Molecular Diversity of Bovine Tuberculosis

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ABSTRACT

Bovine tuberculosis (BTB), the causative agent of which is Mycobacterium bovis, a member of Mycobacterium tuberculosis complex (MTC), poses a major public health hazard globally, especially in developing countries. Mycobacterium bovis having 4200 genes, pathogenicity is associated with proline-glutamate or proline-proline-glutamate genes. The zoonotic nature of infection underscores its significance on both animal and human health. Though several species are affected, cattle serve as the main reservoirs. Multiple strains of the Mycobacterium tuberculosis complex have been revealed by molecular investigations, assisting in comprehending the virulence and evolution of the organism. BTB continues to affect human well-being, trade, and animal health despite efforts to control it in developed countries. To mitigate the effects of this zoonotic disease, effective control measures—such as testing and slaughter—are essential. RFLP is mainly used to study the prevalence of BTB. For this an insertion sequence IS110 is widely used as genetic marker. Multi drug resistant tuberculosis is detected by GeneXpert technique, which identify the rifampicin resistance sequence in MTC. Timely surveillance of a disease is of utmost importance, mostly gamma-interferon and intradermal comparative tuberculin skin test are the valuable tools and used for prevention and control programs. Various national and international measures should be taken to develop good surveillance strategies and strengthen the current ones. Bovine Tuberculosis (bTB), being a zoonotic disease, still raises severe health and economic concerns in developing countries. Therefore, timely surveillance of disease is vital for appropriate containment measures. Keeping in view the objectives, various approaches may be adopted for designing a suitable surveillance plan.

Keywords: Zoonotic Bovine tuberculosis, Mycobacterium bovis, Pathogenicity, Mycobacterium tuberculosis complex, Clonal complexes, Surveillance

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1. INTRODUCTION

Bovine tuberculosis (BTB) is an inflammatory disease characterized by chronic granulomatous, caseous necrotizing lesions in the lungs and associated draining lymph nodes. Other organs may also be affected depending upon the route of infection. Inhalation is the most commonly known route of infection, causing lesions of the nasopharynx and lower respiratory tract (Domingo et al. 2014). The etiological agents of the disease are mainly *Mycobacterium (M.) bovis* and to a lesser extent *Mycobacterium caprae,* the members of the *Mycobacterium tuberculosis* complex (MTC). The disease doesn't affect all the species equally; rather some species serve as maintenance hosts and others as spillover hosts. All warm-blooded animals are prone to the infection. The bovine species such as bison and buffalo being more susceptible and cattle are the most common reservoir of *M. bovis* (Ayele et al. 2004; Pesciaroli et al. 2014; Yahyaoui-Azami et al. 2017). Since it is a zoonotic disease, it is viewed as a public health concern. Due to insufficiency and unavailability of surveillance and control measures in most of developing countries, zoonotic tuberculosis is widespread in animals. Therefore, the epidemiological and public health concerns still remain unknown (Cosivi et al. 1998). In the context of public health concerns, *M. bovis* is categorized as a Risk 3 pathogen (OIE 2005). *M. bovis* has historically been responsible for extrapulmonary tuberculosis in newborns and children, which typically is brought on by the consumption of unpasteurized or un-boiled milk from infected cows (Thoen et al. 2006).

