozen conditions and prescribed temperature of the products. Samples which were not frozen before sampling shall not be frozen after sampling. Dehydrated and dry foods may be shipped and stored without refrigeration and should not be allowed to absorb any atmospheric moisture. These shall be stored in a clean, cool and dust free place. The samples should be protected from direct sunlight or other sources of heat. Meat and meat products, poultry and fish should preferably be transferred under wet ice refrigeration to avoid dehydration at the surface of the sample. Samples having different storage temperature shall be transported in separate transport container. The packaged water sample shall be stored and transported to the laboratory under the ambient conditions avoiding any kind of physical damage and cross contamination.

MONITORING AND DETECTION OF PESTICIDE AND ANTIBIOTIC RESIDUES IN PRODUCTS OF ANIMAL ORIGIN

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In the past few centuries, the global economy has developed in both the industrial and agricultural sectors resulting into exponential production and usage of industrial and agro-chemicals which have entered the global environment as potential contaminants. The contamination of food by chemical contaminants has become a worldwide public health concern and is a leading cause of trade problems internationally. Chemical pollution to the local environment also affects the lives of birds, wildlife, domestic animals, fish and livestock. Therefore, the determination of these contaminants in the environment and in foods is necessary for ensuring that human exposure to contaminants especially by dietary intake does not exceed acceptable levels for health. Lactating cattle may be exposed to pesticides from contaminated feed, fodder and water and thus can accumulate residues in different compartments including milk. However, the contamination of milk by pesticides can also be caused by their application to the cow's body, in the cow barn, or even in the milk processing areas. Due to the persistence and lipophilicity of the pesticides their residues get magnified in lipid rich tissues of the organisms and under certain conditions such as lactation, mobilization of deposited contaminants occurs and results in their excretion into milk. Food of animal origin contamination especially of milk is of extreme concern since milk and milk products have a very special position in the diet of infants, children and elderly for whom these are considered as a perfect natural food. The presences of pesticide residues have been reported in the bovine milk (Sharma *et al* 2007, Nag and Raikwar 2008), fish (Kaur *et al* 2008) and chicken meat (Aulakh *et al.,* 2006).

Antibiotics are substances that are administered to food producing animals, such as cattle, poultry, fish or bees, either for therapeutic, prophylactic or diagnostic purpose or for modification of physiological function or behaviour. Antibiotic residue is defined as detection of antibiotic at or above the tolerance or safe concentration for approved drugs, at any concentration for

unapproved drugs and at any concentration for illegal drugs. Group of antimicrobials that are used in food animals include β-lactams (penicillin, ampicillin), tetracyclines (chlortetracycline, oxytetracycline), sulfonamides (sulfadiazine, sulfamethoxazole, sulfadimidine, sulfadimethoxine), macrolides (erythromycin, spiramycin), aminoglylosides (gentamycin, neomycin, streptomycin) and quinolones (enrofloxacin, ciprofloxacin). The presence of veterinary drug residues in food has become an issue of international concern and debate involving risk assessment which is separated into four steps: hazard identification and characterization, exposure assessment, and risk characterization. Risk characterization is the important stage of risk assessment that brings together hazard characterization and exposure assessment. Therefore, the determination of pesticides and antibiotics residue in the environment and in foods is necessary for ensuring that human exposure to contaminants especially by dietary intake does not exceed acceptable levels for health. Human exposure resulting from consumption of food containing antibiotic residues could lead to allergic reactions, immunopathological effects, autoimmunity, carcinogenicity (sulphamethazine, oxytetracycline, furazolidone), mutagenicity, nephropathy (gentamicin), hepatotoxicity, reproductive disorders, bone marrow toxicity, allergic reactions. However, the greatest threat is the emergence of resistant strains of bacteria in humans through consumption of food containing antibiotic residues.

Estimation of pesticide and antibiotic residues:

Despite the usefulness in the increment of food production, the use of chemicals and antibiotics may lead to environmental contamination and presence of residues in food which have promoted their strict regulation in order to protect consumers, environment and also the users of pesticides. Thus, reliable, and accurate analytical methods are essential to protect human health and to support the compliance and enforcement of laws and regulations *pertaining* to food safety. Pesticide residue analysis is accomplished by means of an adequate sample preparation step followed by estimation with chromatographic technique, either GC or HPLC coupled to universal mass spectrometer (MS, MS/MS) or selective detectors ECD (electron capture detector), NPD (nitrogen phosphorus detector), FPD (flame photometric detector), UV detector, FTD (flame thermionic detector) and FLD (fluorimetric detector).

Multiresidue methods (MRMs) are capable of simultaneously determining more than one pesticide residue in a single analysis. The MRMs concept is raised to a higher dimension when a single extract is examined with

more than one chromatographic determinative step, each providing coverage of residues in a different class e.g., OCPs, OPs, SPs and carbamates. Whenever a sample of unknown pesticide treatment history is analyzed and no residue(s) is targeted, MRM should be used to provide the broadest coverage of potential residues. The presence of pesticide residues is regarded as a potential chemical hazard in several foodstuffs such as fruits, vegetables, and food of animal origin. Based on the increasing consumer's concern about the residue's persistence in their food, a large number of MRMs have been evaluated to ensure accurate residues determination.

Recently, several rapid detection methods have been developed for determining antibiotic residues in food. Microbiological assays and immunoassay techniques have been widely employed as screening assays because of their simplicity and inexpensiveness. However, these methods lack specificity and do not distinguish among members of a given class of antibiotic, provide only semi-quantitative measurements of residues and sometimes give rise to false positives. Foodstuffs containing levels of antibiotics that exceed the tolerance levels must be verified by highly selective and sufficiently sensitive chemical methods. Therefore, some highly selective and sufficiently sensitive methods, such as High-Performance Liquid Chromatography has been developed to replace microbiological assay. LC-MS methods are capable of identifying individual antibiotics within a class and can be valuable when confirmation is required compounds.

Regulations in India

The use of pesticide should be regulated to ensure the minimum residue levels in crops and other food articles which can be considered safe for human beings as well as for environment. The idea to regulate pesticide residues to safe levels was originally introduced by the Joint FAO/WHO Expert committee on Food Additives (1955). In order to implement the joint FAO/WHO Food standards programme, Codex Alimentarius Commission, comprising 120 member nations was established in 1964. The Codex Committee on Pesticide Residues (CCPR) is a subsidiary body of the Codex Alimentarius Commission that advises on all matters related to pesticides residues. Its primary objective is to develop Maximum Residues Limits in order to protect the health of consumer while facilitating international trade. MRL is the maximum concentration for pesticide residues on crop or food commodity resulting from the use of pesticides in accordance with Good Agricultural Practices (GAP). In India for the regulation of pesticide application, government bodies have an important and major role because both producers and users are not likely to

limit themselves in the sales and use of pesticides. The import, manufacture, sale, transport, use, etc. of pesticides is being regulated under a comprehensive statute ‘The insecticides act, 1968’ and the rules are framed to ensure availability of quality, safe and efficacious pesticides to farming community. The act also allows the Board to ban or restrict the use of any pesticide product. At National levels, the maximum residual limits (MRL) are fixed by Food safety and standard authority of India (FSSAI) so that remedial measures could be undertaken through IPM campaign to eliminate the risk of pesticide residues from agricultural commodities with special emphasis on export products.

In India, until 2013 there were no official regulations on the use of antibiotics in food animals reared for domestic consumption when the amendment was made in Drugs and Cosmetics Rules by the Ministry of Health and Family, Government of India. The amendment states that the container of the medicine meant for treatment of food producing animals should be labelled with the withdrawal period of the drug for the species on which it is intended to be used. If the withdrawal period is not specified, it should be at least 7 days for eggs or milk, 28 days for meat including fat and offal, and 500degree days for fish flesh. But still there is no legislation on restricting/banning the use of antibiotics as AGPs in India which have been now banned in many developed countries. In India, the manufacture and sale of antibiotics for human and veterinary purposes are regulated by the Central Drug Standard Control Organization (CDSCO) under the Drugs and Cosmetics Act, 1940. It is revised at times based on the advice of the drugs technical advisory board which is a part of the CDSCO in the Ministry of Health and Family Welfare, India. The most recent is the introduction of Schedule H, a class of prescription drugs in India. These are drugs which cannot be purchased over the counter without the prescription of a qualified doctor. The Food Safety and Standards Authority of India (FSSAI), 2011,(70) under the Food Safety and Standard Act (2006) is the main authority under the Ministry of Health and Family Welfarefor implementing science-based standards for food articles. It also regulates their manufacture, storage, distribution, sale, and import to ensure availability of safe and wholesome food for human consumption. FSSAI’s main roles are to implement good manufacturing practices, good hygienic practices, hazard analysis critical control point, and any new practices which are specified by regulations.

