

Epidemiological and Molecular Investigations of Strangles in Equine Population of Northern India.

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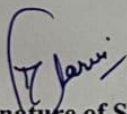
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2026**

DECLARATION

I, hereby declare that the presented work in the thesis entitled **Epidemiological and Molecular Investigations of Strangles in Equine Population of Northern India** in fulfilment of degree of **Doctor of Philosophy (PhD)** is outcome of research work carried out by me under the supervision of Dr. Umesh Goutam, working as Head of Department, in the Department of Molecular Biology and Genetic Engineering of Lovely Professional University, Punjab, India and co-supervision of Dr. Balvinder Kumar of Division of Equine Health, ICAR- NRCE, Hisar. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of another investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.



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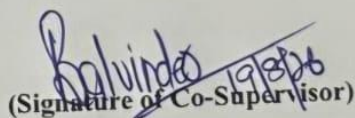
CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled **Epidemiological and Molecular Investigations of Strangles in Equine Population of Northern India** submitted in fulfilment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in the Department of Biotechnology/ School of Bioengineering and Biosciences, is a research work carried out by **Dharvi, 12109611**, is Bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.



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Abstract

One of the most serious infectious bacterial infections majorly affecting horses globally is strangles, which is caused by - *Streptococcus equi subspecies (S. equi)*. The infection mostly affects the head and neck lymph nodes and upper respiratory tract, which results in purulent nasal discharge, abscess formation and frequently systemic consequences. Due to the prevalence of asymptomatic carriers, difficulties in diagnosing the condition, and a lack of knowledge about subclinical infection, strangles persists in equine populations despite having a well-established etiology. The overall goal of the current study was to assess the diagnostic effectiveness of various laboratory methods for precise pathogen detection as well as to investigate the epidemiology of *S. equi* infection in horses using molecular detection, sero surveillance, and carrier status determination. The study was performed on equine populations from organized and unorganized farms across different northern states of India. Random Sampling was conducted, and the required sample size was determined using epidemiological tools to ensure adequate statistical power. Serum and nasal swab samples were collected from clinically healthy, recovered and suspected equines. The serological component of the study employed an indirect ELISA based on the *SeM* specific antigen to detect anti *S. equi* antibodies there by providing insights in to exposure rates and immune response patterns in the population. The molecular component involved the use of both conventional PCR and - multiplex real time PCR assays targeting species specific genes to differentiate - *S. equi subspecies equi*” from related *Streptococcal* species such as *S. zooepidemicus*. The sero- surveillance results showed that the prevalence of antibodies against *S. equi* varied by location, suggesting that the infection may be endemic and that exposure to it was widespread. Real-time PCR molecular detection proved to be more reliable for quickly identifying *S. equi* directly from nasal swabs than traditional PCR, as evidenced by its higher sensitivity and specificity. Together, the findings painted a thorough epidemiological picture of strangles in the explored region, showing both present infections and indications of previous exposure in animals that appeared to be in good health. The carrier detection study constituted a critical component of this research. A total of 200 samples were analyzed to identify sub clinically infected or persistently shedding equines. Of these, 67 animals were seropositive for *SeM*-specific antibodies by ELISA, suggesting prior exposure to *S. equi*. These seropositive animals

were subsequently tested using bacteriological culture, conventional PCR, and real-time PCR. Among them, 20 animals (29.8%) yielded positive results by one or more confirmatory methods, establishing them as true carriers of *S. equi*, representing an overall carrier prevalence of 10% in the total sampled population. The detection of bacterial DNA in recovered but asymptomatic animals confirmed the persistence of *S. equi* and its potential for silent transmission within herds.

Given that antibodies might linger even after bacterial clearance, the results unequivocally showed that seropositivity is not enough to determine carrier status. The most sensitive technique was found to be real-time PCR, which may identify low bacterial loads that traditional PCR or culture could overlook. A successful method for accurately identifying carriers was the combination of molecular confirmation and serological screening. Such a diagnostic approach is essential for preventing recurrence of strangles outbreaks, especially in endemic areas or before animal movement and breeding. This study provides comprehensive epidemiological and diagnostic data on strangles in Indian equines. The integration of serological and molecular techniques improved the accuracy of detection and differentiation of *S. equi* infections. The identification of subclinical carriers underscores the importance of including molecular-serological surveillance as a routine part of equine health monitoring and disease control programme. The outcomes of this research contribute significantly to the understanding of *S. equi* epidemiology, aid in refining diagnostic strategies, and provide a scientific basis for future policy and management interventions aimed at reducing the burden of strangles in the equine industry.

Keywords: - Strangles, *Streptococcus equi*, *SeM* gene, PCR, *Streptococcus* species, Antibodies, Indirect ELISA, Multiplex Real time PCR

**Dedicated To my respected mentor
and Co - supervisor
Dr Balvinder Kumar
and My Beloved Parents**

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“Behind every success there is a team who believed, supported and Inspired”

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Preface

For generations, India's rural life, culture, and economy have relied heavily on horses, ponies, donkeys, and mules. They play a variety of tasks, including helping with agriculture, sports, tourism, and security services, as well as moving people and products through challenging or isolated areas. In many arid, semi-arid, and hilly regions, where mechanization is either impractical or too expensive, Equines continue to be a lifeline for underprivileged communities.

Even though the number of horses in India is decreasing, many households still rely heavily on them for their livelihoods. In brick kilns, agricultural produce transportation, mountainous regions, and smaller communities with inadequate contemporary infrastructure, horses are used for a variety of jobs. In addition to working, they play a significant role in cultural and recreational pursuits such horse racing, traditional riding, tourism, and ceremonial events.

However, despite their importance, equines often suffer neglect in terms of health care, policy support, and research. Diseases affecting equines not only pose serious welfare concerns but also lead to substantial socio-economic impacts. A disease like strangles, which is extremely contagious, can reduce the working capacity of animals, increase veterinary costs, and disrupt the livelihoods of communities dependent on equines.

This thesis is motivated by the recognition that understanding, preventing, and controlling equine diseases is not just a matter of veterinary interest but also one of social justice and sustainable rural development. In India, despite the substantial equine population and their economic importance in agriculture, transportation, and sports, there is a paucity of comprehensive data on the epidemiology of strangles. Limited studies have assessed the prevalence, risk factors, and molecular characteristics of *Streptococcus equi* subspecies *equi* in different regions of the country. This gap in knowledge hampers development of effective control strategies and implementation of preventive measures tailored to the Indian context.

By performing epidemiological research of strangles in horses in Northern India, the current study seeks to close this gap. The study aims to ascertain the disease's prevalence, pinpoint possible risk factors, and describe the strains of *S. equi* that are

circulating in the area through clinical examinations, bacteriological and molecular studies, and evaluation of management procedures. The results are expected to improve disease surveillance, aid in the development of focused intervention measures, and ultimately improve the health and welfare of horse populations and those who rely on them.

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List of Abbreviations

bp	Base pair
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra- acetic acid
Et br	Ethidium bromide
BLAST	Basic local alignment search tool
NCBI	National center for biological information
UV	Ultra violet
COBA	Columbia blood agar
min	Minute.
ml	Milliliter
μl	Micro litre
Mm	Millimoles
Nm	Nanometer
THB	Todd Hewitt broth
ND	Nanodrop
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
<i>Hsp60</i>	Heat shock protein 60
NTI	BINDING BUFFER
NT3	Wash buffer
NTI	binding buffer
IgG	Immunoglobulins
MLST	Multi locus sequence typing
Fb	Fibrinogen
<i>S. equi</i>	<i>Streptococcus equi</i>
TAE	Tris glacial acetic acid EDTA buffer
<i>S. zooepidemicus</i>	<i>Streptococcus zooepidemicus</i>
<i>Sp.</i>	<i>Species</i>

Chapter 1

Introduction

Equines have played a pivotal role in human life since antiquity, serving as indispensable partners in transport, agriculture, and warfare. In the Indian context, they continue to hold significant socio-economic value, particularly in rugged terrains and rural economies. India possesses 0.54 million equines including horses, ponies, mules and donkeys according to the 2019 livestock census (Jose *et al.*, 2024). A significant number of equines, including horses, ponies, mules, and donkeys, support rural and semi-urban communities. The use of equines has special economic significance in difficult terrain in hills, besides arid and semi-arid zones for draught and transport purposes. Major force (approximately 97%) of equines in India, comprises of donkeys, mules and ponies, provides livelihood to the landless, small and marginal farmers and other sections of unprivileged rural and semi-urban society. Remaining equines belonging to the elite group, are used by army, police, para-military forces, racing industry, sports and ceremonial parades etc. Although population of ponies and donkeys has shown slight decline over the years in India, the use of mules for draught and transport and that of thoroughbred and other horses has increased considerably for sports, tracking and racing. Not with-standing their aforesaid use, the equines became victim of increased mechanization resulting in reduction of proper health cover, both in the field of diagnosis as well as immuno- prophylaxis. This brought in catastrophic effect on the economy of equines in the last few decades.

Research work was initiated to contain these effects and also to improve production and work performance for the upliftment of the equines. Amongst infectious diseases, most of the research efforts remained concentrated on viruses rather than the bacterial infections. Among bacterial pathogens, *Streptococcus equi subspecies equi* received attention as one of the most important pathogens (Gowda *et al.*, 2000). Most clinical research and reports involving the beta hemolytic Lancefield group C *streptococcal* infections in equines have been concerned with *Streptococcus equi*, the causative agent of strangles. Strangles is a highly contagious and clinically significant disease that affects the upper respiratory tract of horses and other equines. It is caused

by *Streptococcus equi subspecies equi*, a spherical, Gram-positive, pyogenic, and β -hemolytic bacterium belonging to Lancefield group C. The pathogen is known for its high infectivity, leading to considerable morbidity in susceptible populations, particularly those that have not been previously exposed to the disease. While equines of all ages can contract *S. equi*, younger animals often exhibit more severe clinical manifestations compared to older individuals, who tend to recover more rapidly due to their immune status (Sweeney *et al.*, 2005; Pusterla *et al.*, 2011; Boyle *et al.*, 2018).

The clinical presentation of strangles varies among affected animals but is generally characterized by an acute onset of —pyrexia, followed by pharyngitis and abscess formation in the mandibular and retropharyngeal lymph nodes (Boyle *et al.*, 2018; Sweeney *et al.*, 2005). The disease manifests within 3 to 14 days post-exposure, with initial signs including lethargy and fever ($>40^{\circ}\text{C}$) (Waller., 2014). Progressive lymphadenopathy often leads to dysphagia, reluctance to eat or drink, and abnormal head positioning due to pain and obstruction of the upper airways (Mallicote., 2015). In severe cases, upper airway obstruction may necessitate emergency tracheostomy. Although nasal discharge is a common symptom, coughing is not consistently observed in all infected animals (Mallicote *et al.*, 2015; Sweeney *et al.*, 2005; Boyle *et al.*, 2018). Complicated cases may involve abscess formation exerting pressure on the esophagus and pharynx, further compromising breathing, which is the basis for the disease's name, - strangles. Disease transmission primarily occurs through direct contact with infectious nasal secretions, abscess exudates, or contaminated fomites.

The definitive diagnosis of strangles relies on the isolation of *S. equi* from nasal secretions, abscess material, or lymphoid tissues, as well as molecular detection using Polymerase Chain Reaction (PCR). PCR has emerged as a highly sensitive and rapid diagnostic tool, reducing detection time to a single day (Bekele *et al.*, 2024). It is particularly useful for screening herds, diagnosing acute and chronic cases, and detecting asymptomatic carriers before seroconversion. The primary treatment for strangles involves penicillin; however, the efficacy of antibiotic therapy is dependent on the stage and severity of the disease. In many cases, supportive care is prioritized over antimicrobial intervention to facilitate natural immune clearance of the infection (Bekele *et al.*, 2024). While penicillin remains the preferred antimicrobial agent, treatment failures have been reported due to emerging antimicrobial resistance within

Streptococcus species (Bekele *et al.*, 2024). The isolation and characterization of *S. equi* from clinical cases remain critical for understanding the pathogen's epidemiology, virulence, and antimicrobial susceptibility. Culturing bacterial isolates from infected horses help in confirming the disease and differentiating *S. zooepidemicus*, a closely related commensal organism with opportunistic pathogenicity. Molecular techniques such as polymerase chain reaction (PCR) is widely used molecular diagnostic tool for detecting —*Streptococcus equi subsp. equi* (the causative agent of strangles) in equines. Conventional PCR, though slightly less sensitive than qPCR, remains an effective and reliable method for diagnosing active infections and carrier states. PCR amplifies specific DNA sequences of *S. equi*, allowing rapid and accurate identification of the pathogen, primers targeting the *SeM* gene (a virulence-associated gene encoding the M protein) are commonly used for species-specific detection. Unlike culture-based methods, PCR can detect both viable and non-viable bacteria, comprising its diagnostic value. The quantitative PCR (qPCR) has enhanced the rapid and specific detection of *S. equi*, allowing for improved diagnostic accuracy.

Sero-surveillance plays a vital role in determining the prevalence and spread of strangles in equine populations. The detection of antibodies against major virulence factors of *S. equi*, such as the *SeM* protein, provides insights into previous exposure and herd immunity. Serological screening is particularly useful in regions where clinical cases are underreported or where asymptomatic carriers contribute to disease persistence. Horses that recover from strangles may continue to harbour *S. equi* within the guttural pouches for extended periods, with shedding observed for up to 39 months post-recovery (Boyle *et al.*, 2018; Ivens *et al.*, 2011; Boyle *et al.*, 2009). These asymptomatic carriers play a critical role in maintaining the infection within equine populations and initiating new outbreaks, making disease control and prevention particularly challenging. Historically, outbreak control strategies have relied on a 4-week quarantine period or three consecutive negative nasopharyngeal swabs to confirm bacterial clearance (George *et al.*, 1983). However, due to the intermittent shedding pattern of *S. equi*, culture-based detection alone is often insufficient. As a result, nasal swab/ endoscopic guttural pouch lavages sampling has become the gold standard for carrier identification (Newton *et al.*, 2000; Timoney *et al.*, 1997). Additionally, serological detection of *S. equi* M protein (*SeM*) antibodies via ELISA provides a valuable tool for assessing exposure history and carrier status (De Negri., 2023).

Strangles remains endemic worldwide, with frequent outbreaks reported in Europe, North America, and Asia. Real-time monitoring by the Equine Disease Communication Center (EDCC) and global surveillance networks helps track cases; however, carrier horses, international movement, and vaccine limitations continue to challenge disease control. Despite advancements in PCR-based diagnostics and sero-surveillance, asymptomatic carriers contribute to recurrent outbreaks, making strict biosecurity, quarantine measures, and enhanced screening essential for prevention. *S. equi* is a host-restricted pathogen believed to have evolved from the closely related *S. zooepidemicus*, sharing over 97% genetic similarity. Their divergence is largely attributed to genetic adaptation, functional loss, and horizontal gene transfer mediated by a shared phage pool. The deletion of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) locus in *S. equi* facilitated the acquisition of foreign genetic elements, enhancing its pathogenicity at the cost of genomic stability. A key evolutionary adaptation in *S. equi* is the presence of the equi bactin locus, which enhances iron acquisition and may have played a critical role in its speciation. Additionally, the genome of *S. equi* is larger and more elastic due to the incorporation of multiple mobile genetic elements, increasing its pathogenic potential while restricting its host range exclusively of equids. Given these challenges, controlling strangles requires a combination of advanced diagnostics, improved vaccination strategies, and stringent biosecurity measures to minimize transmission and ensure effective disease management. Although *S. equi* does not persist in the environment for extended periods, eradication of the infection remains challenging. A potent vaccine with DIVA (Differentiating Infected from Vaccinated Animals) capability is considered essential for sustainable disease control. While vaccine development has faced significant challenges, a recombinant protein-based vaccine (Strang vac) has demonstrated protective efficacy in experimental settings (Robinson *et al.*, 2018;2020). However, its field effectiveness and optimal implementation strategies require further investigation (Rendle *et al.*, 2025).

In India, with its substantial equine population engaged in agriculture, transportation, sports, and cultural activities, presents an ideal setting for studying the epidemiology of strangles. The frequent congregation of equines at fairs, races, and trade events, combined with diverse climatic conditions, creates a high-risk environment for disease transmission. Despite the recognized impact of strangles on equine health and

productivity, comprehensive epidemiological and molecular investigations in India remain limited. Advancement in molecular diagnostics, including - PCR, Multi Locus Sequence Typing (MLST) and - Whole-Genome Sequencing (WGS), have revolutionized our ability to track outbreaks, detect asymptomatic carriers, and understand the genetic diversity of *S. equi* strains. These tools are crucial for designing targeted control measures suited to specific epidemiological settings. Given the economic and welfare implications of strangles, there is an urgent need for systematic research to elucidate the prevalence, risk factors, and molecular epidemiology of the disease in India.

Research Gap of the Study

Our understanding of *S. equi* and its pathogenic mechanisms is rapidly advancing. At both national and international levels, the significance of diagnosing and managing persistently infected horses is widely recognized. A review of existing research and literature indicates a scarcity of comprehensive data on this subject. Notably, there is a lack of systematic surveillance to assess the disease prevalence, and no studies have been conducted to determine the carrier status of equines in India. This research aims to bridge these gaps by conducting epidemiological investigations of strangles in equines in northern India. The findings will facilitate the development of targeted intervention strategies to limit the spread of *S. equi*, offering substantial health, welfare, and economic benefits to India's equestrian industry.

Chapter 2

Review of Literature

2.1 History of Strangles

Strangles is one of the oldest recognized infectious diseases affecting horses. The first detailed clinical description dates back to 1251 by Jordanus Ruffus, who noted the characteristic symptoms: fever (pyrexia) followed by abscessation of the lymph nodes in the head and neck (Waller., 2016). The disease got its name because the swollen lymph nodes could obstruct the horse's airway, sometimes causing suffocation (Waller., 2016). The bacterium responsible for strangles, *Streptococcus equi subspecies equi* (*S. equi*) was first identified in 1888 by Schutz (Waller., 2016). Despite centuries of observation, the understanding of its transmission and persistence has evolved only gradually. Genomic studies indicate that while strangles has been present for centuries, the global population of *S. equi* appears to have a common ancestor dating from the 19th or early 20th century (Mitchell *et al.*, 2021). This period coincided with the large-scale use of horses in global conflicts such as World War I, during which millions of horses were mobilized, mixed, and transported internationally. These conditions facilitated the spread and evolution of the fittest strains of *S. equi*, leading to the emergence of the contemporary global population of the pathogen (Waller., 2016).

S. equi enters the horse through the mouth or nose and adheres to the crypt cells of the lingual and palatine tonsils, as well as the follicular-associated epithelium of the pharyngeal and tubal tonsils (Timoney *et al.*, 2008). This attachment is facilitated by various ligands, including surface-exposed proteins (SzPSe), M protein, fibronectin-binding protein, fibrinogen-binding protein, and the hyaluronic acid capsule (House., 2011). Within hours of infection, the bacteria migrate through epithelial cells into subepithelial tonsillar follicles, making nasal or nasopharyngeal cultures unreliable for early detection. The pathogen rapidly spreads to the mandibular and retropharyngeal lymph nodes, which drain the pharyngeal and tonsillar regions.