2. IMPORTANCE OF MOLECULAR DIVERSITY

Regardless of possessing similar 16S rRNA sequences and 99.9% similarity in nucleotide sequences (Boddinghaus et al. 1990), the mycobacteria a group of *Mycobacterium tuberculosis* complex, vary significantly in host specificity, morphology, and pathogenicity. With the assumption that all mycobacteria have been derived from the same ancestor, some of the strains are human-specific (*Mycobacterium tuberculosis, Mycobacterium africanum, Mycobacterium canettii*), some are rodent-specific (*Mycobacterium microti),* while some have a broad spectrum of host infectivity (*Mycobacterium bovis*) (Brosch et al. 2002). The housekeeping genes in *M. tuberculosis* are highly conserved, the reason suggested for the evolution during speciation, which is thought to have occurred some 15 to 20 thousand years ago (Sreevatsan et al. 1997). *Mycobacterium tuberculosis* strains may be categorized as "ancestral" or "modern" strains depending upon the presence or absence of a particular deletion (TbD1) in the sequence of *M. tuberculosis*, the latter being representatives for major epidemics (Brosch et al. 2002). The most prevalent causative agent of human tuberculosis, *M. tuberculosis* has been hypothesized to be evolved from *M. bovis* through precise adaptation of animal's pathogen to human host (Stead et al. 1995). Both of these theories were put out prior to the availability of the whole genome sequence of *M. tuberculosis*, and the revelation of multiple variable regions in the genome of M. tuberculosis complex (Cole et al. 1998). The study of molecular diversity is important to study the genetic organization of the common ancestor of tubercle bacilli, the factors that contributed to host specification and spectrum, and from where *Mycobacterium tuberculosis* evolved. All these help in better understanding of pathogenicity and worldwide epidemiology of disease that can help in predicting future patterns of disease spread (Brosch et al. 2002). Moreover, for effective TB control programs, the genotyping of *Mycobacterium tuberculosis* strains is crucial as it enables the diagnosis of outbreaks, transmission tracking, species diversity monitoring, and the identification of secondary infections (Mozafari et al. 2013).

3. EPIDEMIOLOGY OF BOVINE TUBERCULOSIS

3.1. GLOBAL DISTRIBUTION AND PREVALENCE

Bovine tuberculosis is a highly adaptable and successful infection, having worldwide distribution. It is geographically distributed all over the globe. Between 2015 to 2017, as per the information of OIE's

Worldwide Animal Health Information Database, out of 182 countries, 91 have reported tuberculosis infection in cattle (Interface 2023). It is a major, costly infectious condition in various countries affecting cattle along with other domesticated, feral and wild animals such as badgers, deer, possums, goats, sheep, and camelids (Pollock and Neill 2002; Carslake et al. 2011). The link between the infection and the disease (TB) and the relation among transmission and disease is key to understanding the epidemiology of bovine tuberculosis. Therefore, the risk factors that hypothetically are thought to facilitate the disease spread, such as contact between animals and their movement should crucially be taken into account. The determination of risk environments and risk factors for disease and transmission is also closely associated with the factors affecting susceptibility (Skuce et al. 2012). The deployment of schemes such as test and slaughter, inspection of meat at slaughterhouses, and pasteurization of milk has successfully controlled the disease in most of the developed countries. Regardless of such intensive control regimes, it is still an important concern in some developed countries (Ireland, New Zealand, UK) (Humblet et al. 2009; (Allen et al. 2018), and in a majority of developing countries, where the control strategies are not implemented or at early stages (Teppawar et al. 2018).

4. ECONOMIC IMPACT

Bovine TB not only has detrimental effects on the health of cattle, but it also affects trade and profitability significantly. It can undo years of genetic advancement for desired production traits. The welfare of the affected farming families is also significantly affected (Boland et al. 2010). The overall productivity of animals is reduced such as milk yield, meat production and fertility. It costs human health and is compensated from control programs (Cosivi et al. 1998; Olea-Popelka et al. 2017).

5. *MYCOBACTERIUM BOVIS*

The Mycobacterium tuberculosis complex (MTBC), includes *Mycobacterium bovis* consisting of 11 species of bacteria with varying host tropism and pathogenicity (Gagneux 2017). The principal cause of tuberculosis in humans is *Mycobacterium tuberculosis*, although *M. bovis* has a wider host range and can infect a variety of hosts, mostly cattle but also includes people, with varying populational persistence (Gagneux 2017).

6. MYCOBACTERIUM TUBERCULOSIS COMPLEX

The MTBC is a clonal group (Supply et al. 2003) that evolved from a common ancestor with the *Mycobacterium Canetti*, thousands of years ago (Comas et al. 2013). The horizontal gene transfer and significant amounts of recombination are not thought to have occurred in the genomes of MTBCs, which have >99.95% identity over homologous nucleotide sequences, including the ribosomal RNA genes (Hirsh et al. 2004), however, Single nucleotide polymorphisms (SNPs), indels (small insertions and deletions), deletions of up to 26 Kb, insertion sequences (IS), and duplication of a few paralogous genes are the only way of diversion.