LABORATORY TECHNIQUES USED FOR DETECTION OF ADULTERATION IN MILK AND MILK PRODUCTS

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Milk may be defined as the whole, fresh, clean, lacteal secretion obtain by the complete milking of one or more healthy milch animal excluding that obtained within 15 days before or 5 days after calving. It is a complex mixture of water, proteins, fats, carbohydrates, vitamins, and minerals. The exact composition of milk can vary depending on factors such as the animal species, breed, and diet of the animal, as well as processing methods. It is a popular beverage that is consumed by many people around the world. It is rich source of nutrients such as protein, calcium, vitamin D, and other essential minerals. It can be consumed in a variety of forms, including whole milk, skim milk, and low-fat milk.Whole milk contains a higher percentage of fat compared to skim or low-fat milk. It is used in a variety of food products, such as cheese, butter, yogurt, and ice cream. It is also used as an ingredient in many baked goods and other recipes. It is important to note that some people may be allergic to cow's milk or may have difficulty digesting it due to lactose intolerance. In these cases, alternative types of milk, such as almond milk or soy milk, may be consumed instead.

In general, cow's milk contains about 87% water and 13% solids. The solids are composed of:

- Proteins: The two major proteins present in milk are casein and whey. Casein makes up about 80% of the protein in milk, while whey makes up the remaining 20%.
- Fats: Milk contains about 3-4% fat. The fat is present in the form of small globules, which can vary in size depending on the type of milk.
- Carbohydrates: The main carbohydrate in milk is lactose, which makes up about 4-5% of the milk.
- Vitamins: Milk is a good source of several vitamins, including vitamin D, vitamin B12, and riboflavin.
- Minerals: Milk is also a good source of minerals such as calcium, phosphorus, and potassium.

Overall, the composition of milk makes it a nutritious and important food for human consumption.

Current status of milk production in India

India is the top most milk producer in the world with 23% of the global milk share. It increased to 210 million tonnes in 2020-21. Milk production is growing at the rate of 2% in the whole world. However, in India, its growth rate is more than 6%. Daily milk consumption increased from 107gms to 427gms in 1970 to 2021 as against world average of 322gms in 2021.

Food Safety and Standards Authority of India (FSSAI) advocates that all states and union territories on daily basis should carry out regular testing of milk and milk products. Currently, it has started with mobile vans for testing (Food safety on Wheels). These van are fitted with MilkoScreen machines that uses infrared technology called Fourier Transform Infrared (FTIR) to test milk quality, detect adulteration and perform spot on testing. It also recommends regular testing for fats, solid-not-fat, protein, adulterants like added water, urea, sucrose, maltodextrin, and ammonium sulphate etc.

What is adulteration of milk?

Milk adulteration may be defined as any change caused in the natural level of milk ingredients. These changes may be brought about by addition of some foreign matter to milk or by removing some more valuable ingredients e.g., Fat, Starch, Urea etc. It is the practice of adding impurities or diluting milk with water, synthetic milk or other substances to increase the quantity of milk and decrease the cost of production. Common adulterants used in milk include starch, glucose, urea, formalin, detergent, and vegetable oil. Consuming adulterated milk can pose serious health risks, including food poisoning, allergic reactions, and long-term health problems like cancer and kidney damage. Adulterated milk can also lead to malnutrition as it lacks the essential nutrients that pure milk contains. To prevent milk adulteration, it is important to ensure that the milk you purchase is from a reputable source and undergoes proper testing for purity. It is also essential to be aware of the signs of adulterated milk, such as an unnatural colour or consistency, and to report any suspicious activity to the appropriate authorities.

Common milk adulterants and their detection

Starch, Cane Sugar, Water, Gelatin, Urea, Pond Water/Nitrates, Detergent, skimmed milk powder, Buffalo milk in Cow Milk, Gelatin, Detergents, Neutralizers etc.

Methods for detection of milk adulteration

Chemical Tests: Chemical tests are one of the most commonly used methods to detect milk adulteration. The most common chemical tests for milk include tests for the presence of water, urea, hydrogen peroxide, formalin, and starch. These tests are based on the changes in color, pH, or reaction of the milk to specific reagents.

Organoleptic Tests: Organoleptic tests involve analyzing the sensory properties of milk, such as color, taste, odor, and texture, to detect any deviations from normal standards. For example, if the milk has an unusual color or taste, it may indicate that it has been adulterated.

Microbiological Tests: Microbiological tests are used to detect the presence of harmful microorganisms in milk. Adulterated milk may contain harmful bacteria, such as E. coli or Salmonella, which can cause foodborne illnesses.

Cryoscopy: Cryoscopy is a method that measures the freezing point of milk. The freezing point of pure milk is higher than that of adulterated milk. Therefore, if the freezing point of milk is lower than the standard, it may indicate that it has been adulterated.

Ultrasonic Tests: Ultrasonic tests involve analyzing the sound waves generated by milk to detect any deviations from normal standards. Adulterated milk may produce different sound waves than pure milk.

Qualitative detection of some commonly added adulterants in milk

Starch

Principle: Iodine solution gives intense blue cooler with starch due to formation of an unstable complex starch compound.

Procedure:

1. Take 3ml of defected milk in attest tube.
2. Boil the milk, cool and add few drops of iodine.
3. Adulterated milk will turn blue.

Cane Sugar

Principle: Resorcinol produces red colour solution with sucrose in acidic media.

Procedure:

1. Take 10ml of milk in a test tube.
2. Add 1ml of concentrated hydrochloric acid and mix well.
3. Add 0.1gm of resorcinol powder and mix thoroughly
4. Place the tube in a boiling water bath for 5 minutes.
5. Observe the colour of the contents.
6. Development of red colour indicates presence of cane sugar in milk

Water

It is calculated using lactometer at 20^0c or 15.5^0c. Lactometer is used to check the water dilution of milk. Corrected lactometer reading of cow milk and buffalo milk is 28-30,30-32 respectively. It should not be less than 28^0c for cow milk and 30^0c for buffalo milk. It indicates added water in milk sample.

Gelatin

Principle: The type and colour of the precipitate formed by picric acid and mercuric nitrate in presence of gelatine differ from the precipitate formed in absence of gelatine in milk. Yellow coloured precipitate after the addition of saturated aqueous solution of picric acid in the filtrate confirms the presence of gelatine.

Procedure:

1. Take 10ml of milk in a beaker.
2. Add 10ml of acid mercuric nitrate solution and 20ml of water.
3. Mix properly and filter.
4. If the filter is opaque, it indicates presence of gelatin.
5. Add picric acid.
6. Formation of yellow precipitates indicates presence of gelatin.

Urea

Principle: Urea forms a yellow complex with para-dimethyl amino benzaldehyde (DMAB) in a low acidic solution at room temperature. Urea is generally added in the preparation of synthetic milk to raise the SNF value.

Procedure:

1. Take 2ml of milk in a test tube.
2. Add 2ml of Urea reagent (para-dimethyl amino benzaldehyde).

3. Shake it well. Dark yellow colour indicates presence of urea.

Detergents

In this method, detergent is first extracted in chloroform and then methylene blue solution is added. In presence of detergent blue colour is developed in chloroform layer of the sample, whereas blue colour is observed in milk layer in control (pure milk). Chloroform is heavier (density 1.49 g/ml) than milk (density ~1.030 g/ml), hence settles at the bottom. This implies that observation of blue colour in the bottom layer indicates presence of detergents.

Procedure:

1. Take 2.5 ml of suspected milk sample in a test tube and add 7.5 ml methanol.
2. Filter the content through Whatman No. 1 filter paper.
3. Take 2 ml filtrate in a test tube.
4. Add 2 ml of methylene blue solution and shake well.
5. Subsequently add 4 ml chloroform and shake well again. Allow the chloroform layer to separate.
6. Compare the colour extracted in the chloroform layer in suspected milk with that for pure milk.

If the methylene blue colour extracted from a suspected sample into the chloroform layer is darker than that extracted from pure milk sample, it indicates the presence of detergent in milk.

Nitrates/Pond water

Principle: Pond water is heavier than the tap water, some persons for adulteration usually prefer it. This method actually detects nitrates present in the pond water. In the pond water nitrates may come from fertilizers used in the fields. Deep blue colour will be formed in the presence of nitrate in the milk sample.

Procedure:

1. Take 10ml of milk in a test tube and add 5ml of concentrated sulphuric acid from the sides of the wall without shaking.
2. If a violet or blue ring appears at the intersection of two layers then it shows presence of formalin.

Neutralizers

Principle: In milk neutralizers like hydrated lime, sodium hydroxide, sodium carbonate, sodium bicarbonate is added which are generally prohibited. Rosalic acid develops a rose red colour with milk containing carbonate and bicarbonate.

Procedure:

1. Take 2ml of milk in a test tube.
2. Rinse the tube with the milk and drain the milk from the tube.
3. Add 2-3 drops if the Diphenylamine sulphuric acid along the side of the tube.
4. Deep blue colour will be formed in the presence of nitrate in the milk sample.

Skimmed milk powder

Principle: As per the law, use of skimmed milk powder (SMP) is not allowed for adjustment of SNF in case of sale of cow/buffalo or mixed milk. The method is based on the fact that the coagulum obtained from reconstituted skim milk powder by addition of acetic acid, gives intense blue colour on boiling with phosphomolybdic acid due to certain reducing groups present in the proteins of milk powder which are able to cause reduction of molybdenum blue resulting in formation of blue colour.