Upon infection, complement-derived chemotactic factors interact with bacterial peptidoglycan, attracting large numbers of polymorphonuclear neutrophils (Mukhtar & Timoney., 1988). However, *S. equi* evades opsonization and phagocytosis

through the protective effects of its hyaluronic acid capsule, antiphagocytic *SeM* protein, H factor-binding *Se18.9*, Mac protein, and other unidentified antiphagocytic factors (Timoney *et al.*, 2014). Despite this immune evasion, neutrophil migration to the affected lymph nodes leads to their swelling and abscess formation (Newton *et al.*, 1997). In some cases, the bacteria spread through hematogenous or lymphatic routes, forming abscesses in distant lymph nodes and organs within the thoracic and abdominal cavities (Rao & Gopalkrishna., 2010).

The infectious dose required for disease establishment is significantly higher when bacteria are propagated in artificial media compared to natural transmission, as virulence factors critical for attachment and penetration are optimally expressed in *in vivo* conditions (J.F. Timoney, unpublished data). Inoculation with fewer than 10⁶ colony-forming units (CFU) is often insufficient to induce disease, as lower bacterial loads are effectively cleared by the mucociliary system. The incubation period and severity of the disease are directly proportional to the size of the intranasal inoculum of *S. equi*.

In previously infected but isolated herds, guttural pouch carriers may facilitate ongoing exposure to *S. equi*, contributing to prolonged immunity and extended strangles-free periods. Horses with residual immunity, foals with waning maternal antibodies, and vaccinated animals often develop a milder form of the disease known as "catarrhal" or "atypical strangles." Despite exhibiting less severe symptoms, these individuals can still shed virulent *S. equi*, potentially causing severe infections in highly susceptible, often younger, horses (Sheoran *et al.*, 1997).

Biochemically, *S. equi* is catalase-negative and ferments carbohydrates, producing lactic acid, but it does not grow in high-salt environments or hydrolyze bile esculin. A key virulence factor is its hyaluronic acid capsule, which helps evade phagocytosis, along with the M-protein (*SeM*), which prevents opsonization. Additionally, it produces streptolysins that contribute to tissue damage and superantigens that trigger an excessive immune response. Transmission occurs primarily through direct contact with infected animals or contaminated surfaces, allowing the bacteria to colonize the upper respiratory tract and spread to lymph nodes, leading to abscess formation. In severe cases, the infection can disseminate, causing systemic complications known as "bastard strangles." Despite its virulence, *S. equi* remains highly susceptible to penicillin and

other β -lactam antibiotics, though tetracycline and fluoroquinolone resistance is uncommon. Understanding its bacteriology is crucial for effective prevention and treatment strategies in equine populations.

2.2 General Bacteriology

Streptococcus equi subsp. *equi* is a Gram-positive, spherical (coccus), beta- hemolytic pyogenic bacterium measuring approximately 0.8–1.0 μm in diameter and typically arranged in chains. They are present in chain during division and encapsulated. It needs media containing blood or serum for its growth and multiplication. After 24 hours of incubation at 37°C, it produces clear and mucoid colonies usually less than 4 mm in diameter. It is a catalase negative and facultative anaerobic bacterium that does not ferment sorbitol, lactose, or trehalose (Jorm *et al.*, 1994). It belongs to Lancefield group C *streptococci*.

The environmental survival of *S. equi* is generally limited; however, the organism can persist for several weeks to months in water and in moist or soiled environments (Rao *et al.*, 2010). It remains viable in water for 4–6 weeks but not in faeces or soil. Despite older literature claiming extended survival in the laboratory setting (Jorm., 1992) recent studies using real-world scenarios showed rapid death (1–3 days) of the bacteria on fencing and soil (Weese *et al.*, 2009). *Streptococcus equi* is sensitive to bacteriocins produced by various environmental microorganisms, and its proliferation can be inhibited by the presence of other soil-associated bacterial species.

Transmission of *S. equi* primarily occurs through exposure to purulent nasal exudates from horses exhibiting active infection or those in the convalescent stage of strangles. The spread of infection can take place via both direct and indirect routes. Direct transmission typically involves close physical contact between equines, such as nose-to-nose interaction or the inhalation of aerosolized droplets expelled during coughing by infected animals (Stringer., 2009). Conversely, indirect transmission arises from contact with contaminated fomites, including shared stabling facilities, water troughs, feeding utensils, or equipment such as tack and twitches. Less commonly, transmission may occur through contaminated clothing, tools, or hands of caretakers and veterinarians. Clinically normal animals can also serve as sources of infection, particularly during the incubation period or the convalescent phase when bacterial shedding may still occur (Quinn *et al.*, 2002).

2.3 Epidemiological status of strangles

Strangles can have severe consequences, with some outbreaks affecting hundreds of horses and leading to financial losses exceeding £300,000 (Mitchell *et al.*, 2021). The only known exception to this disease is in Iceland, where the geographically isolated horse population has remained free from infection due to a thousand-year-old ban on horse imports. Horses of all ages are susceptible, though younger and older horses are particularly vulnerable. However, foals under four months of age benefit from passive immunity acquired through colostrum. Elderly horses, with weaker immune systems, often experience more severe and prolonged illness. Some studies suggest that older horses may develop partial immunity after natural infection, leading to milder symptoms in subsequent exposures.

Approximately 10% of horses recovering from acute *S. equi* infections become asymptomatic carriers. These carrier animals, often undetected in routine veterinary examinations, can silently spread the bacteria, triggering new outbreaks. The prevalence of strangles varies significantly across countries. Mitchell and his colleagues conducted a comprehensive analysis of 670 *S. equi* isolates obtained from equine populations across 19 countries using core-genome multi locus sequence typing (cg MLST). The findings provided molecular evidence supporting the global dissemination and intercontinental transmission of *S. equi* among horse populations.

All horses in affected regions are at risk, and in some outbreaks, the fatality rate can exceed 10%. Since 2010, an increase in BAPS-2 strain occurrences in the UK, along with its detection in Europe, the USA, and the Middle East, suggests that this strain may have reached epidemic levels in these regions.

In 2025, Abdu-Lateif and colleagues conducted the first molecular characterization of *Streptococcus equi* strains from horses in Baghdad, Iraq, analyzing 59 *Streptococcus* spp. Clinical isolates. Conventional PCR targeting the *SeM* gene confirmed 49 isolates as *S. equi* (83.1 %). Nine PCR-positive products were sequenced to identify alleles and perform phylogenetic analysis. This work marked the initial report of the *SeM*-97 allele in Iraq, with phylogenetic data showing 100% sequence identity with an Iranian strain, suggesting close regional epidemiologic ties [pmc.ncbi.nlm.nih.gov+1pubmed.ncbi.nlm.nih.gov+1](https://pubmed.ncbi.nlm.nih.gov/1). Their study represented the first *SeM* allele-typing in Iraq and highlights possible transboundary strain dissemination via unsupervised horse

importation, laying a crucial foundation for future surveillance and control measures in the region (Abdul *et al.*, 2025). Nine PCR-positive products were sequenced to identify alleles and perform phylogenetic analysis. This work marked the initial report of the *SeM*-97 allele in Iraq, with phylogenetic data showing 100 % sequence identity with an Iranian strain, suggesting close regional epidemiologic ties. Their study represents the first *SeM* allele-typing in Iraq and highlights possible trans boundary strain dissemination via unsupervised horse importation, laying a crucial foundation for future surveillance and control measures in the region.

In a study conducted in the state of Santa Catarina, Brazil, researchers aimed to determine the prevalence of *S. equi* and *S. zooepidemicus* and assess the antimicrobial susceptibility patterns of the isolates. A total of 420 nasal swabs were collected from randomly selected horses and subjected to phenotypic characterization using 5% sheep blood agar, sugar fermentation profiling (lactose, maltose, sorbitol and trehalose), and PCR confirmation for subspecies identification. The overall prevalence of *S. equi* was reported as 2.38%, with 30% of the isolates identified as *S. equi subsp. equi* and 70% as *S. zooepidemicus*, antimicrobial susceptibility testing using the disk diffusion method against 13 commonly used antibiotics revealed multidrug resistance in 60% of isolates. The highest resistance was observed against clindamycin (70%), followed by penicillin (40%) and ceftiofur (30%), whereas complete sensitivity (100%) was noted for gentamicin, chloramphenicol, levofloxacin, and vancomycin. This study, the first of its kind in Santa Catarina, indicated a low prevalence of *S. equi* but highlighted the emergence of multidrug-resistant strains within the equine population (Veiga *et al.*, 2024).

Between 2014 and 2023, Carpentier and its colleagues, validated a new double-antigen ELISA for *S. equi* serum samples from five groups: 22 horses (symptomatic and asymptomatic) during a Normandy outbreak, 24 horses from Lyon, 464 samples from a longitudinal LABEO cohort in France, 159 negative-control horses from strangles-free Iceland to assess specificity, and eight horses infected with *S. zooepidemicus* to test cross-reactivity. They demonstrated 98.5% specificity (95 % CI: 95.2–99.5 %) and a detection rate of 77% (95 % CI: 66.2–85.1 %) in infected herds, with no positive reactions in the *zooepidemicus* group-supporting its high exclusivity and minimal cross-reactivity (Carpentier *et al.*, 2025).

Weese and colleagues (2023) investigated the correlation between quantitative real-time PCR (qPCR) cycle threshold (Ct) values and bacterial culture detection of *Streptococcus equi* in 158 equine respiratory tract samples. *S. equi* was cultured from only 12 samples (7.6%) overall: 4/9 (44%) when $Ct \leq 30$, 7/30 (23%) when Ct ranged 30.1–35, and just 1/119 (0.8%) when Ct was between 35.1–40, with the highest Ct yielding culture-positive results being 36.9. Receiver-operating characteristic (ROC) analysis established an optimal Ct cut off of 34.2 using Yuden's J statistic—this threshold aligned with cost-sensitive misdiagnosis modelling where false negatives were considered 5 to 10 times more consequential than false positives. These findings indicate that only a minority of qPCR-positive samples contain viable *S. equi*, and that a Ct of 34.2 can serve a practical breakpoint for assessing the likelihood of culture-positive infection, which may aid risk assessment and decision-making in clinical and herd management contexts (Weese *et al.*, 2023)

Recent studies have further investigated the genetic diversity of *S. equi*. Bustos and colleagues employed molecular studies and phylogenetic methods to identify and compare *S. equi* *SeM* genotypes in Argentina. Their research revealed eight *SeM* alleles, with only one (*SeM*-61) previously reported, while the remaining seven (*SeM*-129 to *SeM*-135) were newly identified. These alleles were previously detected in Brazil, highlighting the cross-border spread of the bacteria (Bustos *et al.*, 2022).

Similar studies have been conducted in other countries. A study by Mc Glennon in 2021 analysed data from seven UK laboratories between January 1, 2015, and December 31, 2019. Over this period, 1,617 cases of strangles were reported by 315 veterinarians. Diagnostic methods included swabs (51.6% of cases) and guttural pouch lavages (44.0%). The majority of diagnoses were confirmed via qPCR alone (59.6%), qPCR combined with culture (35.8%), or culture alone (4.6%). The highest concentrations of *S. equi* cases were found in North Yorkshire (75 cases, 4.6%), Staffordshire (71 cases, 4.4%), and North East West Sussex (63 cases, 3.9%). A study conducted in 2021 by Osman and colleagues in the Qassim region of Central Saudi Arabia examined strangles in 29 horses, with 13 showing symptoms and five succumbing to the disease. The study reported a morbidity rate of 44.8% and a mortality rate of 17.24%, with morbidity and mortality varying significantly across age groups, though the case fatality rate remained unchanged. Gender did not appear to

influence disease susceptibility. The clinical signs detected involved high fever, anorexia, a soft non-productive cough, muco-purulent bilateral nasal discharge, and abscessation of the submandibular lymph nodes (Osman *et al.*, 2021)

In five cases, *S. equi* led to metastatic infections, causing abdominal abscessation and acute abdominal pain. The study emphasized the challenge of detecting *S. equi* carriers, who can remain infected for long periods without symptoms, necessitating the development of rapid and cost-effective diagnostic methods, as traditional microbiology may underestimate the disease's true prevalence.

In Egypt, Hassenin and his team analyzed 650 samples from Arabian horses at AL-Zahraa station, various farms in the Shariakia region, and cases presented to the Faculty of Veterinary Medicine at Zagazig University. The study investigated the disease's prevalence across different age groups and seasonal patterns. One of the most concerning findings was the presence of seemingly healthy carrier horses that shed bacteria and infected more susceptible individuals. PCR proved effective in identifying these hidden carriers (Hassenin *et al.*, 2021). Similarly, a case of strangles was diagnosed in a quarantined donkey on a Chinese intensive farm in April 2019. The diagnosis was confirmed using clinical signs, bacterial isolation, histochemistry, PCR, and sequencing. *SeM* gene analysis identified the *S. equi* strain CN190402 as a new genotype, *SeM*-138, based on M-protein typing.

Nasal and guttural pouch lavages have been widely used to detect persistent carriers and confirm strangles-free status in horses. A study linked an outbreak to a specific *S. equi SeM* strain, and follow-up testing three months later revealed a single amino acid variation in *SeM* gene sequences of two isolates. Additionally, two horses remained *S. equi* positive six months later, displaying non-mucoid *S. equi* colonies in culture. Many horses regularly tested positive for *S. equi* through qPCR and culture, highlighting the need for continuous monitoring.

Further sero-epidemiological studies in Iraq assessed *S. equi* antibody levels and bacterial cultures in 462 equines (308 horses and 154 donkeys) across different provinces. The study also examined carrier prevalence among different age groups and genders, emphasizing the necessity of identifying and treating carriers to prevent disease outbreaks. In Iran, Mohammadi and colleagues (2016) evaluated strangles

prevalence in Khuzestan Province, exploring its association with age and geographic factors. They randomly collected serum samples from 184 horses and tested them using ELISA, while 85 nasal swabs were analyzed for *S. equi* subsp. *equi* through bacterial culture. Their findings indicated a high seropositivity rate in the region (Mohammadi *et al.*, 2016).

A 2016 study in Brazil estimated strangles prevalence and identified risk factors through a cross-sectional analysis of nasal swabs. Among 1,010 samples collected from healthy equines across 341 farms, *S. equi* was isolated from 24 horses, with PCR and DNA sequencing confirming the results. The estimated prevalence was 2.3% for individual animals and 5.86% for herds. Similarly, Cordoni and colleagues (2015) developed a —multiplex PCR assay to directly detect *S. equi* and *S. zooepidemicus* from clinical swabs. The test demonstrated 95% sensitivity and 98% specificity, validated against 59 strains of *S. equi* and *S. zooepidemicus* from diverse geographic regions. This rapid and reliable diagnostic method provides an effective tool for identifying and managing strangles outbreaks (Cordoni *et al.*, 2015)

In addition to horses, occurrences of strangles have also been documented in donkeys. A study conducted in Ethiopia investigated donkeys exhibiting clinical signs such as guttural pouch involvement and nasal discharge. A total of 36 nasal swab samples and 12 pus swabs were collected from 36 clinically affected donkeys, from which *S. equi* was successfully isolated and identified (Seyum *et al.*, 2015). Furthermore, a study carried out in the United Kingdom in 2013 utilized genome sequencing data to develop an indirect enzyme-linked immunosorbent assay (iELISA) targeting two *S. equi*-specific recombinant protein fragments. The objective was to detect asymptomatic carrier animals and thereby mitigate potential strangles outbreaks. The diagnostic performance of antigen A and antigen C iELISAs was compared with a commercially available *SeM*-based ELISA (ID vet). While each individual assay demonstrated a trade-off between sensitivity and specificity, the combined interpretation of antigen A and antigen C iELISA results yielded enhanced diagnostic accuracy, achieving a sensitivity of 93% and specificity of 99.3%. This combined assay represented a reliable tool for detecting *S. equi* exposure and identifying persistently infected carriers (Robinson *et al.*, 2013).

2.4 Status in India

Strangles is being one of the major economically affecting infectious diseases gaining attention of researchers and veterinarians. Many studies and research related to strangles have been conducted internationally. Moreover, in India there are various studies related to occurrence and virulence of this infectious disease. One of the studies was performed on molecular characterization of virulence genes of *S. equi*. The target of the study was the existence of *S. equi* in equines. A total 96 samples were collected from both clinically affected animals and two isolates were found resistant to penicillin G " were totally and all rest of the isolates were found sensitive to amoxicillin and streptomycin. The study concluded that the majority of the infection was spread due to *S. equi* and therefore, it is recognized as a very important pathogen of equines (Javed *et al.*, 2016). Senthil and associates in Chennai in 2014 collected nasopharyngeal swabs eight with submandibular edema and four without any symptoms or lesions from 12 horses brought to Madras veterinary college and were cultured in Edwards medium base and tested using conventional PCR for *S. equi* infection. They discovered that three of the eight samples screened from horses with sub-mandibular lymphadenitis were positive for *S. equi*, and one of the four samples taken from apparently healthy horses was positive for *S. equi* by culture and PCR. Their study highlighted the importance of initiating the regular screening process for strangles from both clinically affected as well as apparently healthy horses so as to prevent its spread in equine population and for initiating proper eradication programme (Senthil *et al.*, 2014). One of the studies from Jammu and Kashmir by mir and co-workers in 2013 reported that the overall prevalence of *S. equi sub species equi*, *S. equi subspecies zooepidemicus*, and *S. dysgalactiae subspecies equisimilis* were 2.83, 39.71, and 12.05% of the population, respectively. Detection of *S. equi* species through PCR directly from swab samples was found to be more sensitive (Mir *et al.*, 2013). In a study, a total of 311 samples were collected from Haryana, Uttarakhand, Rajasthan, Delhi, Punjab, Gujrat and Karnataka, 35 *streptococcal* isolates were obtained including 6 - *Streptococcus equi*, 16 *S. zooepidemicus* and 13 *S. equisimilis*. These samples, (185) were collected from apparently healthy equines, which yielded 16 isolates, while samples from clinically affected equines (126) yielded 19 isolates. These samples were collected from both organized farms and unorganized sector. However, the study

included many streptococcal species, many of which are non-pathogenic for equines (Malik & Kalra., 2009).

2.5 Routes of infection and transmission

Streptococcus equi is an obligate bacterium of equines and all infections are caused by infected horses. The most common sources of infection are nasal and abscess discharges from infected horses (Jacobs *et al.*, 2000). Horses that recover from clinical disease may have *S. equi* infection in the pharynx and guttural pouches for the months, and horses with clinically inapparent disease are sources of infection (Hansen *et al.*, 2000). *S. equi*'s environmental existence is limited, but it can survive in water and scalding for several weeks to months (Rao & Gopal Krishan., 2010). It can survive 4–6 weeks in water, but not in faeces or soil. Despite older literature claiming longer survival times in laboratory settings (Jorm., 1992), recent studies have demonstrated bacteria could not survive beyond 3 days (Weese *et al.*, 2009). *S. equi* is susceptible to bacteriocins produced by environmental bacteria and its growth is also affected by the presence of other soil-borne bacteria. Transmission of —*S. equi* occurs through contact with purulent nasal discharge from horses with active or recovering strangles. Transmission may be direct or indirect (Fig. 2.1). Direct transmission involves horse to horse contact, nose to nose touching or coughing of infected secretions into the next stall (Stringer., 2009, Quinn *et al.*, 2002).