7. DIFFERENTIATING REGIONS

Some of the large deletions, in the members of MTBC, called “Regions of Difference” (RD), were initially described through physical mapping and differential hybridization arrays to differentiate between *M. tuberculosis* H37Rv, *M. bovis* BCG Pasteur, and *M. bovis* ATCC 19210 (Gordon et al. 1999). Fourteen

evolutionarily stable regions of difference (RD1–14) are present among these strains that are used to differentiate. These range from 2 to 12.7 kb in size. The identification of these RDs emptied the path for the molecular diagnosis and classification of MTBC species, and they are now regarded as the benchmark for identifying the various individuals in this complex. As a result, the deleted regions RD9 and RD4 and the absence of RD1BCG (which is deleted in BCG strains) allow *M. bovis* to be distinguished from other MTBC members with accuracy (Bespiatykh et al. 2021). Fig. 1 shows the differentiating regions between the different species of MTBC.

8. TAXONOMIC CLASSIFICATION OF *MYCOBACTERIUM BOVIS*

The *M.bovis* was officially named in 1970, since the beginning of the 20th century, albeit called this way (Karlson and Lessel 1970). The type strain was defined as *M. bovis* ATCC 19210, still referenced in the most recent Bergey's Manual of Systematic Bacteriology (Kämpfer 2012), along with CIP 105234 and NCTC 10772. Early taxonomic classification was based on specific phenotypic traits of the isolates, for tuberculous mycobacteria, such as host of origin, virulence in animal models, and biochemical tests (e.g., pyrazinamide resistance, niacin accumulation, nitrate reduction, type of respiration, colony morphology).

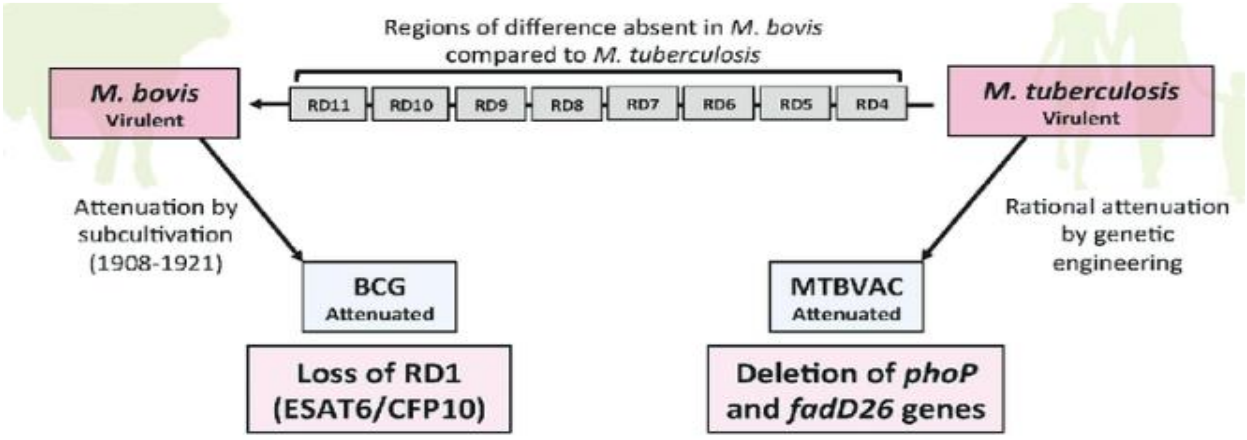

Fig. 1: Differentiating Regions between the different species of MTBC (Gonzalo et al. 2017)

Discussions over the taxonomic categorization of the MTBC species have always been ignited by the close genetic kinship between *M. tuberculosis* and *M. bovis*, as well as among other species. It is sometimes suggested that the MTBC be treated as a single species. However, the epidemiologic and biochemical disparities between diseases, notably between bovine and human infections, bacilli, accentuated the need for differentiating these organisms at some taxonomic level (e.g., species, subspecies, variant).