Procedure:

1. Take 50 ml of milk in a 60 ml centrifuge tube.
2. Centrifuge at 5000 rpm for 15 minutes. Decant the supernatant creamy layer carefully.
3. Add 0.5 ml of 4% acetic acid to skim milk portion for coagulation of protein.
4. Centrifuge the tubes at 5000 rpm for 5 min. Decant the supernatant and wash the precipitate with distilled water twice. Discard the washings. Then, add 2 ml of 1% phosphomolybdic acid to the washed precipitates.
5. Mix the contents thoroughly and heat in a water bath at boiling temperature for 15 minutes and then cool.
6. The curd obtained from pure milk shall be greenish in colour whereas the curd of sample containing skimmed milk powder shall be bluish in colour. The intensity of bluish colour depends on the amount of the skim milk powder present in the sample

Harmful effects of milk adulteration

Health problems: Adulterated milk can cause various health problems such as food poisoning, diarrhea, stomach infections, and even cancer.

Reduced nutritional value: Adulteration reduces the nutritional value of milk, making it deficient in essential nutrients such as proteins, vitamins, and minerals.

Allergic reactions: Adulterated milk may contain substances that can cause allergic reactions in some people, leading to symptoms such as hives, swelling, and difficulty breathing.

Hormonal imbalances: Adulterated milk may contain hormones that can cause hormonal imbalances in the body, leading to health problems such as infertility, early onset of puberty, and breast cancer.

Increased risk of infections: Adulterated milk can be a breeding ground for bacteria, viruses, and other pathogens, increasing the risk of infections.

Adverse effects on children: Adulterated milk can have serious consequences on the health of children, especially infants and young children. It can lead to developmental delays, poor growth, and malnutrition. Overall, milk adulteration is a serious problem that can have significant health consequences. It is important to ensure that the milk we consume is pure and free of contaminants.

Conclusions

In conclusion, milk adulteration is a serious problem that affects not only the quality of the milk we consume but also our health. Adulterants such as water, urea, starch, detergent, and formalin are commonly used by unscrupulous milk sellers to increase the quantity and profit of their milk. These adulterants not only reduce the nutritional value of milk but can also cause serious health problems such as food poisoning, kidney damage, and cancer. To combat milk adulteration, it is important for consumers to be aware of the problem and to choose their milk sources carefully. They can opt for branded milk or milk from trusted local dairy farms that follow quality standards. The government can also play a role in preventing milk adulteration by implementing strict regulations and conducting regular inspections of milk products. It is imperative that all stakeholders, including milk producers, processors, sellers, and consumers, work together to combat this problem and ensure that only pure and nutritious milk is consumed. By taking collective action, we can create a safer and healthier food system for ourselves and our future generations.

LABORATORY TECHNIQUES FOR DETECTION OF ADULTERATION OF MEAT AND MEAT PRODUCT

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Protein is an essential component of food. With the increase in human population the requirement of quality protein for humans cannot be fulfilled by vegetarian foods alone. Animal based proteins, meat is a quality protein for human which contains all essential amino acids (lysine, threonine, methionine, phenylalanine, tryptophan, leucine, isoleucine and valine) and good amounts of micronutrients. (Aida et al. 2005). With the increasing gap between demand and supply of meat and meat products fraudulent adulteration of costly meat with cheap meat has become a common practice throughout the world in-spite of having various national and international laws. Food and Safety Standards Authority of India (FSSAI), defines food adulteration as the addition or subtraction of any substance to or from food, so that the natural composition and quality of food substance is affected. Most meat adulteration are intentional and economically motivated, such as the low-cost addition of duck meat to mutton, however some time unintentional adulteration may be possible during processing of meat. In either way consumption of adulterated meat may lead to serious public health risks (Magiat et al 2019) such as consumption of non-declared meat proteins can induce allergic reaction in consumer. In addition to public health risk of meat adulteration it could also violate religious concern of people; for example, pork or pork-associated products are not acceptable in Kosher and Halal food laws (Lim & Ahmed, 2016). Furthermore, meat adulteration has become acause of concern for all meat industry chains at all levels of the production and distribution. Therefore, the ability to detect less desirable or objectionable species in meat products bas become important not only for economic, health, religious, and ethical reasons; but also to ensure fair trade and compliance with legislation (Spink, 2011). Meat adulteration can be detected by various methods such as (i) Anatomical, (ii) Physical, (iii) Chemical, (iv) Biochemical, (v) Immunological and (vi) Molecular methods, however different methods have their own advantages and limitations.

Anatomical methods

The adulteration of one meat species with another meat species can be differentiated based on the anatomical structures of animal species. By this method species of meat can be differentiated on the basis of anatomical peculiarities, but the method cannot be applied for differentiation of meat species if it is minced or cooked:

(i) ***Colour, texture and odour of flesh*** – the meat of different animal specices varies in their color texture and odour. For example, buffalo meat is darker and coarse when compared to cattle meat. Chevon has typical goaty odour especially in males.

(ii) Osseous tissue like bones or cartilage - Bones or cartilages when present along with the meat can be used for species differentiation based on the anatomical peculiarities of the specific bones. Poultry meat can be identified from other species owing to smaller and soft nature of the bones.

(iii) ***Colour, consistency and distribution of fat*** – Pattern of fat deposition and their coloration varies in different food animal species which can be utilized for differentiation meat from different species. For example, coloration of Cattle fat is yellowish in colour while buffalo fat is creamy white. Fat in pigs is present subcutaneously and in abundant but is not intermixed with flesh, whereas cattle/buffalo fat is uniformly distributed with muscle tissues termed as marbling.

Species	Meat			Fat	
	Colour	Consistency	Odour	Colour & Consistency	Distribution
Sheep (mutton)	Pinkish	Firm	Faint	White and firm	Inter muscular fat present
Goat (chevon)	Pale red	Firm	Goaty	White	Inter muscular fat absent
Cattle (beef)	Red	Fairly firm	-	Yellowish White and very firm	Inter muscular fat present
Buffalo (cara beef)	Dark red	Fairly firm	-	Pure white and firm	-
Pig (pork)	Pinkish grey	Soft	Urine like	White and soft greasy	S/C and Intramuscular

Young cattle (veal)	Pale pink	Not firm	-	White and firm	Very less fat
Horse (Chevaline)	Dark red/ bluish	Soft	Sweetish	Yellow and soft greasy	No inter muscular fat
Poultry (Chicken)	White	Firm	-	Yellow and loose	Abdominal cavity and S/C

Physical methods **-** Based on certain physical parameters meat can be differentiated if intact anatomical structures are not present in the meat

(i) ***Refractive index of fat*** - Refractive index of liquefied fat can be measured by refractometer which differ for different animal species. R.I. of horse 53.5, cattle not above 40 and pig not above 51.9.

(ii) ***Estimation of iodine value***- This is the amount of iodine absorbed by unsaturated fatty acids present in the fat and varies in different animals hence can be useful in differentiating adulterated meat. Iodine value of fat in loin is 71 to 86, in cattle 38 to 46, in sheep 35 to 46 in pig it is 50 to 70.

Chemical methods

Chemical composition of different animal species varies in certain chemicals and the presence or absence of those chemicals in meat can be used for detection of meat adulteration. Some of the chemical used for meat differentiation are:

(i) ***Glycogen content*** – This is found highest in horse flesh than other animals.

(ii) ***Linolenic acid*** - Horse fat contains about 1-2% lenolenic acid, in other animals it is not present in proportions higher than 0.1%.

However, these methods cannot be employed in minced meat, cooked or boiled meat, lean meat mixture of meats having closer indices.

Biochemical methods

These methods use protein characteristic of meat from different animal species. Electrophoresis of meat extract or any other biological material results in separation of various proteins. The separation of protein molecules is specific for different animal species that help in identifying origin of biological materials.

(i) ***Iso-electric focusing (IEF)*** – IEF is considered as a powerful analytical tool for the separation of proteins. This is a technique for separating different protein molecules by differences in their isoelectric point (pI). Proteins negatively charged molecules acidic or alkaline in reaction. Besides, they have a specific pH at which their net electric charge becomes zero and they get precipitated. This precipitation point is termed as iso-electric point (PI). The banding pattern of protein and isoelectric (PI) point at which they are precipitated are very specific to a species of animal which helps in identifying the species origin of meat even in mixtures. This technique can be used for identification of species of animal using many biological materials like blood, serum, milk, meat, organs, serum etc. The results specific, reliable and can be reproduced.

(ii) ***SDS-PAGE*** – in this method proteins are separated based on their molecular weights. Hence proteins of different meat species would give unique banding pattern. Isoelectric focussing and SDS-PAGE technique enable differentiation of raw and partially heated (cooked) meats but could not be used in case of fully cooked meat and meat products.

(iii) **Chromatographic techniques:** Chromatographic methods, with focus on fatty acid composition, histidine dipeptides and protein profiles have been developed for species identification in meat samples. It is less effective in the detection of the cooked or mixed fraud meats, due to the increased complexity of its patterns

Immunological/serological methods

In this technique meat from different animal species can be differentiated based on specific antigen- antibody reaction, though they sometimes give cross reaction also. Raw meat extract is commonly used as antigen for antibody formation in a suitable animal like rabbit or sheep. Also, cooked meat is not suitable for identification by this method. Example of immunological test are Agar gel precipitation test, counter current electrophoresis, ELISA.