Conversely, indirect transmission occurs through contact with contaminated environments, including shared housing areas, water sources, feeding utensils, feed, tack, and twitches, as well as less common fomites such as the clothing and equipment of caretakers and veterinarians. Clinically normal animals may also disseminate the pathogen during the incubation period or throughout the convalescent phase (Quinn *et al.*, 2002). It is increasingly evident that pathogen transmission from clinically normal animals plays a more significant role in initiating new outbreaks than transmission from overtly diseased horses. Such apparently healthy convalescent animals may continue to harbour and shed the organism even after complete clinical recovery (Quinn *et al.*, 2002).

Nasal secretions represent the primary source of *S. equi* transmission in affected animals. Consequently, horses that have clinically recovered should be regarded as potentially infectious for a minimum period of six weeks following the abscessation

of purulent nasal discharge. Intermittent shedding of *S. equi* may persist in clinically normal horses even after apparent full recovery. Such animals are classified as long-term or subclinical carriers of *S. equi*, and substantial evidence indicates that they can act as reservoirs, contributing to new or recurrent outbreaks of strangles within well-managed equine populations (Newton *et al.*, 1997).

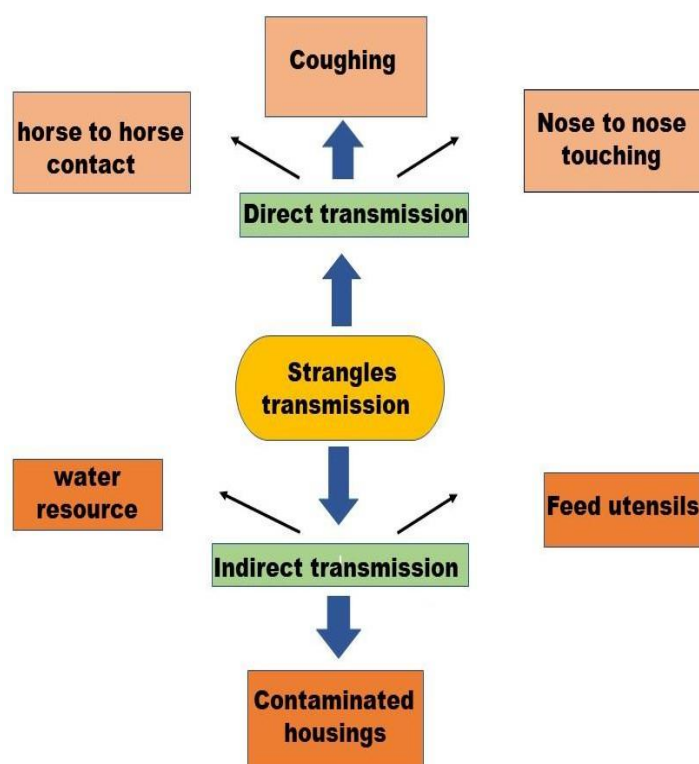


Fig. 2.1: Graphical representation of transmission of strangles through direct and indirect method. (Direct Transmission: coughing, nose to nose touching, horse to horse contact; Indirect transmission: feed utensils, contaminated housings, water resource).

2.6 Pathogenesis

During infection of a susceptible horse, *S. equi* gains entry through the nasal and oral routes and adheres to the epithelial cells lining the crypts of the palatine and lingual tonsils, as well as the follicle-associated epithelium of the pharyngeal and tubal tonsils (Timoney *et al.*, 2008). This adhesion is facilitated by several virulence-associated proteins, including surface-exposed proteins (SzPSe), fibronectin-binding proteins, M protein, and the hyaluronic acid capsule. Following colonization, the organism disseminates to the mandibular and retropharyngeal lymph nodes, where interaction

between its peptidoglycan cell wall and the host complement system leads to the generation of complement-derived chemotactic factors. These mediators recruit large numbers of polymorphonuclear neutrophils to the site of infection (Mukhtar & Timoney., 1988). The hyaluronic acid capsule, the antiphagocytic *SeM* protein, the H-factor– binding *Se18.9* protein, the Mac protein, and several other unidentified antiphagocytic components collectively enable the bacteria to evade opsonization and resist phagocytic clearance (Timoney., 2004). However, as a result of relocation of polymorphonuclear neutrophils to lymph nodes, they undergo inflammation and abscessation (Newton *et al.*, 1997) Further down the line, the pathogen metastasizes to other organs of thorax and abdomen via lymphatic or hematogenous routes causing abscess formation in their lymph nodes. The pathogen spreads after rupture of abscessed lymph nodes. When a retropharyngeal lymph node becomes abscessed, it may rupture into the guttural pouch, allowing purulent material to drain continuously through the nasal passages. In some cases, the exudate undergoes dehydration and consolidation in the guttural pouch, leading to the formation of chondroids, which can act as reservoirs for viable pathogens. They can transfer the infection to naive horses (Rao & Gopal Krishan., 2010) (Fig. 2.2). In nature, to cause the disease the inoculum of bacteria must not be less than 10 colony forming units (CFU) as a smaller number of bacteria are washed off by muco ciliary clearance. However, the infectious dose of pathogen propagated in-vitro is much higher than that required at the time of natural infection since the virulence factors necessary for initial attachment and penetration are best expressed in in-vivo propagated bacteria. The incubation period and severity of infection is directly proportional to the inoculum size. The herds that have been previously infected with strangles develop high immunity against the infection due to ongoing exposure to the pathogen present in the guttural pouch carriers. Older horses with lingering immunity, younger ones with diminishing maternal antibody protection, and vaccinated animals are less susceptible to strangles and can develop a mild variant known as catarrhal or atypical strangles. These animals shed virulent *S. equi* that produce severe disease in more susceptible, often young, horses (Sheoran *et al.*, 1997). The recovered mares produce immunoglobulin such as IgG and IgA in their milk which are similar to the IgGs observed in nasopharyngeal mucus of diseased horses (Galan *et al.*, 1987). Therefore, suckling foals get benefited from the immunity

provided by these antibodies until weaned. Antibodies present in colostrum that is fed during the initial hours after birth recirculate to the nasopharyngeal mucosa providing extra immunity to the foal during its early weeks. If the diseased animal is not subjected to any antimicrobial treatment, it is expected that 75% horses will become immune to re-infection (Hamlen *et al.*, 1994). Immunity wanes over time in the absence of re-exposure. Ironically, chronic carriers help to preserve immunity and minimize relapse of clinical disease in particular groups, despite the fact that the infection will remain endemic.

2.7 Clinical signs

The initial symptoms of equine strangles observed within 24-48 hours of infection are fever (39 °C), catarrh in upper respiratory system and depression. These symptoms occur after approximately 3–8 days of incubation period, and last for 3–4 weeks on average (Timoney *et al.*, 2000). The disease manifests itself abruptly, with thorough depression, fever, anorexia and copious and mucopurulent nasal discharge (Robinson *et al.*, 2009). Enlargement of retro pharyngeal lymph nodes and submandibular enlargement can cause oropharynx obstruction, resulting in dyspnea and dysphagia (Hardy& Leveille., 2003). Generally, the abscess formed in the retropharyngeal lymph node ruptures on the nasopharynx roof which drains into the guttural pouch and resulting in empyema (Safia., 2006). The classic equine —septic sore throat is caused by substantial swelling in the lymph nodes draining the pharyngeal area (the initial infection site), and characterized by completely swollen lymph nodes of submandibular region that commonly drains outside.

In horses, purpura hemorrhagica may be triggered by different streptococcal infections including *S. equi*. This condition is associated with leuco - cytoclastic vasculitis, head and limbs inflammation, petechial hemorrhages in mucosae, musculature and viscera (Heath *et al.*, 1991). At the time of necropsy, anatomical alterations such as infarction of the skeletal muscle, skin, gastrointestinal system, pancreas, and lungs were observed. Frequent hematologic and biochemical changes such as neutrophilia with a left shift, high serum aspartate transferase and creatine kinase levels, hyper proteinemia and hypoalbuminemia in serum are common manifestations (Dixon., 2008). Besides these changes, IgA antibodies complexed with *S. equi* specific antigens are formed due to

autoimmune vasculitis (heath *et al.*, 1991). In horses suffering from —bastard strangles, through metastatic abscesses in brain and lesions in other soft tissues, have been confirmed by magnetic resonance imaging (MRI) (Spoor makers *et al.*, 2003). Deeply placed abscesses are typically undiagnosed because they do not present any well-defined clinical symptoms. Antibiotic penetration is also insufficient to such areas due to their large size (more than 30 cm diameter) (Timoney *et al.*, 1993). In foals that had previously been infected with strangles or came in contact with infected horses, showed neurologic signs such as meningo-encephalomyelitis caused by *S. equi* (Finno *et al.*, 2006). Another reported complication is the development of suppurative necrotic bronchopneumonia as a result of aspiration of pus from burst abscesses or metastatic infection of the lungs (Dixon., 2008). Lymphadenopathy is a major clinical sign. *S. equi* infections affect both the submandibular and retropharyngeal lymph nodes equally, which become engorged and painful around a week after infection. Its initial signs include edema that is intense, diffuse, and painful. As the lymph node abscesses mature, serum may seep from the overlaying skin for several days before rupturing to drain tenacious creamy pus with no bad odour. Other rostral neck lymph nodes (parotid, cranial cervical, and retropharyngeal) are frequently implicated and may form abscesses. Retropharyngeal lymph nodes may drain in to and cause - empyema of the guttural pouch. Normally, it may take a long time for these deeper abscesses to drain to the skin and the inflammation can exert pressure on the larynx, pharynx, oesophagus and trachea leading to severe dyspnoea, dysphagia and stridor. However, retropharyngeal lymph node abscesses do not cause externally visible inflammation but periorbital abscesses cause visible puffiness in the eyelids. Due to formation of abscesses in the lymph nodes of thoracic inlet a horse may suffer from severe tracheal compression, asphyxia and in some cases death. In many cases, coughing is not a noteworthy symptom however; some horses may develop a soft, moist cough that becomes more severe with the disease progression. When the larynx is squeezed, it causes significant pain, stridor, and gagging, causing a retching cough and stretched neck position when the neck is relaxed. Empyema of the guttural pouch is indicated by the expulsion of substantial amounts of pus from the nose or mouth with coughing.

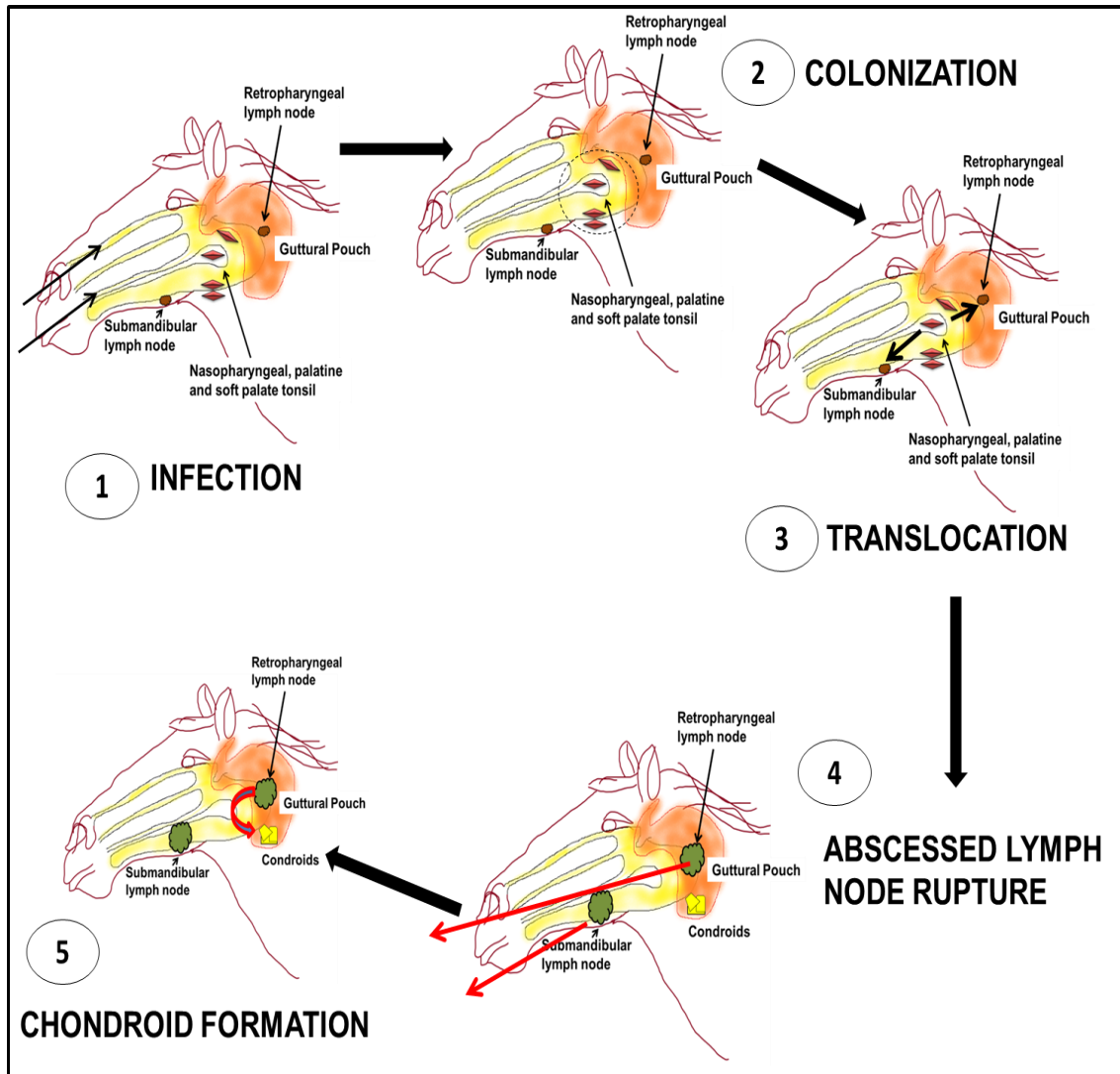


Figure 2.2: Graphical representation of *S. equi* pathogenesis.

1) *Streptococcus equi* enters the healthy horses via inhalation or ingestion. 2) The pathogen colonizes the cells of crypts of the palatine and lingual tonsils and also the follicular associated epithelium of the pharyngeal and tubal tonsils. 3) further translocates to the submandibular and retropharyngeal lymph nodes. 4) The pathogen spreads after rupture of abscessed lymph nodes. An abscessed retropharyngeal lymph node breach into the guttural pouch and from here, the abscess is emptied via continuous nasal discharge. 5) Sometimes, abscess material thickens and transforms into chondroids in the guttural pouch which can store active pathogen. They can transfer the infection to naive horses.



Fig 2.3: Discharge from the abscess of lymph gland of horse affected by Strangles (Manuja *et al.*, 2018)

2.8 Diagnosis and characterization

Strangles is presumptively diagnosed by clinical signs such as fever, mucopurulent nasal discharges, and lymphadenopathy (Chauhan *et al.*, 2001). Endoscopy is another method used to collect tissue sample and diagnose from the pouch contents, guttural pouch empyema and chondroids (Handrill *et al.*, 2002).

2.9 Bacterial culture and differentiation of β -hemolytic streptococci

To test the presence of *Streptococci* in respiratory samples (swabs and lavage fluids), the samples are cultured on blood agar plates supplemented with colistin and oxalinic acid (COBA plates). Hemolysis was also detected using blood agar. *Streptococci* bacteria are aerobic or facultatively anaerobic (Hardie & Whiley.,1995). The different species of *S. equi* can be distinguished by biochemical characterization with different sugar pellets (sorbitol, lactose, and trehalose) (Lancefield., 1933, Quinn *et al.*, 1994). Different species and subspecies of *Streptococci* can also be distinguished through Polymerase Chain Reaction (PCR) (Preziuso *et al.*, 2010, baverud *et al.*, 2007, Alber *et al.*, 2004). One more method for magnifying and detecting minute DNA quantities of different pathogens is the real-time PCR test. Amplification of *S. equi* – specific genes provide a highly useful and rapid diagnosis of *S. equi* infection (Reed *et al.*, 2004).

2.10 Genetic differentiation within the subspecies

Diversity of streptococci at subspecies level can be explored either by sequencing technique or by restriction enzyme analysis technique. The data thus obtained can be used to assess the correlation between bacteria isolated during different outbreaks (Parkinson *et al.*, 2011, Webb *et al.*, 2008, Chanter *et al.*, 1997).

2.11 Sequencing of the *SeM* protein gene

Another method to differentiate genetically related *S. equi* is by the sequencing of *SeM* gene. This gene produces an M-like protein in *S. equi* which is an important virulence factor. Therefore, this gene is probably present in all *S. equi* virulent strains (Kelly *et al.*, 2006, Anzai *et al.*, 2005). Its N-terminal region is highly variable due to selective pressure from the immune system (Waller., 2007) hence; this gene is used to distinguish *S. equi* strains during epidemiological studies (Anzai *et al.*, 2005). The only limitation of this method is that the results are not reliable in case of truncated *SeM* proteins that are sometimes observed in *S. equi* isolated from carrier horses and very rarely from horses suffering from clinical strangles (Chanter *et al.*, 2000).

2.12 Multi - locus sequence typing (MLST)

As an alternative to strain differentiation based solely on sequence variation within the highly variable *SeM* gene, multi- locus sequence typing (MLST) provides a more robust and reliable approach for comparing *S. equi* isolates. In 2008, Webb and colleagues developed an MLST scheme for *S. equi* and *S. zooepidemicus*, utilizing seven conserved housekeeping genes to ensure stable and reproducible strain characterization (Webb *et al.*, 2008). After comparing the sequences of these housekeeping genes to already deposited gene sequences, an online database assigns sequence type (ST) to them (Jolley *et al.*, 2004). Its advantage over other techniques is that its rate of reproducibility is high and it can also compare isolates studied in different laboratories and at different times. However, the technique is somewhat expensive and labour intensive. Also, its ability to differentiate some subspecies is limited for example, through MLST, *S. equi* is divided into 5 STs whereas through *SeM* typing, *S. equi* falls into 128 different types.

2.13 Hematological and biochemical alterations

Strangles infection in horses can cause various hematological and biochemical alterations. According to Neamat-Allah *et al.*, Hematological changes observed in *S. equi* infected horses include a decrease in erythrocyte count, leading to decreased hemoglobin concentrations and packed cell volume (PCV), along with an increase in total leukocyte count (TLC) due to neutrophilia. The authors hypothesized that the suppression of erythropoiesis may be due to iron unavailability for hemoglobin synthesis as ferrous ions release from reticuloendothelial storage is inhibited after infection. Biochemical changes include an increase in total proteins and serum globulins levels due to hyperglobulinemia, a decrease in serum albumin levels, and an increase in aspartate aminotransferase (AST) levels due to local myositis resulting in an open abscess in the infected horse. The disease may also result in respiratory acidosis, leading to a higher respiratory rate in horses. Strangles can also cause a decrease in serum sodium levels and an increase in serum potassium levels, resulting in hyperkalemia and acidosis, which may cause cardiac arrhythmias (Ismail *et al.*, 2013). Zinc levels may also decrease in infected horses, potentially disturbing the immune system (Salem., 2017). The increase in serum globulins levels may be due to increased immunoglobulins in response to bacterial infection, while the decrease in serum albumin levels may be a result of lowered feed intake by the animal during infection. In summary, strangles infection in horses can cause various hematological and biochemical alterations, including changes in erythrocyte count, Hb concentrations, TLC, total protein, serum globulins, serum albumin, AST, sodium, potassium, zinc, and acid-base status.