9. GENOMIC STRUCTURE OF *MYCOBACTERIUM BOVIS*

The M. bovis genome consists of 4.3 Mb, containing approximately 4200 genes, including a single copy of each of the ribosomal RNA genes (5S, 16S, and 23S) and 45 tRNAs. its genome has a high GC content (≈65%), similar to other actinobacteria which suggests the use of suitable sequencing reagents for library preparation in WGS (Tyler et al. 2016). One of the biggest difficulties in analyzing WGS data is the high proportion of repeated sequences in MTBC genomes, especially *M. bovis*. The mobile elements (e.g., insertion sequences—IS), proline-glutamate (PE) or proline-proline-glutamate (PPE) family genes,

integrases, two phage sequences, a CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats), and the 13E12 repeat family genes are few examples. In particular, PE-PPE gene families account for approximately 10% of MTBC genomes and have been associated with TB pathogenesis (Delogu et al. 2017). The most commonly used sequencing platforms generate short reads, usually ranging from 50 to 300 bp, due to this reason, the repetitive elements are tough to handle in genomic studies because the majority of reads are often shorter than the repeats themselves (Tørresen et al. 2019). Some of these repetitive regions are used for traditional genotyping techniques.

10. CLONAL COMPLEXES

Clonal complexes can be defined as groups of short tandems in which every ST shares at least five to seven identical alleles with at least one other ST in the group.
Till now four *M. bovis* CCs have been described including

- African 1 and 2,
- European 1 and 2)

These are determined based on specific deletions ranging few SNPs and spoligotypes. Similarly, to M. tuberculosis lineages, CCs appear to be geographically segregated, with African 1 and 2 restricted to Africa, European 2 usually found in the Iberian Peninsula, and European 1 distributed globally. However, CCs do not represent the whole genetic diversity as all the M. bovis cannot be classified through CCS. One study projected the four distinct global lineages of M. bovis ranging from (Lb1 to Lb4), geographically segregated and not fully represented by CCs (Zimpel et al. 2020).

11. MOLECULAR TOOLS FOR STUDYING BOVINE TUBERCULOSIS

There are different valuable molecular techniques available to study BTB. Restriction fragment length polymorphism (RFLP) is especially used for epidemiological studies and the differentiation of different strains of BTB. An insertion sequence *IS6110* is widely used as a genetic marker. The *IS6110* fingerprinting has been standardized by the use of restriction enzyme *PvuII*, to digest the genomic DNA of BTB. To perform RFLP 20 to 40 days are required to have sufficient DNA and applied to different strains of all mycobacterium species. International consensus regarding the methodology of *IS6110* RFLP of *Mycobacterium tuberculosis* complex and *IS1245* RFLP of *Mycobacterium avium* has been achieved. RFLP is not so feasible because these bacteria are slow growers which can hinder the RFLP results. Secondly, it is laborious, time-consuming, costly, and requires software for advanced analysis.
Polymorphic GC-Rich repeat sequence (PGRS) is another technique probably considered superior over *IS6110* fingerprinting. PGRS-based probes are the most indiscriminatory type for *Mycobacterium bovis* strains.
PCR-based detection is also widely used which is a more sensitive method than culture. Multiplex PCR is faster than culture and even reduces the time to 2 days from 120 days. PCR targets the 16S rRNA gene sequence of the hypervariable region which is specific for *M. avium*, *M. intracellular*, MTB70 gene which is specific for MTBC. s used to differentiate between different MTBC species. Phylogenetic analysis of MTBC strains from animal origin belongs to RD9 while *M. bovis* have RD4. Spoligotyping is a useful method for the detection and typing of MTBC simultaneously.
The GeneXpert technique is also used for the detection of multidrug resistance (MDR) tuberculosis. It is based upon the principle of DNA sequence-specific detection of Rifampicin resistance mycobacterium tuberculosis complex by PCR. It is a rapid, simple-to-use nucleic acid amplification test (Nabeta et al. 2010). It uses the principle of real-time fluorescent probes, and enables the processing of unprocessed sputum samples, resulting in the generation of results within 90 minutes with minimal biohazard and very little technical training.