Molecular methods

Although different methods of detection of meat adulterations are available, however molecular methods targeting DNA/ marker has remained been the most reliable test for detection of adulteration. DNA is the main material for storing, replicating, and transmitting genetic information. DNA exists in all tissues of individual animals and is more conserved than proteins (Kumar et al., 2015 Xiang et al., 2017). DNA fragments have better thermal stability than that of proteins in processed meat, so they could be chosen as

markers for authenticity determination in processed meat, although the variation of DNA content in meat tissues and species could impact the meat speciation. The high sensitivity, specificity and reliability PCR and its derived technologies based on DNA are the most commonly used techniques in the detection of meat and meat product adulteration. The target genes and DNA fragments used as markers for identifying meat product adulteration have mainly been derived from mitochondrial DNA (mtDNA) such as the mitochondrial D-loop region, cytochrome b (CytB) genes, cytochrome c oxidase subunit I, II, and III (COI, COII, and COIII) genes, ATPase subunit 6 and 8 (ATPase6 and ATPase8), 12SrRNA and 16SrRNA as mtDNA are more efficiently arranged and easily accessible, not undergoing recombination (Kumar et al., 2015). Though different types of PCR has been used for identification of meat species DNA barcoding and new generation sequencing are used as a rapid and accurate method for identification of meat of unknown species. This technique can differentiate mixture of several meat species.

RAPID DIAGNOSIS OF FUNGAL TOXINS IN FOODS OF ANIMAL ORIGIN AND MAJOR PUBLIC HEALTH RISKS RELATED TO TOXINS IN CURRENT SCENARIO

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Introduction

Fungi produce variety of substances which differ greatly with respect their structure and function. These include but not limited to certain enzymes (amylase, cellulase, and protease, which help break down complex organic molecules into simpler forms that the fungi can use for food); antibiotics (penicillin, which are used to treat bacterial infections); pigments (red pigment produced by the mold *Neurospora crassa*); polysaccharides (chitin and beta-glucans, which have a wide range of industrial and medical applications); volatile organic compounds (geosmin and 2-methylisoborneol, which can produce characteristic odours in food) and organic acids (citric acid and lactic acid, which are used in food and beverage production). However, certain types of fungi, such as *Aspergillus, Penicillium,* and *Fusarium* produce toxic substances known as mycotoxins. These fungi can grow on a variety of crops, including grains, nuts, and fruits, and can contaminate the food supply. Consumption of such contaminated food leads to disease condition called mycotoxicosis and adversely affect the health of domestic animals and humans.

Mycotoxins in Food

There are many different mycotoxins produced by different types of fungi, but some of the most well-known and studied mycotoxins include:

a) Aflatoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus*, and commonly found in peanuts, corn, and other grains.

b) Ochratoxin A produced by *Aspergillus* and *Penicillium* species, and commonly found in cereals, coffee, and wine.

c) Fumonisins produced by *Fusarium* species, and commonly found in corn and other grains.

d) Trichothecenes produced by *Fusarium* and *Stachybotrys* species, and commonly found in grains, particularly wheat.

e) Zearalenone produced by *Fusarium* species, and commonly found in corn and other grains.

f) Patulin produced by *Penicillium* and *Aspergillus* species, and commonly found in apples and other fruits.

g) Ergot alkaloids produced by *Claviceps purpurea*, and commonly found in grains such as rye and wheat.

h) Citrinin produced by *Penicillium, Aspergillus,* and *Monascus* species, and commonly found in rice, wheat, and barley.

i) Alternaria toxins produced by *Alternaria* species, and commonly found in fruits, vegetables, and grains.

j) Cyclopiazonic acid produced by *Aspergillus* species, and commonly found in nuts, cheese, and other foods.

k) Deoxynivalenol (DON)produced by several species of *Fusarium* fungi, and can contaminate grains such as wheat, corn, and barley.

This is not an exhaustive list, as there are many other mycotoxins that have been identified and studied. Amongst all these, aflatoxins are considered as the most toxic mycotoxin. It is a potent carcinogen and can cause liver damage, immune system suppression, and other serious health effects in both humans and animals. It is estimated that hundreds of millions of people worldwide are at risk of exposure to aflatoxins through contaminated food and feed, particularly in developing countries where food storage and processing practices may be less rigorous. Although mycotoxins are associated with wide variety of foods, the present communication restricts to causes and concerns of mycotoxins in the foods of animal origin.

Sources of mycotoxins in foods of animal origin

a) **Animal feed:** The most common way mycotoxins enter milk and meat products is through contaminated animal feed. If the animal feed contains mycotoxins, the mycotoxins can be absorbed by the animal's digestive system and enter into the bloodstream. The mycotoxins can then be transferred to milk and meat products through the animal's milk or muscle tissue.

b) **Bedding material:** Mycotoxins can also enter milk and meat products if animals are exposed to contaminated bedding material. For example, if the animal's bedding material is made from moldy hay or straw, the mycotoxins can be absorbed through the animal's skin or inhaled, and can then be transferred to milk and meat products.

c) **Water:** In some cases, mycotoxins can enter into milk and meat products if the animal drinks contaminated water.

Dietary exposure of mycotoxins in humans

The dietary exposure of mycotoxins in humans primarily occurs through consumption of plant derived foods such as cereals, nuts, oil seeds, dried fruits and spices as well as their products. Mycotoxin exposure in humans may also be linked to animal-derived foods. When dairy products and meats are improperly stored, they are susceptible to fungal spoilage. Fungal spoilage in animal-derived foods is often visible and therefore ingestion of such mouldy materials is uncommon. However, because mycotoxins are small molecules, they can migrate into deeper layers of food products, and superficial removal of surface areas with visible mould growth during kitchen-food preparation may not eliminate all mycotoxin residues, resulting in involuntary dietary exposure.

The possibility of milk, meat, and eggs being contaminated via the food chain is of particular interest of food toxicologists. Residues of mycotoxins in animal-derived products may contribute to human exposure. The situation becomes unavoidable due to the high chemical and physical stability of most mycotoxins when foods are subjected to common household food processing methods such as boiling and cooking.

Outbreaks associated with mycotoxins.

There have been several outbreaks associated with mycotoxins over the years. Here are some examples:

a) Turkey X disease: In 1960, a large number of turkeys in England died suddenly, and the cause was eventually traced to feed contaminated with aflatoxins.

b) Yellow Rain: In the 1970s, there were reports of a yellow, sticky substance falling from the sky in Southeast Asia, which was associated with a number of health problems, including respiratory problems, blurred vision,

and even death. It was eventually determined that the substance was a mycotoxin called trichothecene.

c) Alimentary toxic aleukia: In the 1940s, a number of outbreaks of a mysterious disease occurred in the Soviet Union, which was eventually linked to the consumption of grains contaminated with the mycotoxin T-2 toxin. The disease was characterized by severe gastrointestinal symptoms, as well as haemorrhaging and leukopenia (a decrease in white blood cells).

d) Ergotism: In the Middle Ages, outbreaks of a disease known as ergotism occurred in Europe, which was linked to the consumption of rye bread contaminated with the mycotoxin ergot alkaloids. The disease was characterized by hallucinations, seizures, and gangrene.

e) Contaminated corn in Kenya: In 2004, an outbreak of aflatoxicosis occurred in Kenya, linked to the consumption of contaminated corn. The outbreak led to several deaths and illnesses, particularly in children.

Adverse health effects of mycotoxins

The harmful effects of mycotoxins on human health can vary depending on the individual's age, health status, and level of exposure. In general, following effects are observed in human due to acute and chronic exposure of mycotoxins.

a) Acute toxicity: Some mycotoxins can cause acute toxicity, which can lead to symptoms such as nausea, vomiting, abdominal pain, diarrhea, and in severe cases, liver and kidney damage, seizures, and even death.

b) Carcinogenicity: Several mycotoxins, including aflatoxins, have been classified as carcinogenic by the International Agency for Research on Cancer (IARC). Long-term exposure to these mycotoxins can increase the risk of liver and other types of cancer.

c) Immunotoxicity: Mycotoxins can also have immune-toxic effects, which can weaken the immune system and make individuals more susceptible to infections and diseases.

d) Teratogenicity: Some mycotoxins, such as ochratoxin A, have been shown to have teratogenic effects, meaning they can cause birth defects and developmental abnormalities in fetuses.

e) Neurotoxicity: Certain mycotoxins, such as fumonisins and trichothecenes, can have neurotoxic effects, leading to symptoms such as headaches, dizziness, and in severe cases, seizures and coma.

Laboratory methods are used for detection of mycotoxins

There are several laboratory methods that can be used to detect mycotoxins in foods. The choice of the method will depend on the specific mycotoxin of interest, the type of sample being tested, and the equipment and expertise available in the laboratory. Here are some of the most common methods:

a) Enzyme-linked immunosorbent assay (ELISA): This method uses antibodies that specifically bind to mycotoxins, allowing for their detection in a sample. ELISA is a relatively simple and quick method that can be used for high-throughput analysis, making it a popular choice in commercial laboratories.

b) Thin-layer chromatography (TLC): TLC is a method for separating and identifying mycotoxins based on their different physical and chemical properties. TLC is a relatively inexpensive method that can be used for rapid screening of mycotoxin contamination.

c) High-performance liquid chromatography (HPLC): This method involves separating mycotoxins from other compounds in a sample using a chromatography column and then detecting the mycotoxins with a detector such as UV, fluorescence or mass spectrometry. HPLC is a widely used method for mycotoxin detection due to its high sensitivity and selectivity.

d) Gas chromatography-mass spectrometry (GC-MS): This method involves separating and detecting mycotoxins by vaporizing them and then separating them using gas chromatography before detection with a mass spectrometer. GC-MS is a highly sensitive and specific method that is often used for the detection of volatile mycotoxins.

e) Liquid chromatography-mass spectrometry (LC-MS): LC-MS combines the separation power of liquid chromatography with the detection power of mass spectrometry. It is a highly sensitive and selective method that can be used for the detection of a wide range of mycotoxins.