2.14 Epidemiological studies in preventive measures for the disease

Epidemiological research underscores that asymptomatic carriers, particularly horses with guttural pouch infections, are key to sustaining transmission. Mathematical models show that unless these carriers are identified and removed from contact with susceptible animals, outbreaks will continue—even when clinical cases are isolate (Duran & Goehring., 2021, Waller *et al.*, 2014).

Epidemiologic data show that to prevent strangles, you must find and manage silent carriers, not just obvious cases. Sensitive diagnostics like qPCR and iELISA have

revolutionized detection and vet-driven decision making. These tools make it possible to identify both symptomatic and carrier animals early—and act accordingly (Chhabra *et al.*, 2023). On the farm, this knowledge is translated into solid biosecurity: quarantine, isolation, and rigorous cleaning. Vaccines help reduce severity, but must be used thoughtfully—never during an outbreak or in horses with high antibody titers. A consistent communication framework, weekly monitoring, and targeted surveillance complete the picture. When applied together, these epidemiology-driven measures form a robust, proactive shield against strangles outbreaks. Epidemiology also gives rise to the - four-ates strategy: Vaccinate, Isolate, Investigate, and Communicate. This approach emphasizes coordinated surveillance, prompt case detection, and clear communication among handlers, veterinarians, and owners especially during outbreaks. It underpins structured outbreak response systems such as traffic-light groupings (clinical vs exposed vs unexposed animals) to prevent spread while continuing essential activity vac (Waller., 2014).

Chapter 3

Hypothesis

Strangles is a highly infectious and serious disease affecting horses, primarily targeting the upper respiratory system and lymph nodes. It is caused by the bacterium *Streptococcus equi* subsp. *equi*, which is a Gram-positive, beta-hemolytic organism classified within Lancefield group C. The disease typically presents with high rates of illness and low rates of death, particularly impacting younger horses who show more severe clinical signs, while older horses often display milder symptoms due to their developed immunity. Common clinical manifestations include fever, nasal discharge, inflammation of the throat, and the formation of abscesses in the mandibular and retropharyngeal lymph nodes, which can cause airway blockage - leading to the term “strangles.” The disease spreads through direct contact with infectious fluids or indirectly through contaminated objects. Molecular techniques such as PCR have emerged as rapid and reliable diagnostic tools, capable of detecting *S. equi* even in non-viable states, while serological assays like *SeM*-protein ELISA are useful in identifying carrier animals. In many countries, including India, strangles is considered endemic, often re-emerging due to increased horse movements and insufficient surveillance. Despite its importance, limited data are available on the epidemiology of strangles in India. There is complete lack of systematic surveillance to estimate the prevalence of disease. No study has been conducted to determine the carrier status of equines in India. Therefore, it is hypothesized that *S. equi* subsp. *equi* remains underdiagnosed in the country due to the constraints of conventional diagnostic methods, and that the true prevalence—including asymptomatic carriers—can be more accurately determined using molecular and serological tools such as “multiplex real-time PCR” and *SeM*-based ELISA. This study will aim to explore the epidemiological status of strangles in northern India using these advanced diagnostic approaches, thereby contributing to the development of effective disease control and prevention strategies.

Accordingly, the following hypotheses were formulated:

Null Hypothesis (H₀):

There is no significant occurrence of *Streptococcus equi* subsp. *equi* infection or carrier state among the equine population of northern India, and the use of molecular and serological diagnostic methods does not significantly improve the detection of infection or carrier status compared with conventional diagnostic methods.

Alternative Hypothesis (H₁):

Streptococcus equi subsp. *equi* is prevalent among the equine population of northern India, including asymptomatic carrier animals, and the combined use of multiplex real-time PCR and SeM protein-based ELISA significantly improves the detection of infection and carrier status compared with conventional diagnostic methods, thereby providing a more accurate understanding of the epidemiology of strangles.

Chapter 4

Research Objectives

- Isolation and characterization of the causative organisms from clinical cases of strangles in equines.
- Sero-surveillance of equine population to determine the prevalence of the disease.
- To detect the carrier state after clinical recovery based on bacterial culture, qPCR and serum antibodies.

Chapter 5

Material and Method

5.1 Ethical Compliance

All animal handling and sampling procedures were performed in strict accordance with the guidelines of the - Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The - Institutional Animal Ethics Committee (IAEC) granted ethical clearance. Before being included in the study, all horse owners gave their informed consent. Every attempt was made to reduce the discomfort to the animals while they were being sampled.

5.1.1 Study Location

The research was conducted across seven northern states of India, each with significant equine populations and diverse agro - climatic conditions:

1. **Jammu and Kashmir:** Characterized by temperate climates, this region has a substantial population of equines, including indigenous breeds adapted to hilly terrains.
2. **Himachal Pradesh:** Known for its mountainous landscapes, equines here are primarily used for transportation in rugged terrains.
3. **Punjab:** With its fertile plains, equines are often employed in agriculture and transportation.
4. **Haryana:** This state has a mix of rural and urban areas where equines serve both agricultural and commercial purposes.
5. **Uttar Pradesh:** One of the most populous states, it hosts numerous animal's fairs and has a high density of equine populations, making it a critical area for studying disease transmission dynamics.
6. **Uttarakhand:** Featuring hilly terrains, equines are essential for transportation and tourism-related activities.
7. **Rajasthan:** Known for its arid climate, equines here are integral to cultural events and traditional transportation methods.

These states were selected due to their substantial equine populations and the frequent movement of animals for work, trade, and cultural events, which can facilitate the spread of infectious diseases like strangles.

Geographically, the northernmost extent of India reaches approximately 37°N latitude, encompassing parts of the Jammu and Kashmir region. The selected states span a diverse range of geographical coordinates. For example, Haryana lies between latitudes 27°39'N to 30°35'N and longitudes 74°28'E to 77°36'E. Uttar Pradesh is situated around latitude 27°40'N and longitude 80°00'E. Rajasthan extends approximately to latitude 27°00'N and longitude 74°00'E. Punjab is located at approximately 30°04'N latitude and 75°05'E longitude. The coordinates for Uttarakhand are around 30°15'N latitude and 79°15'E longitude, while Himachal Pradesh is positioned at approximately 32°29'N latitude and 75°10'E longitude.

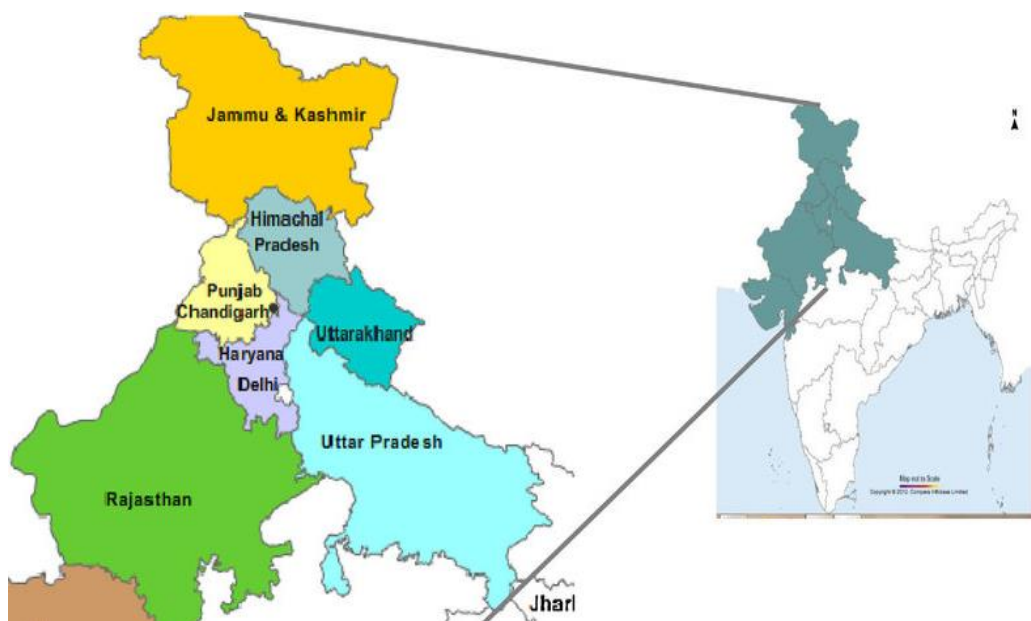


Fig. 5.1: Map showing the seven states of northern India covered under epidemiological studies for strangles

5.2 Equine Population Statistics in Northern India

Equines—comprising horses, donkeys, and mules—have been integral to India's socio-economic fabric for centuries. Their multifaceted roles span agriculture, transportation, cultural traditions, and livelihoods, particularly in rural and semi-urban areas. According to the 20th Livestock Census (2019), the national equine population was recorded as follows:

Table 5.1: State-wise Details of Horse & Ponies by Age, Sex & Use (Rural +Urban)

		Male under 3 years and above				Female under 3years and above				
	State/Union Territory	Under 3 years	Used for cart	Used for sport	Total male	Under 3 years	Used for cart	Used for sport	Total female	Total horse and ponies
1	Jammu and Kashmir	11796	11033	2988	25817	10186	25273	2053	37518	63335
2	Haryana	962	261	171	1394	2506	4706	1077	8289	9683
3	Punjab	1763	249	614	2626	4080	4732	2805	11617	14243
4	Uttar Pradesh	15441	1728	3361	20530	21242	29406	4540	55188	75718
5	Rajasthan	4030	609	1574	6213	11995	11905	3563	27466	33679
6	Uttarakhand	1767	753	206	2726	1001	3600	125	4726	7452
7	Himachal Pradesh	1472	1511	88	3071	998	4706	76	5780	8851

Table 5.2: State-wise Details of Mules and Donkeys by Age & Sex (Rural +Urban)

		Mules			Donkeys						
					Male			Females			
S. No.	State/Union Territory	Under 3 years	3 years and above	Total mules	Under 3 years	3 years and above	Total males	Under 3 years	3 years and above	Total female	Total donkeys
1	Jammu and Kashmir	3156	13566	16722	1013	5010	6023	970	2570	3540	9563
2	Haryana	656	1843	2499	148	204	352	156	292	448	800
3	Punjab	486	1158	1644	76	160	236	78	157	235	471
4	Uttar Pradesh	3556	5377	8933	3422	4610	8032	3578	4406	7984	16016
5	Rajasthan	358	981	1339							
6	Uttarakhand	3452	22841	26293	53	307	360	49	180	229	589
7	Himachal Pradesh	2690	17725	20415	519	2658	3177	311	1309	1620	4797

5.3 Sample size

Sample size to determine a proportion or apparent prevalence with specified precision

The random samples were collected from organized farms. The sample size for different herds was determined using online epitools (<https://epitools.ausvet.com.au/samplesize>). This utility calculated the sample size required to estimate a prevalence) with a specified level of confidence and precision.

$$n_h = \frac{N_h}{N} n$$

n_h = Sample size using proportionate stratified random sampling

N_h = Total Stratum population

N = Total Population

n = Sample size

5.4 Formulae used in the study

No. of samples taken from each state = State wise population/total no. of population x sample size (<https://home.iitk.ac.in/~shalab/sampling/chapter4-sampling-stratified-sampling.pdf>)

5.5 Random sampling Strategy.

Epidemiological and Clinical Data Sheet for Strangles Surveillance and Case Recording Template. Suspected Strangles Cases.

In the study titled a structured data collection form was employed to systematically record information from equine owners and their animals. This form was divided into two primary sections: -

1. **Owner's Details:** This section captured the owner's name, address, and mobile number. Collecting this information was crucial for several reasons:

- Traceability: In the event of a disease outbreak, having accurate owner information allows for efficient contact tracing and implementation of control measures.
- Follow-up: It facilitates subsequent communication for additional data collection, dissemination of test results, or providing guidance on treatment and biosecurity measures.
- Data Validation: Ensures the authenticity of the data collected and aids in cross-referencing with veterinary records.

2. Particulars of Animal: This section documented specific details about each equine, including:

- Name: Identification of the animal.
- Age: Age can influence susceptibility to diseases like strangles.
- Sex (M/F): Sex-based differences may affect disease prevalence and progression.
- Breed: Certain breeds may have varying levels of susceptibility or resistance to specific pathogens.
- Colour: While primarily for identification, coat colour can sometimes be associated with breed-specific traits.
- Symptoms: Recorded clinical signs such as nasal discharge, fever, coughing, and lymph node swelling is essential for diagnosing strangles and assessing disease severity.

By systematically collecting this information, we aimed to establish a comprehensive database to analyse the epidemiology of strangles in the region. This data facilitated the identification of risk factors, assessment of disease prevalence across different demographics, and evaluation of the effectiveness of existing control measures. Moreover, it provided insights into the patterns of disease spread, aiding in the development of targeted interventions to mitigate future outbreaks.

Table 5.3: Represents the Performa of samples collection

Performa for Collection of Data

Sr No.	Owners Details			Particular of Animal					
	Name	Address	Mobile no.	Name	Age	Sex M/F	Breed	Colour	Symptoms
1.									
2.									
3.									
4.									
5.									
6.									
7.									
8.									
9.									
10.									

Table 5.4: Table of to be collected samples from each state

States	State wise population	Sample size	No of samples collected from each state
Jammu and Kashmir	89620	200	56
Haryana	12982	200	8
Punjab	16358	200	10
Uttar Pradesh	100667	200	62
Rajasthan	35018	200	21
Uttarakhand	34334	200	21
Himachal Pradesh	34053	200	22
Total	323032	200	200







अश्वों के लिए लगाया स्वास्थ्य कैंप



हिसार | राष्ट्रीय अश्व अनुसंधान केंद्र द्वारा गांव पाबड़ा में किसान दिवस मनाया गया। आईसीएआर ने पाबड़ा गांव में अश्व और पशुपालकों के लिए एक स्वास्थ्य शिविर का आयोजन किया। एनआरसीई टीम ने अश्व और पशुपालकों को लाभ पहुंचाने के लिए संस्थान द्वारा विकसित विभिन्न गतिविधियों और प्रौद्योगिकियों के बारे में जानकारी दी। स्वास्थ्य शिविर में लगभग बारह घोड़ों और पशुधन मालिकों ने भाग लिया और उनके स्वास्थ्य की स्थिति का आंकलन करने के लिए घोड़ों और अन्य पशुओं की जांच की गई।

Fig 5.2: Showing Sample Collection Images of Equines of Northern India.

5.5 Isolation and characterization of the causative organisms from clinical cases of strangles in equines.

The tests were standardized on available isolates/cultures in the laboratory and performed on collected field samples proposed in the research work.

5.5.1 Sample Collection

Clinical specimens were collected from healthy equines as well as from unhealthy equines exhibiting signs consistent with strangles, such as fever, nasal discharge, anorexia lymphadenopathy, and abscess formation and also from in contact apparently healthy animals. The types of samples collected included:

- Nasal swabs: Sterile cotton swabs were used to collect nasal swabs from the anterior nares.
- Abscess aspirates: Using aseptic techniques, pus was aspirated from enlarged submandibular or retropharyngeal lymph nodes.

All samples were transported to the laboratory under refrigerated conditions and processed within 24 hours of collection.

5.5.2 Bacterial Isolation and Culture

Samples were inoculated on 5% sheep blood agar plates and incubated aerobically at 37°C for 24–48 hours. Colonies exhibiting beta-haemolysis and characteristic morphology were sub cultured for purity.

5.5.3 Identification of Isolates

a. Morphological and Biochemical Characterization

- Gram Staining: Performed to confirm Gram-positive cocci morphology.
- Catalase Test: Negative catalase reaction confirmed the genus *Streptococcus*.
- Sugar Fermentation Tests: Conducted using glucose, maltose, sucrose, lactose, sorbitol, mannitol, and arabinose to determine fermentation profiles.

Principle

Gram staining is a differential staining technique that helps to classify the bacteria into Gram-positive and Gram-negative groups based upon structural differences in their cell

walls. Gram-positive bacteria possess a thick peptidoglycan layer, which retains the crystal violet-iodine complex, appearing purple under a microscope. On the other hand, Gram-negative bacteria have a thinner peptidoglycan layer and an outer membrane, which does not retain the crystal violet-iodine complex after decolorization, resulting in a pink appearance due to the counter stain.

Materials Required

- Clean glass slides
- Inoculating loop
- Bunsen burner
- Crystal violet stain (primary stain)
- Gram's iodine solution (mordant)
- Decolorizing agent (95% ethanol or a mixture of ethanol and acetone)
- Safranin solution (counterstain)
- Distilled water
- Bibulous paper or blotting paper
- Microscope with oil immersion objective lens

Procedure

1. Preparation of Bacterial Smear:

- Placed a small drop of sterile distilled water on a clean glass slide.
- Using a sterile inoculating loop, transferred a small amount of the bacterial culture (from a colony or clinical sample) to the water drop and spread to form a thin smear.
- Allowed the smear to air-dry completely.

2. Heat Fixation:

- Once dry, passed the slide (smear side up) through the flame of a Bunsen burner 2–3 times to fix the bacteria onto the slide. This process killed the bacteria and adhered them to the slide.

3. Primary Staining:

- Flooded the fixed smear with crystal violet stain and let it sit for 60 seconds.
- Gently rinsed the slide with distilled water to remove excess stain.

4. Mordant Application:

- Covered the smear with Gram's iodine solution and allowed it to act for 60 seconds. The iodine binds with the crystal violet, forming a complex that is more firmly trapped in the cell wall.
- Rinsed the slide gently with distilled water.

5. Decolorization:

- Applied the decolorizing agent (95% ethanol or ethanol-acetone mixture) to the smear for approximately 10–30 seconds.
- Immediately rinsed the slide with distilled water to halt the decolorization process.

6. Counterstaining:

- Flooded the smear with safranin solution and let it sit for 60 seconds. This counterstain imparts a pink color to Gram-negative bacteria.
- Rinsed the slide gently with distilled water and blot dried with bibulous paper.

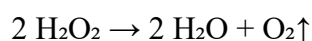
7. Microscopic Examination:

- Placed a drop of immersion oil on the stained smear and examined under a microscope using the 100× oil immersion objective lens.
- Gram-positive bacteria retain the crystal violet–iodine complex during the Gram staining process, resulting in a purple coloration, whereas Gram-negative bacteria do not retain this complex and instead take up the safranin counterstain, appearing pink under microscopic observation.

5.5.4 Catalase Test Procedure

- **Principle**

The catalase test detects the presence of the enzyme catalase in bacterial organism. Catalase catalyses the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen reference.



The rapid formation of oxygen bubbles upon the addition of hydrogen peroxide to a bacterial culture indicates a positive catalase reaction. This test is instrumental in differentiating catalase-positive organisms (e.g., *Staphylococcus* spp.) from catalase-negative organisms (e.g., *Streptococcus* spp.), including *S. equi* subsp. *equi*.

Materials and Reagents required

- Fresh bacterial culture (18–24 hours old)
- 3% hydrogen peroxide solution (H_2O_2)
- Clean glass microscope slides
- Sterile wooden applicator sticks or platinum loops (avoid iron-containing metal loops to prevent false-positive reactions)
- Personal protective equipment (gloves, lab coat, safety goggles)

Procedure

1. Preparation of the Slide:

- Placed a clean, dry glass slide on a flat surface.

2. Application of Bacterial Sample:

- Using a sterile wooden applicator stick or platinum loop, transferred the small amount of the bacterial colony on to the slide, creating a thin smear.
- Ensured that the smear was not too thick to allow for clear observation of bubble formation.