12. IMPLICATIONS FOR DIAGNOSTICS AND CONTROL MEASURES

12.1. DETECTION AND SURVEILLANCE STRATEGIES

The term 'surveillance' is a French word that means 'the act of watching over'. Initially, it was acknowledged as the close monitoring of individuals infected with or exposed to contagious diseases for timely detection and later was improved with the inclusion and implementation of appropriate control measures like quarantine (Rojanaworarit 2015). Progress toward the control and elimination of disease significantly depends on the collection, interpretation, and analysis of health-related data (CDC). Surveillance programs refer to the systematized collection, evaluation, elucidation and well-timed dissemination of generated health data for the appropriate planning and implementation of disease control programs through policies and procedures with the ultimate goal of prevention and/or minimization of disease transmission (Berrada 2006).

13. IMPORTANCE OF SURVEILLANCE

Surveillance of a disease is important not only to improve the effectiveness of prevention and control programs but also to monitor the impact of the most effective interventions. Strong disease surveillance helps to identify gaps in health systems that are met with appropriate changes in policies and practices. Effective implementation of a surveillance system faces several challenges while ensuring complete, timely, and accurate data collection which can be used efficiently at various levels of the health system. The lack of trained personnel for the assessment, analysis, and interpretation of data may be responsible for the failure of program priorities and interventions despite the availability of good surveillance systems (CDC). Various measures should be taken at national and international levels to develop good surveillance programs, including developing guidelines and tools for the respective Task Force, vital registration systems, data collection tools and guidance, training workshops, and national-level meetings. Surveillance programs must be strengthened with the assistance of international organizations to ensure that authentic data is used for program improvement, expansion of access to treatment and development of new disease indicators (CDC).

Bovine Tuberculosis (bTB), a chronic zoonotic disease, imposes a serious economic impact on animal production and raises public health concerns. Timely surveillance of disease is important for the appropriate containment measures. In developed countries, single intradermal comparative tuberculin skin test (CITT) and gamma-interferon test are valuable tools for health surveillance and monitoring of animals at the farm level which has resulted in a dramatic decrease in the prevalence of the disease (Berrada 2006; Gonçalves et al. 2022). Many factors like poverty, close association between humans and animals, sociocultural habits and HIV/AIDS are responsible for the widespread dissemination of *M. bovis* infection among humans in developing nations like Africa. Inadequate documentation is available on the zoonotic implications and epidemiology of bovine tuberculosis in developing countries, even though the prevalence of human tuberculosis is quite high in these areas. Several factors account for this situation including the insidious nature of tuberculosis, the simultaneous existence of other more devastating diseases, the lack of cooperation between veterinary and medical experts to deal with the public health significance of bovine tuberculosis, and the limited capacity of planning and implementing surveillance and control programs in developing countries (Berrada 2006).

Various countries tackled the bovine tuberculosis challenge by designing different control strategies for raising awareness, capacity building and skills development (Berrada 2006). Since 1991, Portugal has had an eradication program, PETE, which aims to limit the infection in susceptible animal hosts and hence

reduces the risk of human infection (Gonçalves et al. 2022). The Kingdom of Morocco, a developing country, built organizational and technical capacities at regional and international levels for the surveillance of bovine tuberculosis through a well-designed TCP/MOR/2904 project. At the regional level, this project recommended the establishment of collaborating and reference centers for research, training, diagnosis, and surveillance of bovine tuberculosis. At the international level, the provision of assistance and financing approaches for developing countries to strengthen institutional capabilities and human resources is highly recommended (Berrada 2006).

14. OBJECTIVES OF SURVEILLANCE

The following are the three main objectives of surveillance:

- Description of dynamic disease occurrence patterns that are linked to public health actions
- Elucidation of the historical and epidemiological profile of a disease
- Provision of relevant information and baseline data (Rojanaworarit 2015)
- Identification of current burdens and trends
- Detection of circulating strains and their antimicrobial resistance (Murray and Cohen 2017)

15. METHODS OF SURVEILLANCE

Various approaches for surveillance are based on the objectives of surveillance, clinical presentation of disease, and epidemiology (Murray and Cohen 2017). Bovine tuberculosis (bTB) surveillance may be conducted by two methods, namely, active and passive surveillance. Voluntary reporting to district veterinary staff by cattle herdsmen and peri-urban dairy farms refers to passive surveillance. Whereas, active surveillance includes meat inspection, laboratory testing by Ziehl-Neelsen staining, and targeted screening of cattle in a reported area.