Further, there are emerging technologies that are not yet widely available commercially. These includes biosensor-based methods, lab on a chip and microarray-based methods.

RECENT DIAGNOSTIC TOOLS FOR EMERGING AND RE-EMERGING ZOONOTIC DISEASES AND THEIR USE IN MANAGEMENT OF ZOONOTIC DISEASES

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Infections or diseases naturally transmissible between man and lower vertebrates are called as zoonoses. Six of ten cases of human infections are of animal origin. 75% of new or emerging infections of man have origins in animals. Most of the infections of man that have been discovered in the last twenty years are shared with lower animals. A number of diseases previously thought to be limited to man are now classified as zoonoses. These diseases are among the most frequent and dreaded risk, to which mankind is exposed. An early and accurate diagnosis of zoonoses in animals is essential for designing disease management strategies and reducing the burden of such infections in human beings as well as in animals.

Diagnostic tests of infectious disease are based on either detection (e.g., culture, staining, and polymerase chain reaction etc.) of the pathogen or immune response (agglutination or precipitation tests, complement fixation test and enzyme linked immunosorbent assays) elicited by the host against the agent. According to World Health Organization for Animal Health (WOAH/ OIE), a diagnostic test in animal can be employed to:

1. determine the freedom of the population from infection.
2. determine freedom of individual animal from infection.
3. implement the eradication policies.
4. confirm status of a animal suffering from suspected infection or clinical case.
5. conduct surveillance of herd/flock for the prevalence of infection.
6. determine the immune status of the individual animal or the population post vaccination.

A diagnostic test should be accurate, reliable and should provide valid reliable results. Accuracy is measure of overall performance of a test and gives extent of exactness of the results. Reliability of the test depends upon repeatability (agreement between the results recorded on same sample/individual/animal by same observer) and reproducibility (agreement between the results recorded on same sample/individual/animal by different observers). Validly of the test depends upon its sensitivity (ability to detect diseased animals/patients as positive), specificity (ability to detect non-diseased/healthy animals as negative) and predictive values of the test. Predictive values are the probability of reflecting the true disease status. Positive predictive value is probability of having the disease when test is positive whereas negative predictive value is probability of absence of disease when test is negative.

An emerging zoonosis is defined as a new infection/disease transmissible between animals and man resulting from the evolution or change of an existing pathogen resulting in a change of host range, vector, pathogenicity or strain; or the occurrence of a previously unrecognized infection or disease. The examples of emerging zoonoses are Hendra virus infection, Nipah virus infection, Australian bat laysa virus, Middle East respiratory syndrome (MERS), avian influenza, bovine spongiform encephalopathy (BSE), feline cowpox, rotavirus infection, norovirus infection, Ebola, Hanta virus infection, West Nile fever, canine leptospirosis and methicillin resistant *Staphylococcus aureus* infection.

A re-emerging zoonosis is an already known zoonosis that either shifts its geographical setting or expands its host range, or significantly increases its prevalence. The examples are rabies, brucellosis, Japanese encephalitis and tuberculosis.

Avian Influenza

Influenza viruses belong to the family Orthomyxoviridae which includes enveloped negative-strand RNA viruses with segmented genomes. There are seven influenza genera but only influenza A viruses are known to infect birds. Wild waterfowl are believed to be the natural reservoir of influenza A viruses. influenza A viruses are maintained predominantly by asymptomatic infections among aquatic birds are termed as low pathogenic avian influenza (LPAI). The infection caused by LPAI virus strains are either without clinical symptoms or show mild clinical disease. However, mutation in haemagglutinin H5 or H7 results in evolution of highly pathogenic avian influenza (HPAI) strains that are associated with high mortality in domestic poultry.

HPAI in susceptible birds may lead to clinical outcomes ranging from sudden death to respiratory signs, such as ocular and nasal discharges, coughing, difficulty in breathing, blood-tinged discharge from nostrils, swelling of the sinuses, cyanosis of the skin, wattles and comb, in coordination, nervous signs, marked reduction in feed and water intake, and diarrhoea. Post-mortem lesions may include swelling of the face and clear straw-coloured fluid in the subcutaneous tissues.

Avian influenza is primarily considered as a disease of birds but infections can occur in human beings. Two commonly reported avian influenza strains from human clinical cases are H5N1 HPAI viruses and H7N9 LPAI viruses. The symptoms in human beings include fever, malaise, cough and muscle aches. Other early symptoms may include abdominal pain, chest pain and diarrhoea. The infection may progress to severe respiratory illness with symptoms including difficulty in breathing or shortness of breath, pneumonia, acute respiratory distress syndrome and neurological changes involving altered mental status or seizures.

Diagnostic tests: Agent detection by real time RT-PCR and isolation of virus in embryonated eggs (9-11 days). Haemagglutination inhibition (HI) for the detection of immune responses.

Other tests: Antigen detection (polyclonal or monospecific sera), agar gel immunodiffusion (AGID)

Note: A combination agent identification methods should be applied on same clinical specimen.

Rabies

Rabies is caused by neurotropic viruses of the genus Lyssavirus in the family Rhabdoviridae and is transmissible to all the mammals. Rabies virus (RABV) represents the taxonomic prototype species of Rabies lyssavirus in the Lyssavirus genus, which includes other genetic and antigenically-related lyssavirus species. RABV is found worldwide, and is responsible for the majority of the reported animal and human rabies cases. The rabies virus causes acute encephalitis in all warm-blooded hosts with fatal clinical outcomes. As no clinical sign or gross post-mortem lesion can be considered pathognomonic and constant clinical finding are absent in rabies, the diagnosis of rabies has to rely on laboratory testing.

Rabies is a major zoonosis for which diagnostic techniques have been standardized internationally. Laboratory techniques are preferably undertaken

on central nervous system (CNS) tissue removed from the cranium (for example brain stem, Ammon's horn, thalamus, cerebral cortex, cerebellum and medulla oblongata). A composite of CNS samples should be tested and the brain stem is the most important component of the sample. Laboratories should follow appropriate bio-safety and containment procedures as determined by bio-risk analysis.

Diagnostic test: Direct fluorescent antibody (DFA) test is the most widely used test for rabies diagnosis, recommended by both World Health Organization (WHO) and OIE. This test is performed directly on a brain impression smear. Impression smears should be prepared from a composite sample of brain tissue. Slides with impression smears of brain tissue are labelled with fluorescein isothiocyanate (FITC) anti-rabies conjugate.

The slides are examined by fluorescence microscope capable of excitation at 488 nm, using an excitation filter with narrow passband windows in the blue spectrum (475–490 nm). Each impression is observed for rabies-specific fluorescence (indicating the presence of viral antigen) at a magnification of 200× or greater.

Specific fluorescence is denoted by bright 'apple' green fluorescence generally in the peri-nuclear area of cells, or longer 'string-like' neurons and is read as positive. Dull green or red/green auto-fluorescent granules should not be counted as positive antigen.

Other tests: Direct rapid immunohistochemistry test (dRIT), cell culture (virus isolation), mouse inoculation test (MIT) and real time reverse transcription (RT-PCR). Virus neutralization test (VNT) and enzyme-linked immunosorbent (ELISA) assays are suitable tests to monitor immune responses in vaccinated animals.

Note: Histological techniques such as Seller staining (Negri bodies) are no longer recommended for diagnosis.

Japanese Encephalitis

Japanese encephalitis virus (JEV) is a Flavivirus and causes encephalitis, principally in horses and humans. Approximately 60% of the world's human population lives in regions endemic for Japanese encephalitis (JE). The disease has been observed in large parts of Asia including India and in the western Pacific region.

JEV is maintained in nature between mosquitoes, pigs and water birds ardied birds (e.g., cattle egrets, pond herons etc.). The major vectors of JEV are mosquitoes of *Culex* species especially *Cx. tritaeniorhynchus* and *Cx. vishuni* however other species may be locally important**.** Pigs act as important amplifiers and birds can also be involved in its amplification and spread in the environment.

In pigs, abortions and stillbirths can occur when pregnant sows are infected with JEV for the first time. Infected pregnant sows usually show no clinical signs. In horses, the infection is usually in apparent. Affected horses show clinical signs that include pyrexia, depression, muscle tremors, and ataxia. In human beings, infections are usually asymptomatic. In severe forms of the disease; CNS is involved with a range of clinical manifestation ranging from acute encephalitis to acute flaccid paralysis and mental retardation especially in children. The case fatality rate can be as high as 25–30% and CNS damage is recorded in 30–50% survivors.