3. Addition of Hydrogen Peroxide:

- Placed 1–2 drops of 3% hydrogen peroxide directly onto the bacterial smear.

4. Observation:

- Immediately observed the slide for the appearance of oxygen bubbles.
- A positive reaction indicated by rapid and sustained bubbling.
- A negative reaction showed no or minimal bubbling

5.5.5 Biochemical Characterization

Biochemical procedure was performed with two methods:

- **Phenol red base agar with sugar pellets**

Biochemical characterization was performed on available cultures in the laboratory and field samples using phenol red base agar supplemented with specific sugar pellets for fermentation analysis. Bacterial colonies were selected from agar plates and inoculated into Todd Hewitt Broth (THB) for culture preparation. Agar plates were prepared by pouring solidified phenol red base medium into sterile petri dishes. Once the medium had solidified and dried, bacterial cultures were evenly spread onto the agar surface. Sugar pellets were then placed onto the plates, which were incubated at 37°C for 24 hours. Fermentation results were recorded based on color changes in the phenol red medium, indicating acid production. The fermentation assay was conducted using phenol red base agar with the following sugar substrates: maltose, sorbitol, lactose, and trehalose. The ability to ferment sorbitol and lactose was indicative of *S. zooepidemicus*, while fermentation of maltose was used to identify *S. equi* at the subspecies level.

- **Hi strep™ Identification Kit**

Material required for the procedure

1. **Inoculation loops, pipettes**
2. **Enrichment medium / Isolation media**

Preparation of inoculum

The suspected organism was initially cultured on Columbia Blood Agar to obtain pure growth. A single, well-isolated colony was selected and inoculated into 5 ml of Brain Heart Infusion (BHI) broth (M210). The inoculated broth was incubated at 35–37°C for 4–6 hours until the turbidity of the suspension reached 0.1 optical density (OD) at 620 nm, ensuring an appropriate bacterial inoculum for further testing. In cases where the required turbidity was not achieved within 6 hours, incubation was continued until the inoculum reached the desired 0.1 OD at 620 nm. The KB005A kit was opened under aseptic conditions and the sealing foil was carefully removed. Each well of the test strip was inoculated with 50 µl of the prepared bacterial suspension using the surface

inoculation technique. Alternatively, inoculation could be performed by gently introducing a loopful of the bacterial suspension into each well. After inoculation, the kit was incubated at $35 \pm 2^{\circ}\text{C}$ for a period of 18–24 hours. Following incubation, the biochemical reactions were recorded and interpreted according to the identification index provided by the manufacturer. Specific reagents were added to well numbers 1 and 3 at the end of the incubation period (18–24 hours) as per the manufacturer's instructions to obtain the final reaction results.

Table 5.5: Identification kit used for differentiation of *Streptococcus* species with twelve conventional Biochemical tests.

KB005A

Tests	Species	Species	Species
	<i>Streptococcus equi</i> <i>subspecies equi</i>	<i>Streptococcus equi</i> <i>subspecies</i> <i>zooepidemicus</i>	
Voges Proskauer's	-	-	
Esculin hydrolysis	-	-	
PYR	-	-	
ONPG	-	-	
Arginine	+	+	
Glucose	+	+	
Lactose	-	+	
Arabinose	-	+	
Sucrose	+	+	
Sorbitol	-	+	
Mannitol	-	-	
Raffinose	-	-	

These tests aided in differentiating *S. equi* subsp. *equi* from other closely related species

5.6 Sero-surveillance of equine population to determine the prevalence of the disease.

The study was conducted in various equine-dense regions of northern India, including Jammu & Kashmir, Punjab, Haryana, Uttarakhand, Uttar Pradesh and Himachal Pradesh. Horses of different age groups, breeds, and sexes from organized farms, riding clubs, and rural set ups were included. A total of 200 clinical samples were collected across seven Indian states to assess the distribution and potential incidence of *Streptococcus equi*, a key pathogen responsible for equine strangles. The highest number of samples (62/200, 31.0%) were collected from Uttar Pradesh, which has the largest equine population among the surveyed states (100,667 animals, 31.2% of total population). This reflects appropriate targeting of surveillance relative to population density. Jammu & Kashmir, despite having the second-highest equine population (89,620; 27.7% of total), also contributed significantly with 56 samples (28.0%), aligning well with its equine distribution. Haryana, with a comparatively small equine population (12,982; 4.0%), contributed 8 samples (4.0%), reflecting a balanced sampling effort relative to its equine base. From Punjab, we collected 10 samples (5.0%) from a population of 16,358 equines (5.1%), showing consistent proportional representation. Rajasthan, with 35,018 equines (10.8%), contributed 21 samples (10.5%) - very close to proportional representation. Uttarakhand and Himachal Pradesh showed similar trends, each contributing 21 (10.5%) and 22 samples (11.0%), respectively, from equine populations of 34,334 (10.6%) and 34,053 (10.5%). Overall, the sample distribution appears proportional to state-wise equine populations, with Uttar Pradesh and Jammu & Kashmir appropriately prioritized due to their higher animal densities. These sampling efforts are representative and suitable for drawing meaningful epidemiological conclusions about the distribution of *S. equi* infection across major equine belts in northern India.

5.6.1 Study Design

A cross-sectional sero-surveillance study was conducted from [November 2022] to [July 2025] to estimate the seroprevalence of *S. equi* infection among equines. Sampling was stratified to ensure representation across various geographical and management conditions.

5.6.2 Sample Size Estimation

The sample size was calculated using the following formula:

$$n = \frac{Z^2 \times P(1-P)}{d^2}$$

Where:

- n = required sample size
- $Z = 1.96$ (at 95% confidence level)
- P = expected prevalence (based on prior studies, e.g., 10–20%)
- d = desired precision (typically 5%)

Based on this, a total of 200 equines were sampled.

5.6.3 Sample Collection

- Blood samples (approx 10 ml) were collected aseptically from the jugular vein using sterile tubes.
- Samples were labelled, kept on crushed ice, and transported to the laboratory within 4–6 hours.

5.6.4 Serum Isolation

- Collected whole blood (approx. 10ml) in a centrifuge tube.
- After collection of the blood sample, allowed the blood to clot by leaving it undisturbed at room temperature for 15-30 minutes.
- Removed the clot by centrifuging at 4500x g for 15 minutes in a centrifuge.
- The resulting supernatant was serum.
- The serum samples were maintained at 2-8°C while handling.

5.7 Serum Dilution Protocol for ELISA

5.7.1 Materials Needed:

- Equine serum samples
- ELISA diluent (as specified by the kit, e.g., PBS, BSA, or proprietary buffer) kit
- Calibrated micropipettes and sterile tips
- Sterile microcentrifuge tubes

5.7.2 Dilution Steps:

- Labelled a microcentrifuge tube appropriately.
- Added 1 μL of the equine serum into the tube.
- Added 100 μL of the ELISA diluent buffer to the same tube.
- Mixed thoroughly by gently vortexing or pipetting up and down to ensure homogeneity.

5.7.3 Wash Buffer Preparation

Allowed the **Wash Concentrate (20X)** to reach room temperature (approximately $21 \pm 5^\circ\text{C}$).

Mixed thoroughly to ensure complete solubilization.

5.7.4 Diluted the Concentration

- Prepared the 1X wash solution by diluting the 20X concentrate at a 1:20 ratio with distilled or deionized water.
- Mixed 500 μL of the 20X concentrate with 9500 μL of distilled or deionized water to make 10ml of 1X Wash Solution.

5.7.5 Conjugate Composition and Storage

- The kit provided an **anti-horse IgG–HRP conjugate** at **10X concentration**.
- Stored the conjugate at **2–8 °C** and protected from light.
- Bought the conjugate to **room temperature** ($21 \pm 5^\circ\text{C}$) before dilution to avoid precipitation and ensure uniform activity.

5.7.6 Preparation of 1X Conjugate

- Pipetted 100 μL of the 10X conjugate into the tube.
- Added 900 μL of the provided dilution buffer (often called “Dilution Buffer 3” or similar) to achieve a 1:10 dilution (resulting in 1 mL of 1X conjugate).
- Mixed gently by inverting 5–10 times or by gentle pipetting.
- Used the prepared conjugate immediately;

5.8 Serological Testing

The serological analysis was carried out using an indirect enzyme-linked immunosorbent assay (ELISA) method, specifically targeting antibodies against the SeM protein of *Streptococcus equi* subsp. *equi*. All reagents and materials were equilibrated to room temperature before use. Serum samples were diluted appropriately and incubated with coated antigen wells, followed by washing and the addition of conjugated secondary antibodies. A commercially available ELISA kit (ID vet - ID Screen *Streptococcus equi* Indirect Strangles ELISA Kit, Innovative Diagnostics, France) was used for this purpose. The assay was conducted according to the manufacturer's protocol.

5.8.1 Procedure of ELISA.

5.8.2 Sample and Control Addition

Dispensed **100 µL** of:

- Negative control (duplicate wells)
- Positive control (duplicate wells)
- Test sera into designated wells.

5.8.3 First Incubation

- Incubated for **45 minutes at room temperature (21 ± 5 °C)**.

5.8.4 Washing

- Washed each well **3 times** with **1X wash solution** (e.g., 300 µL/well).
- Removed all residual liquid after the final wash (tap dry or blot).

5.8.5 Conjugate Addition

- Added **100 µL** of freshly prepared **1X HRP-conjugated anti-horse IgG** to each well.

5.8.6 Second Incubation

- Incubated for **30 minutes at room temperature (37°C)** and protected from direct light.

5.8.7 Washing

- Washed wells 3–5 **times** with **1X wash solution**.
- Ensured no residual liquid remains.

5.8.8 Substrate Addition

- Added 100 µL of “**TMB substrate**” to each well.
- Incubated for 15–20 minutes at room temperature in the dark.

5.8.9 Stopping the Reaction

- Added **100 µL** of **stop solution** to each well.

5.8.10 Reading the Results

- Read absorbance at “450 nm” using an ELISA plate reader within 30 minutes of stopping the reaction.

5.8.11 Interpretation of the Results.

- Calculated **S/P ratios** or use **OD cut-off values** according to the kit manual.
- Results were compared to positive and negative controls to determine the presence of antibodies against *S. equi*.
 - **Positive**
 - **Doubtful**
 - **Negative**

5.8.12 Data Management and Statistical Analysis

All data were entered into Microsoft Excel spreadsheets and subjected to statistical analysis using SPSS Version/Graph Pad software

- Descriptive statistics were used to calculate the overall seroprevalence and stratified prevalence by age, sex, breed, and management system.
- Chi-square test was employed to determine associations between categorical variables and seropositivity.
- Multivariable logistic regression was applied to identify potential risk factors associated with strangles seropositivity, with p-values < 0.05 considered statistically significant.

5.8.13 Data Analysis

- Seroprevalence was calculated as the percentage of horses testing positive out of the total tested.
- Statistical analysis was performed using software such as Graph Pad software
- Associations between seropositivity and variables like age, sex, breed, and management practices were analysed using chi-square or logistic regression tests.
- A p -value of <0.05 was considered statistically significant.

5.9 To detect the carrier state after clinical recovery based on bacterial culture, qPCR and serum antibodies.

5.9.1 Study Design and Rationale

This longitudinal observational study was designed to detect and characterize the carrier state of “*Streptococcus equi* subsp. *equi*” in horses that had clinically recovered from strangles. The research incorporated bacteriological culture, conventional PCR quantitative real-time PCR (qPCR), and serological antibody profiling to assess microbial persistence and immune response. This multi-modal approach was chosen to improve detection sensitivity and specificity across different phases of pathogen carriage.

5.9.2 Study Location and Animal Enrolment

The study was conducted in equine-dense regions of Northern India, encompassing veterinary hospitals, stud farms, riding schools, and community stables across states such as (Jammu & Kashmir, Himachal Pradesh, Punjab, Haryana, Uttar Pradesh, Uttarakhand and Rajasthan) Horses were enrolled between [November 2022] and [July 2025] based on the following inclusion criteria.



Fig 5.3: A surveillance plan was developed for the systematic sampling and analysis of epidemiological data across organized and unorganized livestock farms in seven northern Indian states. Random sampling was conducted using a web-based randomization tool (<https://www.randomizer.org/links/>).

A total of 200 equines were enrolled for carrier detection analysis.

5.9.3 Sampling Strategy

Nasopharyngeal Swabs

Sterile rayon-tipped swabs were inserted via the ventral nasal meatus to reach the nasopharynx. Swabs were rotated gently for 10 seconds to collect epithelial secretions, then immediately immersed in Amies transport medium and transported to the laboratory at 4°C.

5.9.4 Carrier Status

A total of 200 equines were enrolled for carrier detection analysis.

5.9.5 Bacteriological Culture

Nasopharyngeal samples were inoculated onto 5% sheep blood agar and colistin-nalidixic acid agar plates. Plates were incubated aerobically at 37°C for 24–48 hours.

Suspected colonies exhibiting β -haemolysis and characteristic morphology were subjected further confirmed by PCR targeting the *SeM* gene.

5.9.6 DNA Extraction and qPCR

DNA extraction was done using boil-chill & commercial kit method (Zymo Research, USA). Nasal swab was dipped to a new, sterile vial containing 200 μ l nuclease free water and boiled for 10 minutes in water bath and chilled at -20°C. After chilling, 2 μ l of supernatant was used as a template either immediately for real time PCR reaction and rest stored at -20°C (Zu *et al.*, 2024).

- DNA was extracted from the suspected bacterial *Streptococcus* cultures stored in glycerol supplemented Todd Hewitt Broth (THB). The Isolates were sub cultured twice on 5% Columbia blood agar before DNA isolation. A loopful of bacterial colony was suspended in 5 ml of THB supplemented with 2% of horse serum incubated at 37°C for bacterial growth. DNA was isolated by using the Quick bacterial and fungal DNA extraction Kit (Zymo research) according to the manufacturer's instructions.
- The isotonic buffer (phosphate buffer saline) was added to the pellet in amount of 200 μ l.
- The pellet was vortexed and allowed to stand for 2 min.
- The amount of 700 μ l of washing bead buffer was added in the tube and tightly closed it.
- All the material were transferred in to the ZR bashing bead lysis tube.
- Vortexed them again and allowed to stand for 5 to 10 min.
- Centrifuged the ZR Bashing bead Lysis tube in a micro centrifuge at 10,000g for 1 min.
- The supernatant (800 μ l) was taken in to the zymo spin III filter in a collection tube.
- Centrifuged it at 8000g for 1 min and discarded the spin III filters.
- Genomic lysis buffer was added to the supernatant in a quantity of 1200 μ l.

- The Mixture (800 µl) approx. was transferred in to zymo spin 1C column and centrifuged at 10,000 g for 1 min.
- Discarded the flow through out and repeated the above step.
- DNA pre wash buffer was added in zymo spin 1C column in a collection tube in a volume of 200 µl.
- Centrifuged the tube again at 10,000 g for 1 min.
- Discarded the flow through.
- DNA wash buffer was added to Zymo spin 1C approx. 500 µl.
- Centrifuged again at 10,000 g for 1 min.
- Transferred to zymo spin 1C in to 1.5 ml microfuge tube.
- The amount of DNA elution buffer was added.
- The tube was incubated for 5 min at room temperature.
- The suspension was centrifuged at 10,000 g (Spin win MC03 TARSONS) for 1 min and the eluted DNA was used as template in the quantitative PCR reactions, and stored at -20°C .

5.9.7 Quantity and Quality of DNA sample

The quality and quantity of DNA was determined by Bio spec - nano spectrophotometer (Pico drop, life science) by taking the absorbance at 260 nm and 280 nm. DNA samples with A260/A280 ratios ranging from 1.7 to 1.8 were considered of sufficient purity and selected for further experimentation. To confirm further, 5 µl of each sample was electrophoresed on a 1 % of agarose gel in TAE buffer containing 0.5 µg/ml. of ethidium bromide.

5.9.8 PCR Amplification with *ICESe2* and *SeM* gene.

Polymerase chain reaction was performed with different primers of *SeM* gene to amplify the fragment of different base pairs. PCR was performed with *SeM* gene primers according to (Kelly *et al.*, 2006). The primers were ASW73 forward primer and ASW74 reverse primer and the procedure was done and to amplify the *SeM* gene of 541bp. The PCR reaction was performed according to (Kelly *et al.*, 2006) with a final

volume of 25 µl which contained the 12.5 µl of green dream Taq which was a master mix 1 µl of forward Primer and 1 µl of reverse primer and 8.5 µl nuclease free water and 2 µl of DNA template and the amplification was performed with following Conditions, 35 cycles denaturation for 5 min at 95°C , 30 sec at 95°C , 55°C at 30 sec annealing temperature and extension was done for 40 sec at 72 °C and the final extension of 5 min at 72° C (Libardoni *et al.*,2013)

Table 5.6: Cycling conditions for PCR for *SeM* gene

Cycling Steps	<i>SeM</i> GENE
Initial DNA denaturation	95°C for 5 min.
DNA denaturation	95°C for 30 sec
Primer annealing	55°C for 30sec.
DNA extension	72°C for 40sec.
Number of cycles	35
Final extension	72°C for 5 min.

Table 5.7: List of primers or oligonucleotides for detection of *SeM* gene and ICESe2 (Libardoni *et al.*, 2013 and Cordoni *et al.*, 2016)

PRIMER	NUCLEOTIDE SEQUENCE	PRODUCT SIZE
Forward primer	5'CAGAAACTAAGTGCCGGTG3'	541bp
Reverse primer	5'ATTCGGTAAGAGCTTGACGC3'	
Forward primer	5'TTACCTCCATTACTTGACAATCCAT3'	201bp
Reverse primer	5'GATTTGCAACATGAAACATTTACAG3'	

5.9.9 Polymerase Chain Reaction (PCR) for *ICESe2* gene Amplification

PCR amplification of ICESe2 gene of *S. equi* was performed using the primer set described by (Libardoni *et al.*, 2013). The forward primer and reverse primer were used to amplify a 201 bp fragment corresponding to the *eqbE* (ICESe2) gene and for the *zooepidemicus* a set of forward and reverse primers were used to differentiate from *S. equi*. The PCR reaction was carried out in a total volume of 25 µL, with 12.5 µL Dream Taq Green Master Mix (Thermo Fisher Scientific), 1.0 µL forward primer (final

concentration: 10 μ M), 1.0 μ L reverse primer of both the species as listed below (final concentration: 10 μ M), 2.0 μ L DNA template (102 ng/ μ L) and 8.5 μ L nuclease-free water. Amplification was performed in a thermal cycler (Bio Rad) using the following conditions 35 cycles denaturation for 2 min at 95°, 30 sec at 95°C, 54°C for 15 sec and extension was done for 10 sec at 72 °C and the final extension of 2 min at 72° C (Cordoni et al., 2015). PCR products were analysed by agarose gel electrophoresis (1.5 % agarose gel in 1 $\times$ TAE buffer, run at 80V for 90 min) and visualized under UV transillumination following staining with ethidium bromide. A DNA ladder 100 bp (Promega) was included for fragment size estimation.