16. SURVEILLANCE SYSTEM ESTABLISHMENT

Surveillance systems propose plans including national activities that should be carried out and updated by State involvement (Connie et al. 2021). Due to resource constraints and additional burdens on the operating system, rational selection of diseases for which a surveillance system is established is of utmost importance. Diseases having severe health outcomes (e.g., rabies), high transmission rates (e.g., Ebola), and epidemic or pandemic nature (e.g., influenza) are generally considered for surveillance (Rojanaworarit 2015).
A surveillance system includes several consecutive methodical steps that are illustrated in Fig. 2 and are subsequently elaborated.

16.1. DATA COLLECTION

In terms of investing in the budget and obtaining quality data during surveillance, data collection is the most crucial step. Several factors account for the adaptation of data sources in terms of public health surveillance. These include accessibility and availability of data, features of health service system, budget constraints, personnel, availability, and quality of facilities. In his article, (Rojanaworarit 2015) provided guidelines that surveillance data should include important information like data on mortality and morbidity, data for epidemic detection and laboratory surveillance, disease occurrence and associated risk factors, data for healthcare and health system surveillance as well as environmental data.

Text

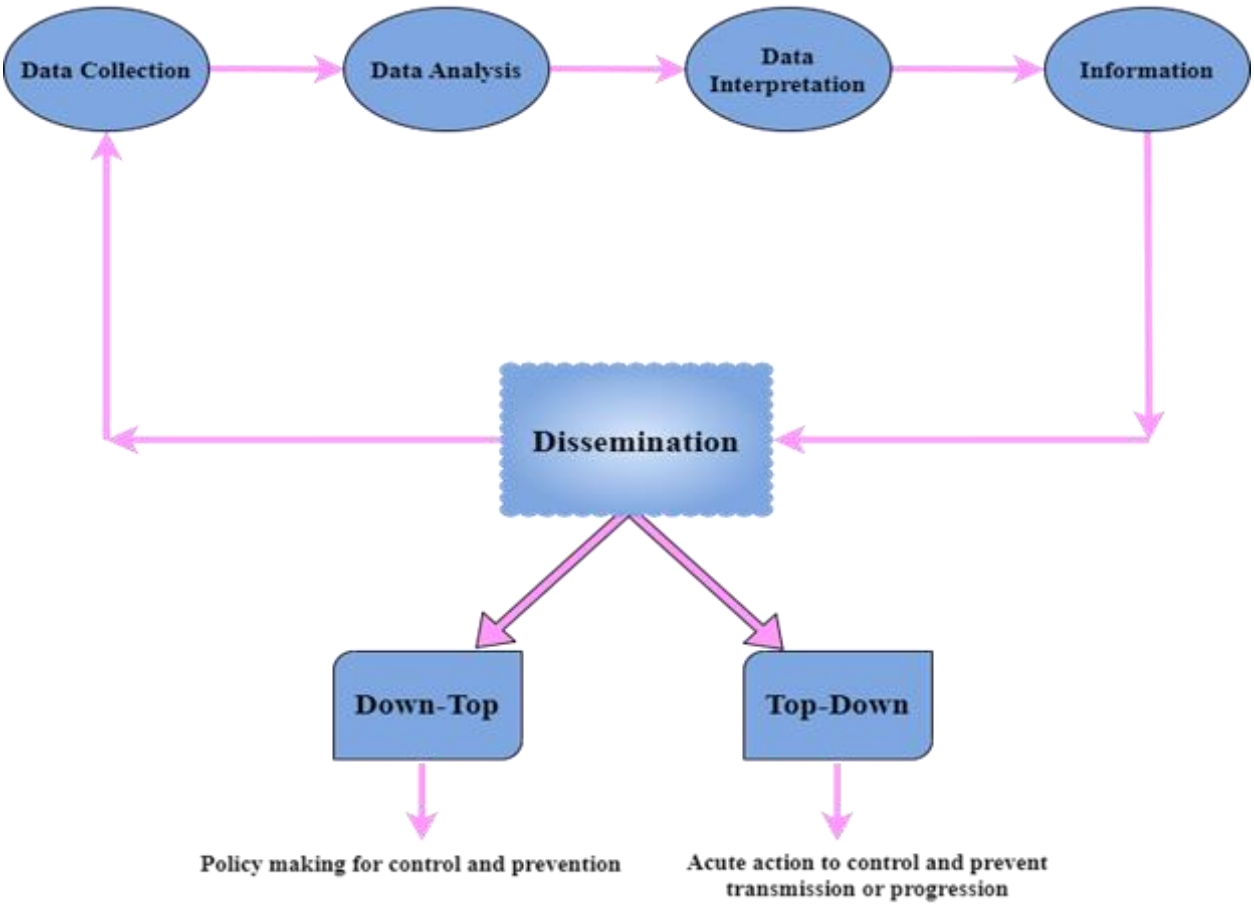

Fig. 2: Surveillance System Workflow

16.2. DATA ANALYSIS

The data analysis approach is adopted to reveal the pattern, magnitude, and trend of a certain health problem. For this, appropriate indicators like prevalence and incidence are used to identify the magnitude of a problem. Elements required in the analysis of surveillance data are:

- Measuring the 'magnitude' of the problem
- Description of pattern and trend
  - Analysis by 'person' characteristics (age, gender, ethnic groups, marital status, occupation, etc)
  - Analysis by 'place' characteristics (international vs intra-country comparison, local disease distribution, etc)
  - Analysis by 'time' characteristics (time onset, secular trend, seasonal pattern, point epidemic, etc (Rojanaworarit 2015).

16.3. DATA INTERPRETATION

Surveillance data interpretation is crucial as it leads to further considerations of whether appropriate public health action is required. The key point at this step is the accurate identification of higher-than-usual disease occurrences that need additional prompt public health action to timely control the disease. Many factors may influence the increase in observed disease occurrence. These include:

- The larger size of the population being investigated
- Altered disease screening campaign
- Improved diagnostic methods
- Better reporting system