Diagnostic test: HI widely used test for the diagnosis of Japanese encephalitis, but cross-reactivity can occur with other flaviviruses. For this test, the sera must first be treated with acetone or kaolin, and then adsorbed with homotypic red blood cells (RBCs) to remove any nonspecific haemagglutinins in the test sera. The RBCs of geese or of 1-day-old chickens are used.

Other tests: Virus isolation and real time RT-PCR are the recommended tests for the detection of the JEV. The virus can be isolated in primary cell cultures made from chicken embryos, porcine or hamster kidney cells and established cell lines such as African green monkey kidney (Vero), baby hamster kidney (BHK-21), or mosquito (C6/36) cells. Detection of immune responses can be done by VNT, complement fixation test (CFT) and ELISA.

Note: JEV has serological cross reactivity with other flaviviruses. The plaque reduction VN test can be used to differentiate JEV infection from other flavivirus infections.

Nipah Virus

Nipah virus (NiV) belongs to genus Henipavirus and family Paramyxoviridae and. NiV appeared in the human population between September 1998 and April 1999 in Malaysia as the cause of fatal acute encephalitis, after spreading primarily as a respiratory disease of unknown aetiology in the pig population. The name Nipah virus is derived from the village of Sungai Nipah in the state of Negeri Sembilan, Malaysia. Apart from

Malaysia, disease is reported from Bangladesh, India (West Bengal & Kerala), Philippines and Singapore. Fruit bats (flying-foxes) in the genus *Pteropus* are the reservoir hosts. Infection of pigs is highly contagious and characterized by fever with respiratory and sometimes neurological involvement, but many infections are subclinical. Some infected pigs display an unusual loud barking cough and abortions can occur.

Human beings become infected after coming in contact with infected swine and Pteropid bats. Human-to-human transmission has been reported in Bangladesh and India. Disease in human beings varies from from asymptomatic infection to acute respiratory infection (mild, severe), and fatal encephalitis. Patients also experience atypical pneumonia and acute respiratory distress. Encephalitis and seizures occur in severe cases, progress to coma. The case fatality rate is estimated at 40% to > 90%.

Diagnostic test: NiV is biosafety level 4 pathogen and all the tests should be performed under appropriate conditions of biological risk management. The VNT is accepted as the reference standard and confirmatory test for NiV diagnosis. VNT results are considered positive if virus neutralization is observed at any of the dilutions used in the test

Other tests: Detection of the agent by real tine RT-PCR and propagation of virus in cultured cells. Immunohistochemistry (IHC) can be applied on formalin-fixed tissues to detect NiV antigen. ELISA and bead bases assay can also be used to detect immune responses.

Note:

1. NiV is present in respiratory epithelium of pigs.
2. Virus isolation from field samples should be attempted, but only in situations where operator safety can be assured.
3. A combination of agent identification methods should be applied on the same clinical specimen.
4. Confirmation of positive ELISA & bead-based assay results should be done by a validated VNT assay

West Nile virus

West Nile virus (WNV) is a single-stranded RNA neurotropic virus belonging to the family Flaviviridae. The virus was first reported in the West Nile province of Uganda in 1937. WNV is a neurotropic pathogen maintained

through a mosquito–bird–mosquito transmission cycle. It primarily involves mosquitoes belonging to *Culex* species as vectors and birds as natural reservoirs (amplifying) hosts. In India, WNV has been isolated from *Cx. vishnui*, *Cx. quinquefasciatus* and *Cx. tritaeniorhynchus*. Birds of the order *Passeriformes*, *Charadriiformes* and *Falconiformes* are the major amplifying hosts. Human beings and horses are the dead-end hosts and do not develop viremia.

In horses, clinical signs of WNV infection arise from viral-induced encephalitis or encephalomyelitis. Affected horses frequently demonstrate mild to severe ataxia. Signs can range from slight in coordination to recumbency. Some horses exhibit weakness, muscle fasciculation and cranial nerve deficits.

In human beings, infection with WNV is asymptomatic in around 75-80% of infected people. About 20% of patients are presented with flue like illness. Symptoms include fever, headache, tiredness, and body aches, nausea, vomiting, occasionally with a skin rash (on the trunk of the body) and swollen lymph glands. Less than <1% patients develop neuroinvasive disease. Acute cases with neurological complications are classified as encephalitis with symptoms of include headache, high fever, neck stiffness, stupor, disorientation, coma, tremors, convulsions, muscle weakness, respiratory failure and paralysis.

Diagnostic test: Real time RT-PCR for the demonstration of viral nucleic acid or virus antigens in the tissues.

Other tests: Detection of the agent by IHC and isolation of WNV by cell culture (mammalian). Antibody can be identified in equine serum by IgM capture ELISA, HI, IgG ELISA, plaque reduction neutralization (PRN) or VN tests.

Note: Bird tissues generally contain higher concentrations of virus than equine tissues. Brain and spinal cord are the preferred tissues for virus isolation from horses. In birds, kidney, heart, brain, liver or intestine can yield virus isolates. Virus can also be isolated from mosquitoes.

Bovine tuberculosis

Bovine tuberculosis is a chronic bacterial disease caused by *Mycobacterium bovis.* In a large number of countries bovine tuberculosis is a major infectious disease among cattle, other domesticated animals, and wildlife populations. Aerosol exposure to *M. bovis* is considered to be the most frequent route of infection among cattle, but infection by ingestion of contaminated material also occurs. Drug resistance [multi drug resistance (MDR) and

extensive drug resistance (XDR)] along with HIV has led to the re-emergence of tuberculosis in man.

Infection is often subclinical; when present, clinical signs are not specifically distinctive and can include weakness, anorexia, emaciation, dyspnoea, enlargement of lymph nodes, and cough, particularly with advanced tuberculosis. On necropsy, characteristic nonvascular nodular granulomas (tubercles) are found in the lungs and the retropharyngeal, bronchial and mediastinal lymph nodes. Tubercles are also found in the mesenteric lymph nodes, liver, spleen, on serous membranes, and in other organs.

M. bovis is transmitted to human beings through contaminated milk and milk products. The incidence of pulmonary tuberculosis caused by *M. bovis* is higher in farm and slaughterhouse workers. It can cause both extra pulmonary and pulmonary forms of the disease. Lungs and pleura are the two major sites of infection. Breathing difficulties, low grade fluctuating fever, night sweats bloody sputum, cough, extreme fatigue, chest pain and progressive emaciation are the characteristic features of the disease. Renal tuberculosis results in necrosis of renal medulla, pelvis, ureters and bladder. Tuberculosis of bones and joints causes paralysis and sensory loss. Tuberculous meningitis can result into mental deterioration, permanent retardation, blindness and deafness.

Diagnostic test: Delayed hypersensitivity test (tuberculin skin test) is the standard method for detection of tuberculosis in live cattle, small ruminants, deer, pigs and camelids. The single test involves measuring the skin response after intradermal injection of tuberculin (purified protein derivative – PPD).

The comparative tuberculin skin test with bovine (PPD-B) and avian (PPD-A) tuberculin is used to differentiate between animals infected with the *M. tuberculosis* complex and those sensitized to tuberculin due to exposure to other mycobacteria or related genera. The recommended dose of bovine PPD-B in cattle is at least 2000 International Units (IU) and in the comparative tuberculin test, the doses should be no lower than 2000 IU each. The test involves injecting bovine tuberculin intradermally into the measured area and measuring any subsequent swelling at the site of injection 72 hours later. The results were interpreted as negative (<2 mm), inconclusive (2-4 mm) and positive (>4 mm).

Other tests: Acid fast staining along with isolation *M. bovis* can be done using egg based bacteriological media (Lowenstein–Jensen, Coletsos base or Stonebrinks). Since isolations may take 10-12 weeks therefore, other methods

of diagnosis are preferred. Real time PCR (direct from specimen), gamma interferon assay, lymphocyte proliferation assay, ELISA and histopathology.

Brucellosis

Brucella genus comprises of 12 different species. Brucellosis is the generic name used for the animal and human infections caused by several species of the genus *Brucella*, mainly *B. abortus*, *B. melitensis* and *B. suis*. Brucellosis in cattle is usually caused by *B. abortus*, less frequently by *B. melitensis*, and occasionally by *B. suis*. In sheep and goats, *B. melitensis* is the main causative agent of infection. In swine, disease is primarily cause by *B. suis*. Clinically, infection with *Brucella* in animals is characterized by one or more of the following signs: abortion, retained placenta, orchitis, epididymitis and, rarely, arthritis, with excretion of the organisms in uterine discharges and in milk.

B. melitensis, *B. suis* and *B. abortus* are highly pathogenic for human beings. In addition to these three species, human infections can occur due to *B. neotome* (desert rat), *B. canis* (dogs), *B. ceti* (dolphins), *B. pinnipedialis* (seals) and *B. papionis* (human beings). The epidemiology of the disease has changed in recent years due to identification of new species and new animal reservoirs.

The infection in man occurs through direct or indirect contact with infected animals or their tissues (aborted foetus, placenta and associated tissues), cultures or more importantly due to consumption of contaminated animal milk or milk products not adequately heat-treated/ pasteurized. Clinical symptoms in human beings, include weakness, biphasic fever, depression, generalized bodyaches, chill, profuse sweating, headache, anorexia, arthritis, lymphadenopathy and hepatosplenomegaly, epidydimatis and orchitis. Other complications include spondylitis, sacroiliitis, osteomyelitis, meningoencephalitis, endo or pericarditis, bronchopneumonia and pleural effusions.