5.10 Agarose Gel electrophoresis

The PCR product was confirmed by electrophoresis on 1.5% agarose gel. The gel was set by heating molecular grade agarose (Amresco, Biotechnology) in Tris acetic acid ethylene diamine tetra acetic acid buffer (TAE, pH 8.0) to melt it properly. After that the gel was cooled to 50°C, 15 μ l of ethidium bromide was added to 1.5% of the gel. The gel casting tray was placed on a flattened surface after sealing the open sides with adhesive tape. The gel comb was then placed across the gel casting tray. The melted gel was poured onto the gel-casting tray and kept undisturbed until hardened. The comb was taken out and tape was removed. The gel-casting tray with the gel was then placed in the electrophoresis tank. Five microliters of the amplified product and 5 μ l of ladder (100 bp ruler) were loaded into designated wells. Electrophoresis was carried out at 80V in 1X TAE buffer till the dye reached the last third of the gel. After the electrophoresis, the gel image was photographed under UV Transilluminator (Syngene). Molecular size of amplicons was predictable by comparison of their movement with respect to that of standard molecular size marker.

5.11 Quantitative real-time PCR (qPCR)

Quantitative real-time PCR (qPCR) targeting the *SeM* gene of *S. equi* was employed for the molecular detection of the pathogen in nasopharyngeal swab and guttural pouch lavage samples. A TaqMan-based “multiplex real-time PCR” assay was performed in a 96-well plate format using the Applied Biosystems real-time PCR system integrated with Quant Studio™ software (Thermo Fisher Scientific, USA).

The assay was designed to simultaneously detect and differentiate *S. equi*, *S. zooepidemicus*, and the broader *Streptococcus spp.* group using organism-specific primers and Taq Man probes, as previously described by (Libardoni *et al.*, 2013).

The total reaction volume for each well was 10 µL, comprising the following components:

- 5.0 µL of TaqMan Fast PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific)
- 1.7 µL of nuclease-free water
- 0.5 µL of 10 µM forward and reverse primers specific for each target organism
- 0.3 µL of 10 µM TaqMan probes for each organism
- 2.0 µL of template DNA extracted from the sample

Amplification was carried out under optimized thermal cycling conditions for multiplex detection: Initial denaturation at 95°C for 20 seconds, followed by 40 amplification cycles of denaturation at 95°C for 2 seconds, annealing and extension at 60°C for 20 seconds. All reactions were performed in triplicate, and appropriate positive and negative controls were included in each run. The cycle threshold (Ct) values were automatically calculated by the software. Interpretation was based on predefined criteria: samples with Ct values <35 was considered positive, those with Ct 35– 36 was classified as weak positive, and Ct >35 as negative.

- **Positive: Ct < 35**
- **Negative: Ct > 35**

Table 5.8: Represents the Primer details for identification of *Streptococcus species*

Primers and Probes (PCR/Real time)	Sub -species	Sequence (5' - 3')
Conventional PCR (FP)	SEE	TTACCTCCATTACTTGACAATCCA
Conventional PCR(RP)	SEE	GATTTGCAACATGAAACATTTACAG
Conventional PCR (FP)	SEZ	CTTTTCTTCACCGCCCACT
Conventional PCR(RP)	SEZ	TGAGCTTTGGAGAAATGGAA

Real time FP	SEE	CTTTCATCTCTGTTTTTTTGCAAAC
Real time RP	SEE	GAAGCTGGATATTTGAAAGATGAGATA
Real time FP	SEZ	CACCGCCCACTGCTTATATACC
Real time RP	SEZ	GATTTTGCATGAAATGAGAGTATATGC
Real time FP	<i>Streptococcus</i>	GACGCTTTTGGTTCCTTTGC
Real time RP	<i>Streptococcus</i>	GCCAAAACGACCCGTAGCT

Table 5.9: Showing Probes for detecting *Streptococcus equi* and *Streptococcus zooepidemicus* using Real time PCR.

Genus /Subspecies	PROBES	Sequence (5' -3')
<i>Streptococcus Species</i>	<i>Streptococcus</i> Pb	6FAM- CTTTAAAGAGCAATTCACMGBNFQ
<i>Streptococcus equi subspecies equi</i>	SEE Pb	VIC-ATAAGCGGTTATACTATTGTCMGBNFQ
<i>Streptococcus equi subspecies zooepidemicus</i>	SEZ Pb	NED-TATAGGAAGGCTTAAGATTACMGBNFQ

5.12 Evaluation of Analytical Sensitivity and Specificity of the Multiplex Real-Time PCR Assay

To determine the analytical sensitivity of the developed multiplex real-time PCR (qPCR) assay for detection of *Streptococcus* spp., including “*Streptococcus equi* subsp. *equi*” and “*S. zooepidemicus*”, a limit of detection (LOD) study was conducted. The assay was tested using serial tenfold dilutions of DNA extracted from a known *S. equi*-positive clinical sample. A total of eight tenfold serial dilutions were prepared, ranging from 35.6 ng/μL to 0.035 pg/μL. Each dilution was subjected to the multiplex qPCR in

triplicate under previously described optimized conditions. Additionally, a non-template control (NTC) containing nuclease-free water was included in each run to monitor for potential contamination or nonspecific amplification. The sensitivity of the assay was assessed by determining the lowest concentration of DNA that consistently yielded a positive amplification signal (Ct value < 35) in all replicates. A standard curve was generated by plotting the Ct values against the logarithm of DNA concentration to assess linearity (R² value) and amplification efficiency. Furthermore, to assess the analytical specificity and rule out potential cross-reactivity, the assay was evaluated using genomic DNA extracted from non-target bacterial species, specifically *Staphylococcus* spp. The qPCR reaction was conducted under identical conditions with DNA from *Staphylococcus* spp. as the template.

5.13 Statistical Analysis of Sensitivity and Specificity

To evaluate the diagnostic performance of the developed multiplex real-time PCR assay, its sensitivity and specificity were statistically compared to a reference conventional PCR assay targeting the *SeM* gene of “*Streptococcus equi subsp. equi*”. The reference assay was considered the gold standard for this comparison. The performance metrics were calculated using 2×2 contingency tables, and results were expressed in terms of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). The following formulae were used:

Formulae used to determined Sensitivity and Specificity

- Sensitivity = $\frac{a}{a+b} \times 100$

where:

a = number of true positives

b = number of false negatives

- Specificity = $\frac{d}{c+d} \times 100$

where:

c = number of false positives

d = number of true negatives

- Positive Predictive Value (PPV) = $\frac{a}{a+c} \times 100$

- Negative Predictive Value (NPV) = $\frac{d}{b+d} \times 100$

To assess the association between the diagnostic method and test outcomes, a Chi-square (χ^2) test of independence was performed. A p-value less than 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism (version 9.0)

Chapter 6

Results

6.1 Sample size

The random samples were collected from organized as well as unorganized farms. The sample size for different herds was determined using online epitools (<https://epitools.ausvet.com.au/samplesize>). This utility calculates the sample size required to estimate a proportion (or prevalence) with a specified level of confidence and precision.

- **Estimated true proportion:** 0.5
- **Desired precision ($\pm$):** 0.07
- **Confidence level:** 0.95 (95%)
- **Population size (finite):** 323,032

Epitools sample size calculation interface was used to determine the number of samples required for estimating a proportion or apparent prevalence with a given level of statistical precision.

From the inputs:

- $\text{inp2} = 0.95 \rightarrow$ This represented the confidence level of 95%, meaning the study results were expected to reflect the true population values with 95% certainty.
- $\text{inp4} = 323,032 \rightarrow$ This was the total population size under consideration (e.g., total number of animals in the population).
- The tool calculated that for both a "large population" assumption and the actual finite population of 323,032, the required sample size was 196.
- This means that to estimate the disease prevalence in this population with the given parameters (likely 50% expected prevalence and a certain precision), at least 196 samples must be collected to achieve reliable and statistically valid results.

In short, it confirms that 196 samples were sufficient whether the population is considered infinite (large population) or finite (323,032 animals) because the difference in required sample size is negligible at this scale.

EPITOOLS

Home Prevalence Freedom Studies Diagnostics Sampling

Sample size calculations

Sample size to estimate a proportion or apparent prevalence with specified precision

Estimated true proportion: 0.5

Desired precision (+/-): 0.07

Confidence level: 0.95

Population size (for finite populations): 323032

[Submit](#)

Activate Windows
Go to Settings to activate Windows

Results

Sample size required for specified inputs

Large population	196
Population = 323032	196

[Sample sizes for varying prevalence and precision values](#)

Activate Windows
Go to Settings to activate Windows

Fig 6.1: Represents the sample size estimation to determine prevalence in a large population using Epitools.

Table 6.1: Represent number of samples to be collected from seven states

States	State wise population	No of samples collected from each state
Jammu and Kashmir	89620	56
Haryana	12982	8
Punjab	16358	10
Uttar Pradesh	100667	62
Rajasthan	35018	21
Uttarakhand	34334	21
Himachal Pradesh	34053	22
Total	323032	200

* The details of collected samples have been given in supplementary file.

6.2 Isolation and characterization of the causative organisms from clinical cases of strangles in equines.

6.2.1 Revival of cultures from the NRCE Laboratory

A total of 45 microbial cultures were utilized in the present study, all of which were sourced from the culture repository maintained at the ICAR- National Research Centre on Equines (NRCE), Hisar, Haryana, India. The most isolates were isolated from nasal swabs, while five originated from wound or lesion swabs, two from abscess material, and two from culture stocks. Nasal swabs, particularly those processed from the states of Haryana and Rajasthan, were found positive for *Streptococcus equi*, corroborating earlier findings reported by Vaid *et al.* (2020). Regarding the host distribution, a higher number of isolates (n = 20) originated from mares, six from ponies (pony mares), and nineteen were from animals with unspecified identity. Geographically, most isolates were obtained from Haryana, followed by Rajasthan (8 isolates), Gujarat (1 isolate), Uttar Pradesh (4 isolates), and Uttarakhand (3 isolates). The species *S. equi* was the most frequently recovered, particularly from nasal swabs collected from Haryana and Rajasthan, consistent with previous reports (Lindahl *et al.*, 2013; Delph *et al.*, 2019). All the isolates were revived and sub cultured on Columbia blood agar (CBA) containing 5% defibrinated sheep blood and incubated at 37°C for 24–48 hours. Colonies exhibiting β -hemolysis were further purified by sub cultured on fresh 5% blood agar plates and subsequently transferred into - Todd Hewitt Broth (THB) supplemented with yeast extract and 2% horse serum, followed by incubation at 37°C (Chhabra *et al.*, 2024).

6.2.2 Phenotypic and Biochemical Characterization

The isolates were initially identified as *Streptococcus* spp. based on hemolytic pattern, colony morphology, Gram staining, and catalase reaction. Colonies presumptively identified as *S. equi* were β -hemolytic, mucoid, and translucent with smooth, glistening surfaces on Columbia blood agar. The colony size varied from 1.0–3.0 mm in diameter after 24–48 h of incubation at 37°C. Microscopically, Gram-stained smears revealed Gram-positive cocci arranged in short and long chains, with a negative catalase reaction (Figure 6.2 a, b, g). According to phenotypic identification, two isolates displayed distinct β -haemolytic, minute colonies on blood agar and were identified as *S. zooepidemicus*. These colonies appeared smaller and less mucoid, consisting of Gram-

positive cocci in pairs and chains (Fig 6.2 c, d). Two additional isolates produced translucent, pearl-like β -hemolytic colonies (1.5 - 2 mm) on sheep blood agar and were depicted as *S. equisimilis* (Fig 6.2 e, f).

Further biochemical characterization was carried out to confirm the identity and differentiate among - *S. equi*, *S. zooepidemicus*, and *S. equisimilis* isolates. Subspecies differentiation was based on the fermentation pattern of specific sugars, following the method described by Bannister *et al.* (1985). *S. equi* is biochemically characterized by its ability to ferment maltose, and non-fermentative for various other (sugar pellets) carbohydrates, including lactose and sorbitol, trehalose. The majority of isolates were non-fermenters of lactose, sorbitol, and trehalose, a characteristic biochemical profile of *S. equi* (Kumamoto *et al.*, 2001). In contrast, isolates identified as - *S. zooepidemicus* utilized lactose and sorbitol, but not trehalose, which clearly distinguished them from —*S. equi* (Quinn *et al.*, 1994; Silva *et al.*, 2007; Harrington *et al.*, 2002). Two other isolates showed variable lactose fermentation but were non-fermenters of sorbitol, consistent with —*S. equisimilis*.

Representative sugar fermentation results are shown in Figure 6.3, with accuracy confirmed using appropriate positive and negative controls. Despite minor variations, the overall fermentation profiles were consistent with traditional biochemical characteristics of *S. equi*. Notably, a higher rate of maltose fermentation was observed among the *Streptococcus* isolates under the given experimental conditions (Fig 6.4). The majority of isolates were thus confirmed as *S. equi*, with a few identified as —*S. zooepidemicus* and —*S. equisimilis*.

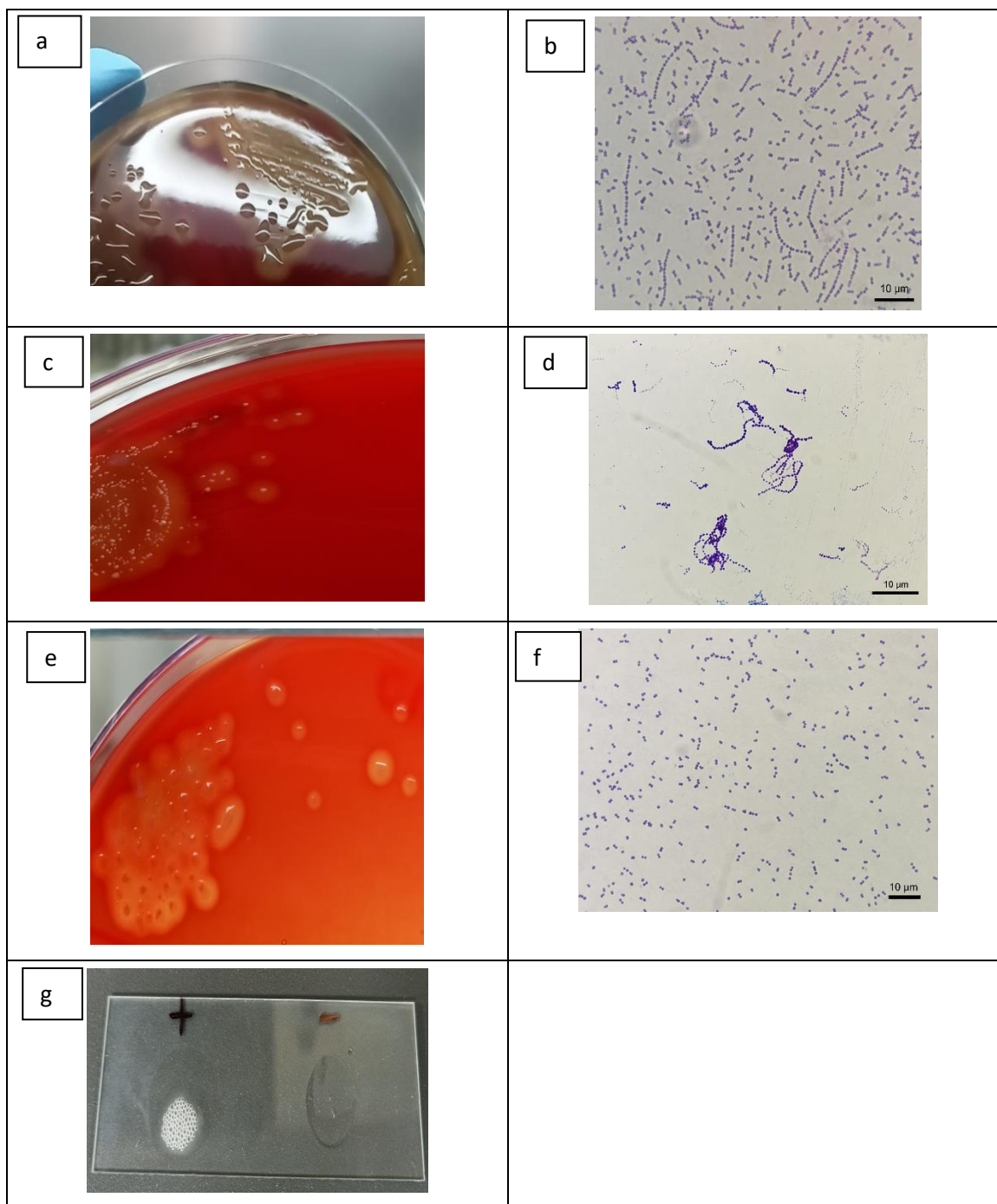


Figure 6.2: Microscopic examination of *S. equi*, *S. zooepidemicus* & *S. equisimilis* at (1000X) view magnification. a) β -hemolytic, mucoid colonies of *S. equi* on sheep blood agar media. b) Gram staining of *S. equi* showing Gram-positive cocci in short chains. c) *S. zooepidemicus* showing β -haemolysis on sheep blood agar media. d) Gram's stain of *S. zooepidemicus* showing cocci in chains. e) Colony of *S. equisimilis* on blood agar. f) Gram positively stained cocci of *S. equisimilis*. g) Catalase test showing positive and negative test results.

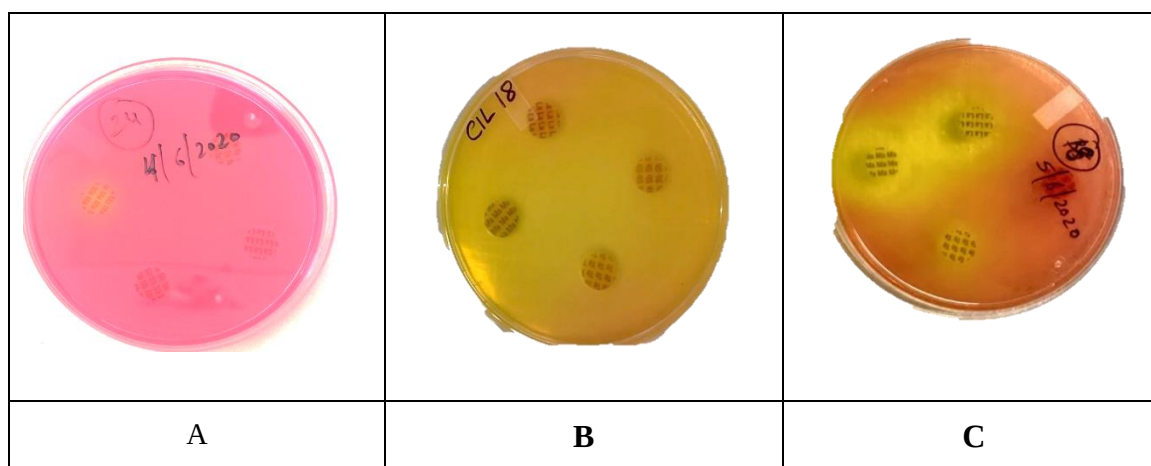


Figure 6.3: Representative Images of characteristic sugar fermentation. a) *S. equi* as non- fermenter of lactose (La), sorbitol (Sb) & trehalose (Tr). b) *S. zooepidemicus* showing fermentation of La & Sb but non- fermenter of Tr. c) Fermentation pattern of Sb non-fermenter *S. equisimilis*

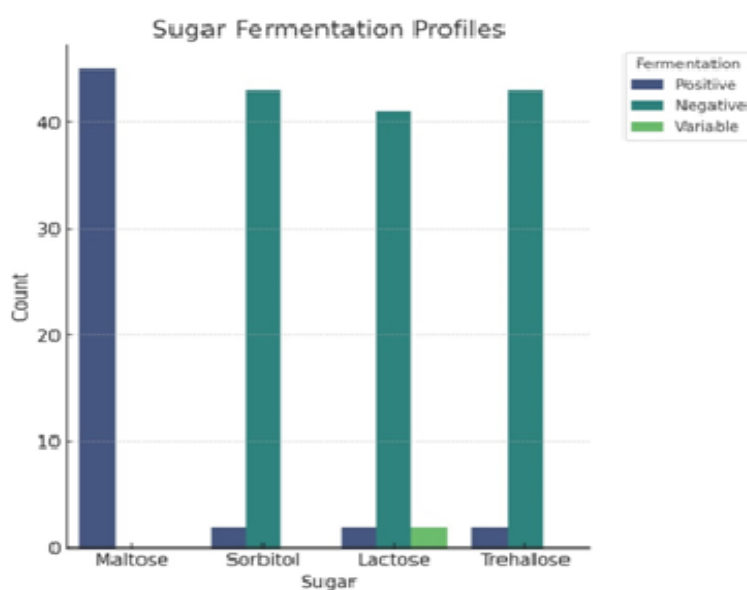


Figure 6.4: Sugar fermentation profiles of maltose, sorbitol, lactose & trehalose of *Streptococcus* spp.