Before concluding whether the disease occurrence has increased or not, the above-mentioned factors should be rationally ruled out. Commonly used epidemiological measures like incidence proportion, incidence rate, point prevalence, period prevalence, and case fatality rate must be carefully selected. The interpretation of these measures should be scientifically sound as they are used in the generation of different implications (Rojanaworarit 2015).

16.4. DATA DISSEMINATION

At this stage, the 'who needs to know?' question is addressed. Authoritative health personnel are the ones who must be informed since they require this information for deciding whether to act or not. There are two patterns for the dissemination of data; 'down-top' and 'top-down'. Both of these approaches are important in planning the control and prevention of disease as well as for providing feedback and improving the reporting of surveillance data at the local level (Rojanaworarit 2015).

17. RESEARCH CHALLENGES AND FUTURE DIRECTIONS

Various complications limit the complete eradication of bovine TB. These include: (1) breakdowns due to an anergic carrier or breach in the herd security, (2) complications in administrative and public relations due to non-visible reactor (NVL), (3) large herds, (4) the presence of wildlife reservoirs, (5) lack of diagnostic tests to differentiate between vaccinated and infected animals. International organizations highly endorsed the One-Health approach for TB to address these challenges at the human-animal interface (Borham et al. 2022).

The livestock sector majorly impacts the economy of developing countries by alleviating malnutrition and poverty in rural areas. The rise in the human population has increased the demand for animal-origin foods that are met with the intensification of animal production leading to a higher risk of zoonotic diseases in humans (Berrada 2006). Cattle and other food animals are reservoirs of several diseases of public health importance. Bovine tuberculosis has great potential to infect humans and has thus been considered a neglected zoonotic disease by WHO. Developed countries have greatly reduced the incidence of bTB through efficient eradication and milk pasteurization programs. However, this incidence is still high in developing countries where human TB cases are also on the rise. Several international forums like WHO, World Organization for Animal Health (OIE), and the Food and Agricultural Organization (FAO) have stressed to adoption of strong disease surveillance programs for the prevention and control of both human and bovine tuberculosis in developing and developed countries. There is a need for strong intersectoral collaboration between veterinary and medical professionals to combat zoonotic bTB (Rahman et al. 2015).

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