Diagnostic tests: Confirmatory diagnosis of brucellosis can be made by isolation and identification of *Brucella* organism. PCR methods are additional means for detection of the presence of Brucella DNA in a sample. Indirect ELISA and CFT are the recommended test for the herds/flocks and individual small ruminants, camelids and bovines (cattle and buffaloes).

Other tests: The buffered *Brucella* antigen tests (rose bengal test and buffered plate agglutination test), competitive ELISA or the fluorescence polarisation assay, are suitable tests for screening. The brucellin skin test (delayed hypersensitivity test) can be used in unvaccinated ruminants, camels and swine

as either a screening or a confirmatory herd test when positive serological reactors occur in the absence of obvious risk factors. 2-Mercaptoethnol tube agglutination (measures IgM titers) and standard tube agglutination (measures IgG titres) tests are used to measure titres.

Notes:

1. Uterine discharges, aborted foetuses especially stomach contents, udder secretions or selected tissues, such as lymph nodes and male and female reproductive organs are used for Brucella isolations.
2. Serological tests indicate exposure to Brucella species, but cannot identify the aetiological agent to the species level.
3. No single serological test is appropriate in each animal species and all epidemiological situations.

Leptospirosis

Leptospirosis is a transmissible disease of animals and humans caused by infection with any of the pathogenic members of the genus *Leptospira*. Acute leptospirosis should be suspected in the following cases: sudden onset of agalactia (in adult milking cattle and sheep); icterus and haemoglobinuria, especially in young animals; meningitis; and acute renal failure or jaundice in dogs. Chronic leptospirosis should be considered in the following cases: abortion, stillbirth, birth of weak offspring (may be premature); infertility; chronic renal failure or chronic ac tive hepatitis in dogs; and cases of periodic ophthalmia in horses. Leptospira spp. are classed in Risk Group 2 for human infection.

In human beings' disease manifests by with fever, hemorrhagic complications, and renal failure. Symptoms include aseptic meningitis, iridocyclitis, jaundice, renal failure (azotemia, oligo- or anuria), anemia, thrombocytopenia, hemorrhages (petechiae to severe bleeding in skin, lung, kidneys and intestine), conjunctival suffusion (red eye) hemorrhagic pneumonia with acute respiratory distress syndrome, interstitial myocarditis with arrhythmia, and circulatory collapse.

Diagnostic tests: Isolation of leptospires from clinical material and identification of isolates is difficult and is generally done in specialized reference laboratories. The microscopic agglutination test (MAT) and ELISA are recommended diagnostic tests.

MAT using live antigens is the most widely used serological test. It is the method of choice for the detection of the antibodies. The MAT is the reference test for the evaluation of other serological tests. It involves use of live or fomalized *Leptospira* culture and filtered sterilized complement inactivated serum. The dilutions are prepared in microtitre plate and examined by dark-field microscopy. The endpoint is defined as that dilution of serum that shows 50% agglutination, leaving 50% free cells compared with a control culture diluted 1/2 in phosphate buffered saline.

A titre of 1:100 is taken as a positive indicting past or present infection. Higher titres may be recorded for vaccinated animals.

Other test: PCR

Note: MAT will yield positive results at the earliest on the 5th to 9th day of illness. It should be followed up by repeat testing within 8 to10 days, with a fourfold increase in titer being diagnostic.

Q fever

Query (Q) fever (or Coxiellosis) caused by *Coxiella burnetii*, is a zoonosis that occurs in most countries. Humans generally acquire infection through air-borne transmission from animal reservoirs, especially from domestic ruminants, but other domestic and wildlife animals (pets, rabbits, birds, etc.) can be involved. *C. burnetii* is a bio-safety level 3 pathogen. All manipulations with potentially infected or contaminated material must be performed at an appropriate biosafety and recommended containment level to prevent aerosol generation.

In domestic ruminants, Q fever is mostly associated with sporadic abortions or outbreaks of abortions and dead or weak offspring, followed by recovery without complications. *C. burnetii* infection persists for several years, and is probably lifelong. Sheep, goats and cows are mainly subclinical carriers, but can shed bacteria in various secretions and excreta.

In man, Q fever caused sudden onset of illness following incubation period of 14-28 days. It is marked by sharp rise in temperature (39-42^{0}C), a severe frontal headache (characteristic symptom) and photophobia. Acute Q fever may manifest itself as meningoencephalitis, myocarditis, pericarditis, pneumonia, thromboses in several organs, granulomas and necrotic foci in the bone marrow, orchitis, chorioamnionitis. Recovery may take several months.

C. burnetii infections in pregnancy may lead to prematurity, abortion, or stillbirth. In chronic cases, hepatitis, endocarditis or interstitial lung fibrosis may also occur. Infection in human beings may persist asymptomatically throughout life.

Diagnosis test: PCR is recommended test for the pathogen identification. ELISA has a high sensitivity and a good specificity according to comparative evaluations between methods. ELISA is convenient for large-scale screening and is preferred to other tests detecting immune responses. *C. burnetii* ELISA antigen is prepared by growth of standard strains in either embryonated hens' eggs or in cell culture.

Other diagnostic tests: Culture [embryonated chicken eggs, Human embryonic (HEL) fibroblasts], laboratory animal (mouse or guines pigs) inoculation, staining (Stamp, Gimenez, Macchiavello, Giemsa and modified Koster), CFT, indirect immunofluorescence assay (IFA) and genotyping [restriction endonuclease of genomic DNA, pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP) and sequence analysis].

Echinococosis/Hydatidosis

Human cystic echinococcosis, caused by *Echinococcus granulosus*, and alveolar echinococcosis, caused by *E. multilocularis* are important public health threats in many parts of the world. Disease is asymptomatic both in animals and man. Symptoms develop when cyst(s) becomes large or ruptures. Hydatid cyst fluid contains protein antigens and results in anaphylactic shock and sudden death.

Diagnostic tests: Diagnosis of echinococcosis in dogs or other susceptible carnivores relies on the detection of adult cestodes of the *Echinococcus* genus or their eggs in the faeces or small intestine. In intermediate hosts, diagnosis is dependent on post-mortem detection of the larval cyst form that can infect almost any organ, particularly the liver and lungs.

Under normal conditions of faecal examination, the eggs of *Echinococcus* cannot be differentiated from those of *Taenia* spp. The eggs of *E. granulosus* and E. *multilocularis* can now be identified and differentiated from other *Taenia* spp by PCR.

Coproantigen and coproDNA assays have proven useful for safe, fast and accurate diagnosis of echinococosis. Adult *Echinococcus* worms inhabiting the intestine will release either surface or secretory molecules (corpoantigens) and

DNA (coproDNA, within eggs). These molecules can be detected by assaying faecal samples. Coproantigen ELISA or coproELISA detect somatic or excretory/secretory (ES) antigens. These tests are generally not available on a commercial basis and are developed within individual research laboratories.

Other tests: coproDNA based RT PCR assay, PCR, necropsy in intermediate hosts.

Notes: In coproELISA, the positive to negative threshold is taken as 3 standard deviations above the mean absorbance value of control negatives, or against reference standard control positive using absorbance units equivalence.

Cysticercosis

Cysticercosis is caused by the larval stages (metacestodes) of cestodes of the family Taeniidae (tapeworms), the adult stages of which occur in the intestine of humans, dogs, cats or wild Canidae and Mustellidae (weasels, badgers and otters etc.). Bovine cysticercosis (primarily in muscle) and porcine cysticercosis [primarily in muscle and the central nervous system (CNS)] are caused by the metacestodes (cysticerci) of the human cestodes *T. saginata* (*Cysticercus bovis*) and *T. solium* (*Cysticercus cellulosae*), respectively. *Taenia asiatica* (Asian tapeworm) is a less widespread cause of cysticercosis in pigs, with the cysts locating in the liver and viscera and the adult tapeworm occurring in humans.

Cysticercosis in bovines and pigs is usually not associated with apparent clinical signs. In cattle, developing cysts may cause myocarditis and heart failure. Pigs with neurocysticercosis can develop clinical signs such as excessive salivation, blinking and tearing, and may also suffer from tonic muscle contractions and seizures. Cysticercosis causes economic loss through condemnation of infected meat and offal.

In human beings, adult forms cause intestinal disease ‘taeniasis’ and that caused by metacestode (cysticerci) ‘cysticercosis’. Taeniasis is usually asymptomatic. Some infected individuals may exhibit clinical signs of abdominal pain, intermittent diarrhoea or constipation, loss of appetite and weight, and generalised allergic manifestations, including urticaria, anal pruritus and eosinophilia.

Cysticercosis has variable incubation period and may remain asymptomatic for many years. The clinical manifestation depends on the affected organs and the number of cysts in the body. The cyst in muscles does not cause any apparent clinical manifestation but over the period of time turn

into tender and painful lumps. Cysts in the eyes cause blurry or disturbed vision, deviations in gaze and blockage of visual fields. Neurocysticercosis (NCC, cysts in the brain and/or spinal cord) varies from asymptomatic infections to symptoms including severe headache, blindness, convulsions, epileptic seizures, difficulty with balance, cerebral hypertension and excess fluid deposition around the brain (hydrocephalus). It is estimated that 30% of all epilepsy cases in endemic regions where people and pigs co-exist is due to neurocysticercosis.