6.2.3 Field samples collected from equines in northern India.

A total of 200 samples collected from 7 states from healthy as well as clinically affected animals were collected under complete hygienic conditions as proposed in the research work. For standardization, experiments were conducted on cultures/isolates available in the Central Instrument Laboratory of —ICAR-National Research Center on Equines, Hisar.

6.2.4 Isolation of bacterial organisms and colony Characteristics

Isolation of the organisms was done on —Columbia blood agar plates. Out of the 200 nasal swab samples collected from equines exhibiting clinical signs consistent with strangles, 14 samples (7%) yielded mucoid, white to amber, β -hemolytic colonies on Columbia blood agar supplemented with 5% sheep blood. These colony characteristics were presumptively suggestive of —*S. equi*”. On further examination, Gram-positive cocci in chains were observed in Gram staining, and catalase tests were negative, which supported the possibility of *Streptococcal* growth. However, pure isolation of *S. equi* could not be achieved from any of the samples, primarily due to mixed microbial growth, overgrowth of commensals, and possibly low viability of *S. equi* in the specimens.

From the field samples, no definitive isolate could be purified. The inability to isolate *S. equi* through culture underscores the limitations of conventional microbiological methods, particularly when dealing with subclinical, chronic, or partially treated infections and emphasizes the necessity for molecular techniques such as PCR and qPCR for the confirmation of infection.



Fig 6.5: *Streptococcus* cultures on blood agar plate.

6.2.5 Biochemical characterization of field samples

Biochemical characterization of the bacterial isolates obtained from field samples was performed using traditional sugar fermentation method and Hi Strep Identification kit. The analysis revealed the presence of organisms belonging to the *Streptococcus* genus in several samples. However, due to the presence of mixed bacterial cultures in most of the samples, definitive identification of *Streptococcus equi* was not possible at this

stage. While colony morphology and biochemical reactions were suggestive of *S. equi*, the results remained inconclusive without pure culture isolation. Further confirmatory tests such as molecular diagnostics (qPCR) and Hi Strep™ identification kit were employed to strengthen the identification process.

6.2.6 Hi strep™ Identification Kit

Fourteen presumptive β -hemolytic streptococcal isolates—characterized by smooth, translucent, shiny colonies with β -haemolysis on blood agar and Gram-positive cocci were subjected to biochemical profiling using the Hi Strep™ Identification Kit (KB005A). The kit comprised 12 tests including “Voges–Proskauer, esculin hydrolysis, PYR, ONPG, arginine” utilization, and fermentation of “glucose, lactose, arabinose, sucrose, sorbitol, mannitol, and raffinose”. Of the 200 isolates 14 isolates exhibited the biochemical profile consistent with *S. equi* (i.e., positive/negative reactions characteristic of this species).

Table 6.2: Result Interpretation Chart of Hi Strep Identification kit

Test	Reagents to be added after Incubation	Principle	Original color of the medium	Positive Reaction	Negative Reaction
“Voges Proskauer’s	1-2 drop of Baritt reagent A and 1 -2 drops of Baritt reagent B		Colourless/light Yellow	Pinkish red	Colourless
Esculin Hydrolysis	-	Detects esculin hydrolysis	Cream	Black	Cream
PYR	-	Detects PYR enzyme activity	Cream	Cherry Red	Cream
ONPG	-	Detects beta galactosidase activity	Colourless	Yellow	Colourless

Arginine Utilization	-	Detects arginine decarboxylation	Olive green to light purple	Purple /Dark	No Change in color or yellow
Glucose	-	Carbohydrate utilization	Pinkish Red/Red	Yellow	Red/Pink
Lactose	-	Carbohydrate utilization	Pinkish Red/Red	Yellow	Red/Pink
Arabinose	-	Carbohydrate utilization	Pinkish Red/Red	Yellow	Red/Pink
Sucrose	-	Carbohydrate utilization	Pinkish Red/Red	Yellow	Red/Pink
Sorbitol	-	Carbohydrate utilization	Pinkish Red/Red	Yellow	Red/Pink
Mannitol	-	Carbohydrate utilization	Pinkish Red/Red	Yellow	Red/Pink
Raffinose	-	Carbohydrate utilization	Pinkish Red/Red	Yellow	Red/Pink”

In the present study, biochemical identification of “*S. equi*” using the Hi-Strep kit yielded inconclusive and highly variable results. Several reactions, including “Voges–Proskauer’s, Esculin hydrolysis, ONPG”, and carbohydrate fermentation tests, displayed inconsistent patterns across isolates, with many reactions recorded as variable or not detected. This contrasts with the characteristic biochemical profile of *S. equi*, which is typically negative for “PYR, Voges–Proskauer’s, Esculin hydrolysis, ONPG”, and most carbohydrate fermentation tests (lactose, sorbitol, mannitol, raffinose), but positive for arginine utilization, sucrose, and glucose fermentation. The variability observed in the current isolates therefore did not align with the expected unreactive pattern of “*S. equi*”, making reliable identification through the Hi Strep kit alone impractical. These findings highlight the limitations of conventional biochemical methods in distinguishing *S. equi* from closely related species such as *S. zooepidemicus*, and underscore the necessity of employing molecular diagnostic assays for accurate confirmation.

*The table of tests has been given in Supplementary section.

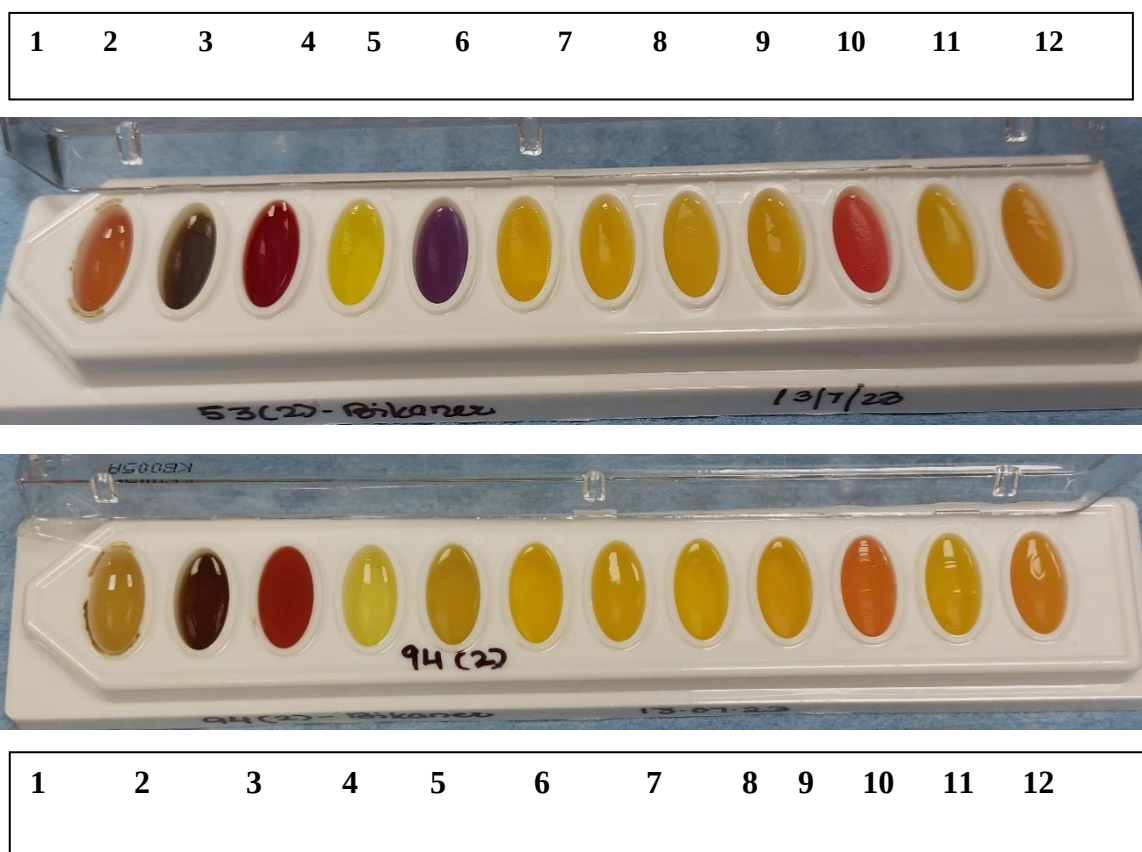


Fig 6.6: Representative figure showing biochemical characterization of *Streptococcus equi* with hi strep Identification kit.

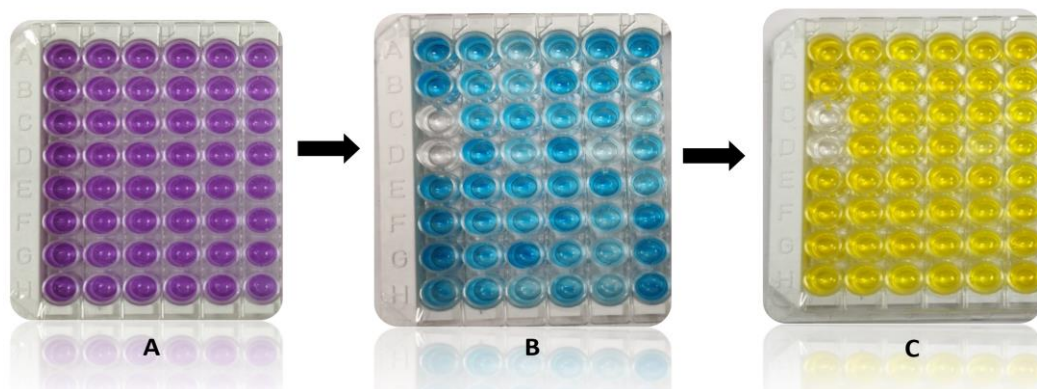
6.3 Sero-surveillance of equine population to determine the prevalence of the disease.

A total of two hundred blood specimens were collected from the jugular vein of each equine using sterile vacutainer tubes. The samples were kept chilled at approximately 4 °C during transport to the laboratory. Upon arrival, the blood was centrifuged at 4500 rpm for 15 minutes to isolate the serum.

An indirect ELISA was performed on the collected serum samples to detect antibodies against *Streptococcus equi* using the commercially available ID Screen® *Streptococcus equi* Indirect ELISA kit (IDvet, France). Serum samples were tested at a 1:100 dilution according to the manufacturer's instructions. Each ELISA plate included two positive control wells and two negative control wells supplied with the kit to validate the assay. The positive control wells consistently produced the expected colour reaction, confirming proper assay performance, whereas the negative control wells remained

colourless (or showed negligible colour development), confirming the absence of non-specific reactions.

The figures below illustrate the sequential enzymatic reactions of the ELISA. In Step A, the HRP-conjugate was added to the antigen-coated wells containing the diluted serum samples together with the positive and negative controls, allowing antigen–antibody binding to occur in reactive samples. In Step B, following incubation and washing, tetramethylbenzidine (TMB) substrate was added. The two positive control wells, along with antibody-positive serum samples, developed a blue colour due to the enzymatic reaction, whereas the two negative control wells and antibody-negative serum samples remained colourless. In Step C, the reaction was terminated by the addition of stop solution, resulting in the conversion of the blue colour to yellow in the positive wells, while the negative control wells remained colourless to pale yellow. The optical density (OD) of each well was measured at 450 nm, and the Sample-to-Positive (S/P) percentage was calculated for interpretation of the results according to the manufacturer's recommendations.



A. - Conjugate B. - Color developed after adding Substrate C. - Color developed after adding stop solution

Fig 6.7: Indirect ELISA for the detection of antibodies against *Streptococcus equi* using the commercially available ID Screen® *Streptococcus equi* Indirect ELISA kit. Test serum samples were analysed at a dilution of 1:100 together with the positive and negative controls supplied with the kit. (A) Microplate after addition of horseradish peroxidase (HRP)-conjugated antibody. (B) Development of blue colour following the addition of TMB substrate, indicating enzyme activity. (C) Development of yellow colour after addition of stop solution, after which optical density (OD) was measured at 450 nm and results were interpreted according to the manufacturer's instructions.

6.4 Geographical distribution and prevalence of strangles

Serum samples were analysed using the indirect ELISA of these, 67 samples tested seropositive 33.5%, with an overall seroprevalence of 35 % in organized farms and 5.6% in unorganized farms. The study also emphasized the importance of stringent bio security measures and regular testing to manage outbreaks effectively. Seroprevalence differed across the sampled regions, with the highest prevalence observed in Haryana 100% and the lowest in Uttar Pradesh 13%, 62 % in Uttarakhand, 34% in Jammu and Kashmir, 38% in Rajasthan, 13.6 % in Himachal Pradesh and 80% in Punjab. This variation may be attributed to differences in equine population density, management practices, and bio security measures.

Table 6.3: The prevalence rate of strangles in seven states of north India.

States	State wise samples Collected	Total samples found positive from each state	Prevalence rate (State wise)
Haryana	8	8	100
Jammu and Kashmir	56	19	34%
Rajasthan	21	8	38%
Uttar Pradesh	62	8	13%
Uttarakhand	21	13	62%
Punjab	10	8	80%
Himachal Pradesh	22	3	13.6%
Total	200	67	

6.5 Sample Percentage Ratio Interpretation

Out of 200 equine serum samples analysed, 67 samples tested seropositive, exhibiting an S/P ratio greater than 0.3, while 8 samples were classified as borderline, and 125 samples tested negative. According to the manufacturer's interpretation criteria, the Sample Percentage Ratio (S/P) was calculated using the formula:

$$S/P = (\text{Optical Density of Positive Control} / \text{Optical Density of Sample}) \times 100$$

The S/P ratio serves as a quantitative measure for detecting antibodies against "*Streptococcus equi*" in horses. Samples with an S/P ratio below 0.3 are classified as negative, indicating the absence of significant antibody levels, with 125 samples falling into this category. This suggests that the horses are either uninfected or in the early

phase of infection. Samples exhibiting an S/P ratio between 0.3 and 0.5 are categorized as borderline or inconclusive, observed in 8 cases. In such instances, retesting after 2–3 weeks is recommended to assess disease progression, as these horses may be in the early stages of infection or undergoing recovery. Samples with an S/P ratio exceeding 0.5 were considered seropositive, with 67 cases identified in this category. This indicates the presence of *S. equi*-specific antibodies, suggesting either a recent or past infection or the potential for a carrier state within the population.

Percentage

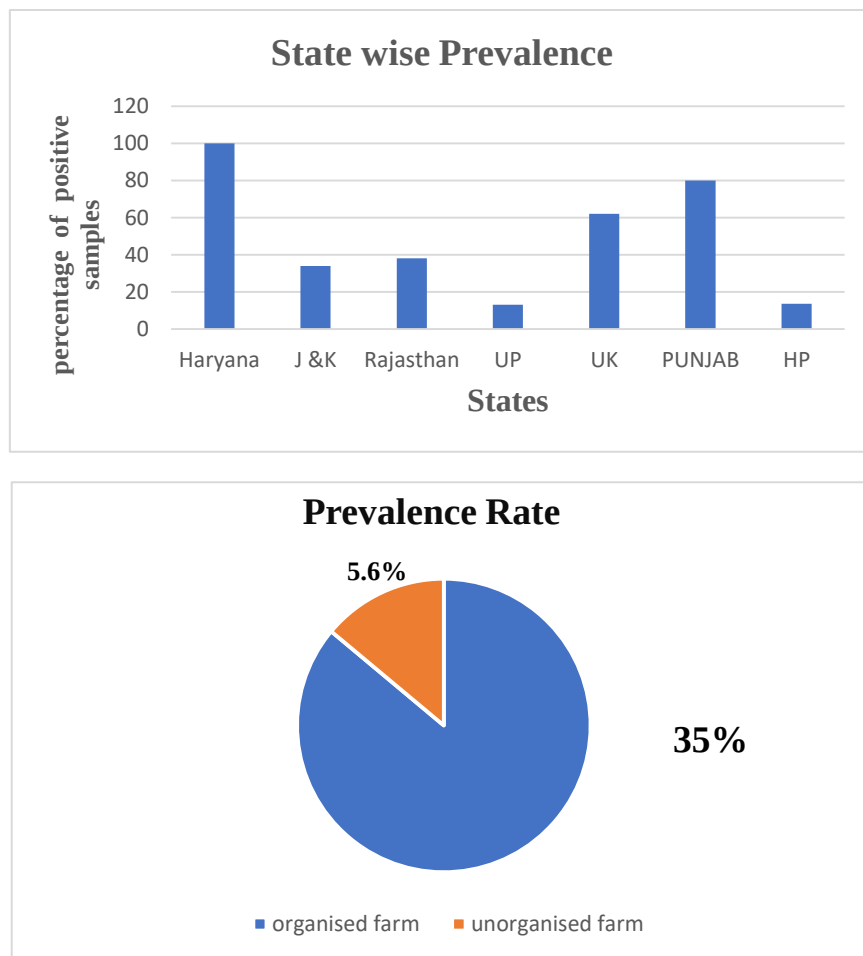


Fig 6.8: Prevalence rate of strangles in equine populations, showing a significantly higher occurrence in field animals (35%) compared to those in organized farms (5.6%). State-wise, Haryana exhibited the highest prevalence at approximately 100%, followed by Punjab at 80% and Uttarakhand at 62%. In Rajasthan, the prevalence rate is 33%, while Uttar Pradesh records a lower prevalence of 13%, while Himachal Pradesh reported the lowest rate in prevalence according to its population highlighting regional variations in disease occurrence.