Diagnostic tests: Demonstration of eggs or gravid segments, coproantigen ELISA, enzyme linked immunoelectrotransfer blot, tissue biopsy, imaging techniques.

Notes: In human beings for NCC imaging techniques such as by X-ray, computed axial tomography (CT) scan, magnetic resonance imaging (MRI), and demonstration of specific antibodies is useful.

Toxoplasmosis

Toxoplasma gondii is an obligate intracellular parasite that has a sexual cycle in Felidae and a two-stage asexual cycle in all warm-blooded animals. It infects all warm-blooded animals including birds. It causes acute life-threatening disease particularly sheep and goats and pigs. Clinical signs may include lymphadenopathy, hepatomegaly, interstitial pneumonia, neural signs, infertility and stillbirths or abortions few days earlier than the predicted end of pregnancy.

The most likely sources of human infection are ingestion of raw or lightly cooked meat containing live *T. gondii* tissue cysts or ingestion of raw or lightly cooked vegetables contaminated with oocysts or exposure to oocysts derived from cat faeces. Toxoplasmosis is now also recognized as a water-borne zoonosis and human infections are higher in regions with ineffective or non-existent water treatment.

In human beings, *T. gondii* infection rates may be as high as 30% depending on the age and environment but clinical disease is relatively uncommon. Pregnant women, immunosuppressed/immunocompromised patients, young children and elderly are more susceptible and can develop acute lethal infection. On occasions, people with no apparent immune deficiency may develop an illness characterized by general malaise, fever and lymphadenopathy. In infected pregnant women abortions occurs and children

born alive suffer from fever, adenopathy, splenomegaly, hepatomegaly, hydrocephalus, microcephaly and psychomotor disturbances.

Diagnostic tests: The dye test (Sabin Feldman dye test) is the established serological method. It is considered as gold standard in human beings but has proven unreliable in some species. The test uses live *Toxoplasma* and carries a potential risk of human infection as well as being expensive to conduct.

The IFA test is safer and gives titres comparable with the dye test. It can be used to differentiate IgM and IgG antibodies. It is rapid and does not require complex laboratory facilities.

a) Positive test serum: The parasites will fluoresce red and at least 80% of them within a given well will be surrounded by an unbroken band of green fluorescence.

b) Negative test serum: The whole parasites will appear red due to the auto-fluorescence of the Evan's blue dye. They may also present with a green fluorescent cap at the parasite pole (nonspecific polar fluorescence).

In an adult sheep/goat a positive titre could be defined as ≥1/64 and a negative titre as ≤1/32. For lamb/kid and fetal sera, respective titres could be defined as ≥1/32 and ≤1/16

Other tests: Demonstration of the parasite in tissues, direct agglutination test, latex agglutination test, ELISA, PCR

Cryptosporidiosis

Cryptosporidiosis is the pathological condition caused by infection with the protozoan *Cryptosporidium*. There are at least 46 *Cryptosporidium* species, some of which cause disease in human beings, livestock, poultry and game birds, and companion animals. *Cryptosporidium parvum* infects mainly the gastrointestinal tract and is an important cause of diarrhoea in young and unweaned livestock. Transmission occurs either directly through faecal oral route or indirectly through contaminated food and water. Weaned and adult animals do not normally exhibit signs of disease, but can excrete oocysts that may contaminate the environment facilitating onward transmission. The clinical signs in infected calves may include yellow watery faeces, reluctance to feed and dehydration, and in severe cases the animal may die of infection.

C. parvum is one of the major causes of zoonotic human cryptosporidiosis. Clinical course of cryptosporidiosis in humans depends on the immune state. Most infection in healthy individuals remains asymptomatic.

In immunocompetent individuals, the disease is self-limiting. The gastrointestinal symptoms among include diarrhoea (usually voluminous and watery), nausea, vomiting, fever and abdominal discomfort that usually resolve within 2 weeks. In immunocompromised patients, diarrhea is profuse and watery, with as many as 71 to 171 stools per day. Abdominal pain, nausea, vomiting and low-grade (<39°C) fever are less frequent than diarrhea, and symptoms may last for more than 30 days in the immunocompromised.

Diagnostic test: Convectional microscopy and staining, antigen detection ELISA, PCR and FAT

Other tests: Immunochromatography and antibody detecting ELISA

Cryptococcosis

Cryptococcosis is caused by two worldwide distributed species of *Cryptococcus* are *C. neoformans* and *C. gattii*. *C. neoformans* is mainly associated with avian environment especially pigeons and *C. gattii* was isolated from *Eucalyptus* spp. or *Syzygium cumini* trees. In some cases, *C. neoformans* and *C. gattii* have been reported from the same sources such as bird feces or tree. *C. neoformans* is also isolated from bark, tree-trunk hollows and decayed wood. Thus, domestic or wild animals and birds are carrier for *C. neoformans*. The cryptococcal infection is usually acquired by inhalation of the yeast from soil contaminated with bird droppings or eucalyptus trees and decaying wood.

A wide variety of domestic and wild (terrestrial and aquatic mammals, reptiles, amphibians and fish) are affected. Cats and dogs produce granulomatous lesion in oral and pharyngeal mucosa. Cats suffer from cryptoccal conjunctivitis, nasal swelling and fatal meningitis. Cattle and sheep suffer from mastitis while in bird's granulomatous lesions occur in liver lung and intestine.

In human beings, cryptococcal infections are major concern in immunocompromised patients with more than 80% mortality in patients with non-protective immune responses. The disease manifests itself in five forms in human beings; pulmonary, nervous, visceral, cutaneous and osseous. Cryptococcal meningitis (CM) is the most common CNS infection in immunocompromised patients. The neurological complication due to CM increases the intracranial pressure, recoverable neurocognitive impairments, non-recoverable deafness, and blindness. Approximately 5% of CM patients are reported to suffer from cutaneous cryptococcosis. Cryptococcus is the most

common cause of fungal meningitis and leading cause of death in AIDS (acquired immunodeficiency syndrome) patients higher than tuberculosis.

Diagnostic tests: Staining (Indian ink staining, Alcacian blue, Gomori methenamine silver, colloidal iron, periodic acid- Schiff, Masson- fontana silver stain, ram's stain, new methylene blue and Wright's stain), culture [Sabouraud dextrose agar, brain heart infusion agar and Niger (birdseed) agar]. Brown color colonies are observed on Niger (birdseed) agar.

PCR, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS), gene chips, sequencing techniques and radiography are widely used .

Notes: Samples of body fluid aspirates -blood, cerebrospinal fluid (CSF), nasal secretions, bronchial washings, skin exudates and urine might can be used for the diagnosis of Cryptococcus spp.

Scrub typhus

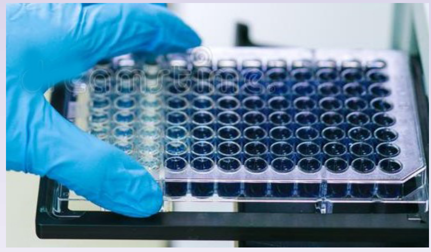
Scrub typhus is a serious under diagnosed/underreported public health problem of Asia Pacific region affecting one million people each year. Scrub typhus is an acute febrile, arthropod borne infectious disease caused by *Orientia tsutsugamushi*. The trombiculid mites act as both vectors and reservoirs. Disease is transmitted to mammalian hosts including humans by the bite of larval stages of mites, chiggers. The vector involved in the transmission of the disease thrives in 'scrub' (terrain between woods and clearings) type of vegetation. Among vertebrates, only monkeys, gerbils, hamsters and human beings suffer clinically with scrub typhus. Rodents are often implicated in scrub typhus transmission.

Human beings initially exhibit non-specific flu-like symptoms. An eschar at the site of chigger bite is a classic clinical feature of scrub typhus but not present always. Complications due to scrub typhus include jaundice, acute renal failure, pneumonitis, acute respiratory distress syndrome, myocarditis, septic shock, meningoencephalitis, pericarditis, gastrointestinal bleeding and multiple organ failure. CNS involvement results delirium, seizures, psychiatric disturbances and visual hallucinations. Involvement cranial nerve results in acute hearing loss or hearing impairment, diplopia, esotropia and profound weakness of ocular muscles. Case fatality rate is between 7% and 30%, but can be more (up to 70%) if cases are presented late and diagnosis and treatment are delayed.

Diagnostic tests: IgM ELISA, IgG ELISA, microimmuno fluorescence/ immunofluorescence assay, PCR, real time PCR, Weil Felix's reaction

Notes: Weil Felix's reaction is not a preferred test due to low sensitivity and specificity.

Management of the infectious/zoonotic diseases to a large extent is dependent upon the choice of diagnostic tests. It is important that the tests employed for the diagnosis of zoonoses should be accurate and reliable. Uniformity in the diagnostic test procedures, allows comparison between different prevalence/incidence studies across different geographical regions and populations. It helps better implementation of the surveillance and disease management programmes.



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