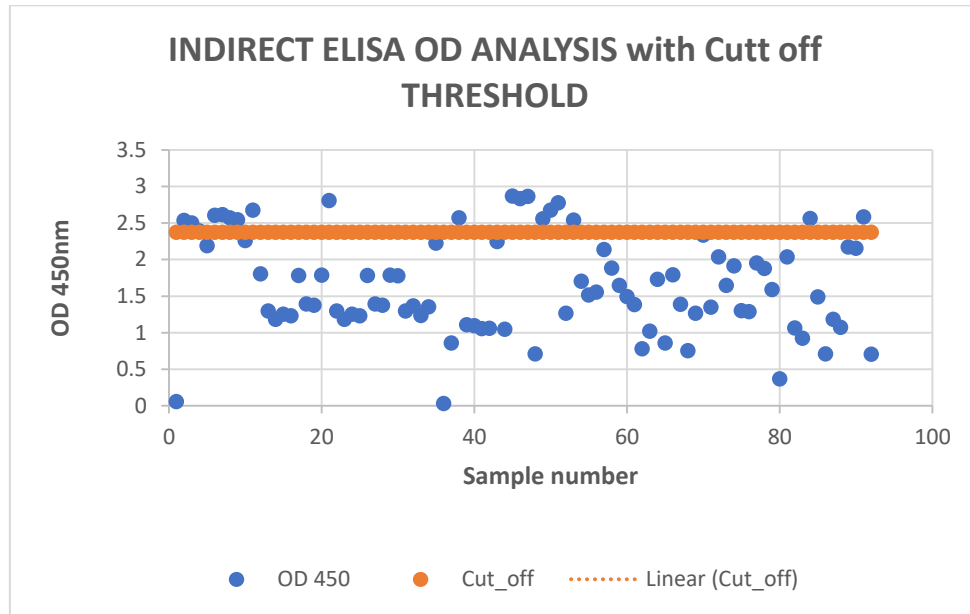


Fig 6.9: Represents the Indirect ELISA OD450 analysis for detection of antibodies against *Streptococcus equi* in equine serum samples. The scatter plot represents the optical density (OD) values measured at 450 nm for individual serum samples. The blue circles indicate OD450 values of the tested equine serum samples. The orange circles represent the calculated cut-off threshold, determined based on the negative control value. Samples with OD values above the cut-off line were considered seropositive, indicating the presence of antibodies against *Streptococcus equi*, whereas samples below the cut-off were considered seronegative. The dotted line represented the cut-off trend line across all analyzed samples.

6.6 Statistical analysis

Statistical analysis was conducted using one-way ANOVA to compare the mean optical density (OD) values among different experimental groups. Post hoc comparisons were performed using Tukey's multiple comparison test. A significance level of $p < 0.05$ was considered statistically significant. Data were expressed as mean $\pm$ standard deviation (SD) and analysed using (Med calc software). A one-way ANOVA was performed to compare the optical density (OD) values between the two experimental groups. The analysis revealed a statistically significant difference in mean OD values between the groups ($F(1,12) = 12.03$, $p = 0.0046$), exceeding the critical F-value ($F_{crit} = 4.75$), indicating a significant variation. Method revealed significant associations between seropositivity among different states.

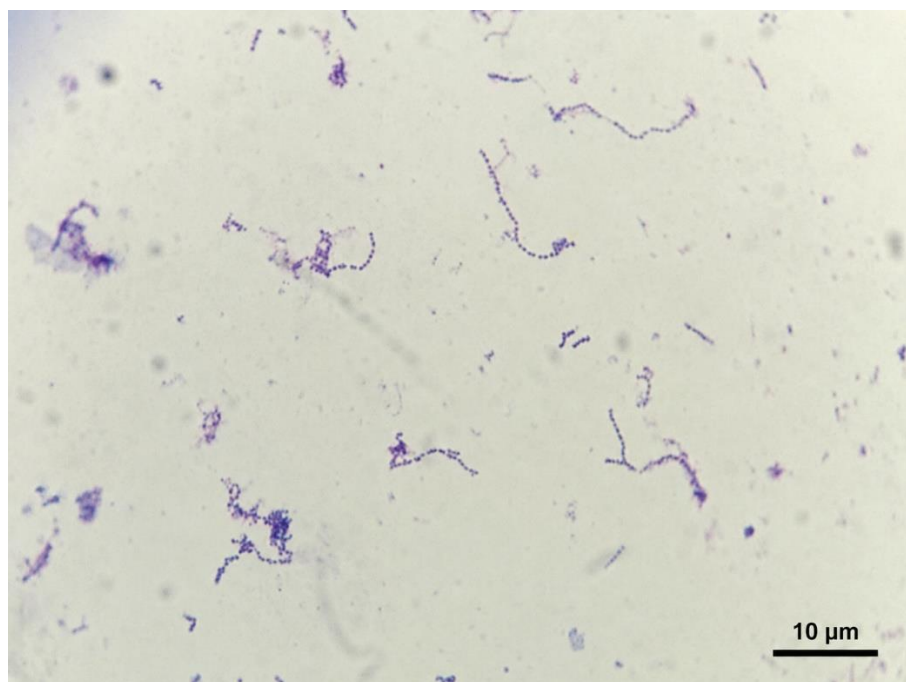
6.7 To detect the carrier state after clinical recovery based on bacterial culture, qPCR and serum antibodies.

Morphology and colony Characteristics

A total of 200 samples were collected from 7 northern states, were examined for the presence of *S. equi* and differentiated from other *Streptococcus* species including *S. zooepidemicus*. Nasal swabs were cultured on solidified blood agar and incubated at 37°C for 24 to 48 hours in aerobic conditions. In a total of 14 samples beta haemolysis was detected using blood agar. Beta-hemolytic colonies were sub cultured on 5% Columbia blood agar by inoculation and incubated at 37°C for 24 hours (fig 6.10a). Colony diameters ranged from 0.5 to 2.0 mm, displaying color from greyish to whitish, often appearing amber or mucous-like without pigmentation. Clear zones surrounded the colonies, indicating RBC haemolysis. The Gram staining of the isolates revealed Gram +ve cocci present in pairs, some in short chains and few in long chains as shown in figure 6.10b.



a



b

Fig 6.10: a Representative figure shows colonies of *Streptococcus equi* on 5% Sheep Blood Agar: b *Streptococcus* species exhibiting cocci morphology confirmed by Gram's staining observed under optical microscope (1000x).

6.8 Polymerase chain reaction with *SeM* gene

Optimization of PCR conditions demonstrated that no amplification was observed at an annealing temperature of 54.2 °C; however, a distinct and specific band was obtained at 55 °C, which was subsequently selected as the optimal annealing temperature for further assays. All streptococcal isolates tested positive for *Streptococcus* spp. through direct DNA analysis, yielding a detection rate of 100%. Among these, 34 isolates were previously identified as *S. equi* and were further confirmed by conventional PCR. In addition, out of 200 nasal swab samples collected from the field, 18 (16.7%) were confirmed positive for *S. equi* by PCR targeting the *SeM* gene. Amplification of the *SeM* gene consistently produced a 541 bp product under the optimized PCR conditions, confirming the specificity of the assay. The obtained PCR products originated from *S. equi* isolates of different horses using gene-specific primers, and the results were consistent with earlier reports of *S. equi* gene expression using similar primers (Chhabra *et al.*, 2024). Nevertheless, it is important to note that PCR-positive results should be regarded as presumptive unless confirmed by bacterial culture (Laus *et al.*, 2016).

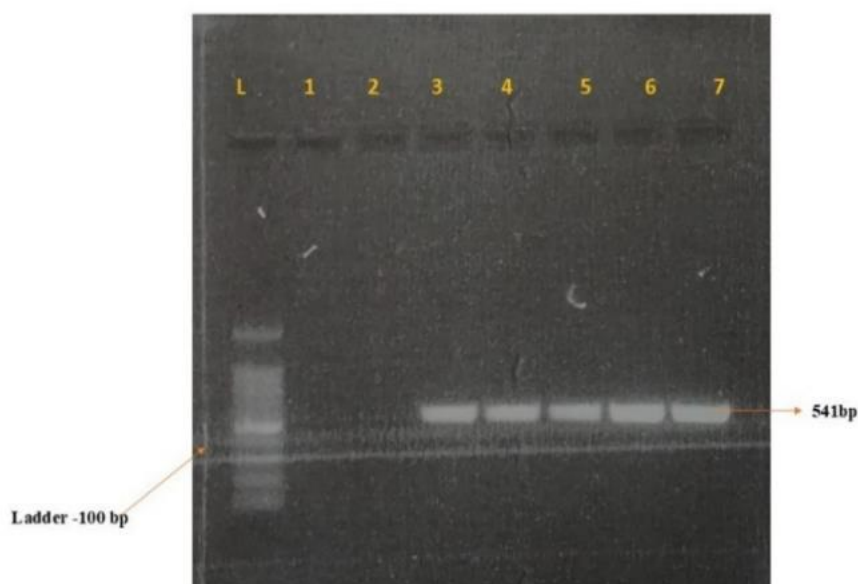


Fig 6.11: Representative gel image of gradient PCR for *SeM* gene amplification. No band was observed at the annealing temperature of 54.2°C, whereas a clear and specific amplification band was obtained at 55°C, indicating the optimal annealing temperature for the assay.

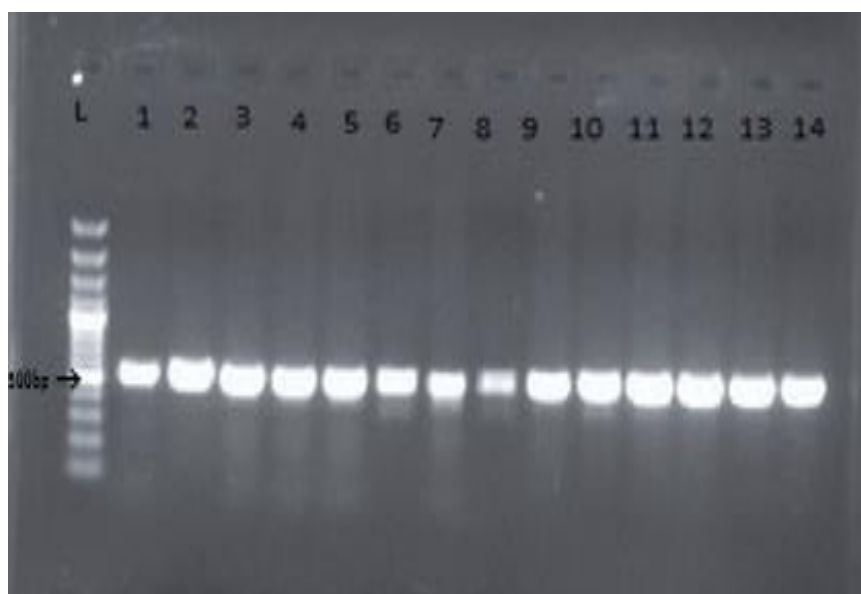


Fig 6.12: Representative Image depicting the amplification of the *SeM* gene of *S. equi* demonstrates the successful amplification of 541 bp fragment.

6.8.1 Amplification of *eqbE* (*ICESe2*) gene

The PCR product with gene yielded 201 bp product when subjected to PCR conditions. The primary PCR products generated from *S. equi* samples of different equines using gene specific primers (Cordoni *et al.*, 2015). However, there was no amplification in samples representing *S. zooepidemicus* (Fig 6.13).

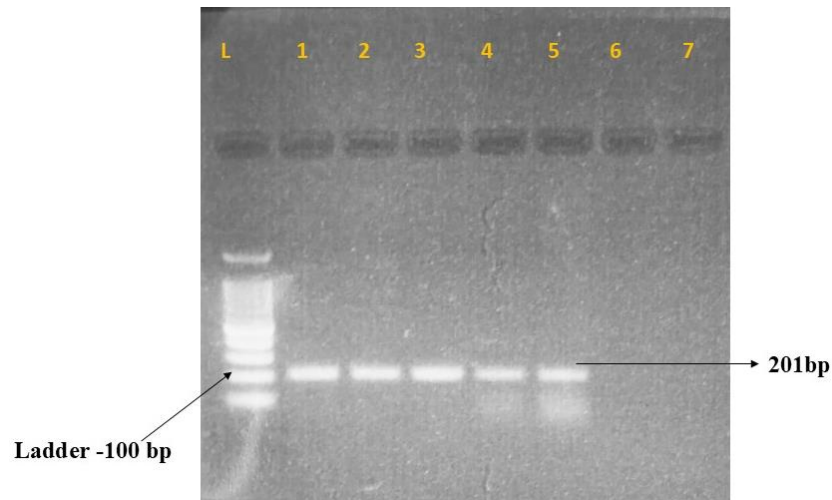


Fig 6.13: Representative image depicting the amplification of the *eqbE* (*ICESe2*) gene of *Streptococcus equi* demonstrates the successful amplification of a 201 bp fragment (1–5). In contrast, no amplification was observed in (6–7), which correspond to *Streptococcus zooepidemicus*.

6.8.2 Multiplex Real-time PCR of *Streptococcus* cultures

All the *Streptococcal* cultures demonstrated positive results for *Streptococcus* species (100 %), which comprised samples positive for *S. equi* with conventional PCR as well as with real time PCR and samples for “*S. zooepidemicus*” and 1 for *Streptococcus* species other than “*S. equi/S. zooepidemicus*”. The method detected the VIC, NED and FAM (reporter dyes) used the “primers and probe” to amplify a fragment as described previously section. FAM reporter dye detects the genus *Streptococcus* (fig.6.14a) while VIC indicates that the species is *S. equi* (fig. 6.14b) and NED indicates that the species is *S. zooepidemicus* (fig. 6.14c). Multiplex real-time PCR reactions with *Staphylococcus* and NTC did not exhibit any amplification, while the reaction with *S. equi* and *S. zooepidemicus* DNA showed the amplification curves for respective species. The multiplex assay successfully detects and differentiates *Streptococcus* species, *S. equi* and *S. zooepidemicus* simultaneously and saving the detection time Fig. 5d. The minimum detection limit for detection of *S. equi* was found as 0.35

picograms/μl of genomic DNA. No amplification signal was observed in the *Staphylococcus* spp. reactions, confirming the high specificity of the multiplex assay for *Streptococcus* species and its lack of cross-reactivity with closely related Gram-positive cocci. This validation step reinforced the diagnostic robustness of the assay for field application in equine samples. Based on a genome mass of ~2.2 fg, the lowest concentration corresponded to ~16 genome copies/μl; with 2 μl template input, this equated to ~318 genome copies per reaction, indicating moderate assay sensitivity. Cross-Reactivity Testing was performed using DNA from a panel of non-*Streptococcus equi subspecies equi* bacterial species commonly associated with equine respiratory or systemic infections. The panel included *S. zooepidemicus*, *Staphylococcus aureus* VTCCBAA19, *Escherichia coli* VTCCBAA8, *Rhodococcus equi* VTCCBAA61, *Pasteurella multocida* VTCCBAA106. None of these organisms showed any detectable amplification signal in the multiplex real time PCR assay across all replicate reactions, and no melt peaks corresponding to specific amplicons were observed. In contrast, all *S. equi* isolates produced strong, characteristic amplification curves with the expected melt temperatures. These results demonstrate that the assay exhibited 100% analytical specificity for *S. equi* among the bacterial species tested, with no detectable cross-reactivity under the assay conditions.

Table 6.4: Dilution series used for analytical sensitivity assessment of the multiplex real time PCR assay.

Dilution level	DNA concentration	DNA concentration	Estimated genome copies/μl*	Template input (μl)	Genome copies/reaction
10 ⁰	35.6 ng/μl	35,600 pg/μl	1.6 × 10 ⁷	2	3.2 × 10 ⁷
10 ⁻¹	3.56 ng/μl	3,560 pg/μl	1.6 × 10 ⁶	2	3.2 × 10 ⁶
10 ⁻²	356 pg/μl	356 pg/μl	1.6 × 10 ⁵	2	3.2 × 10 ⁵
10 ⁻³	35.6 pg/μl	35.6 pg/μl	1.6 × 10 ⁴	2	3.2 × 10 ⁴
10 ⁻⁴	3.56 pg/μl	3.56 pg/μl	1.6 × 10 ³	2	3.2 × 10 ³
10 ⁻⁵	0.356 pg/μl	0.356 pg/μl	1.6 × 10 ²	2	3.2 × 10 ²
10 ⁻⁶	0.0356 pg/μl	0.0356 pg/μl	1.6 × 10 ¹	2	3.2 × 10 ¹
10 ⁻⁷	0.00356 pg/μl	0.00356 pg/μl	1.6	2	3.2

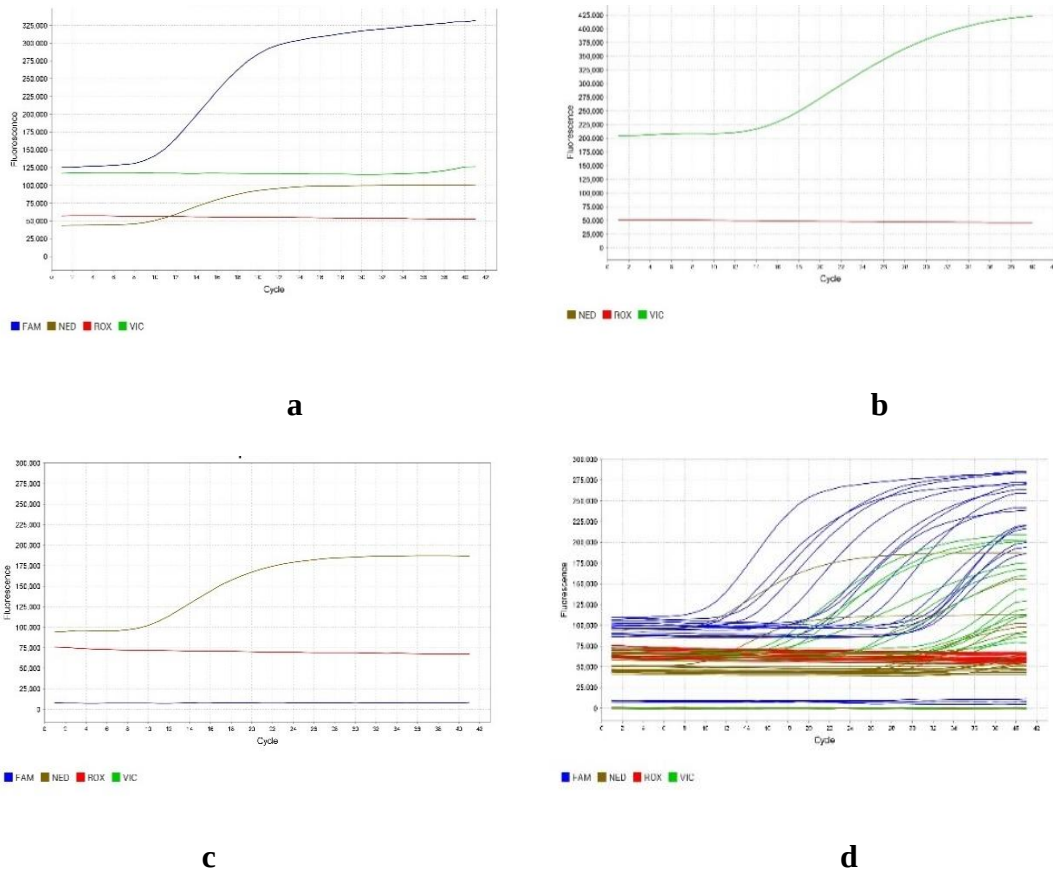


Fig 6.14: In Fig 6.14 a, the blue graph indicates classification at the genus level as *Streptococcus*. Fig. 6.14b presents a graph identifying the species as *Streptococcus equi*, while Fig. 6.14c corresponds to *Streptococcus zooepidemicus*, Fig. 6.14 d displays a amplification plot of multiplex real time PCR, where the blue curve represents the genus *Streptococcus*, and the green curves indicate the species *S. equi* and the brown curves corresponds to *Streptococcus zooepidemicus*.

6.8.3 Testing of field samples by Multiplex Real-Time PCR.

The study was conducted in 7 states of northern India. DNA extracted from nasal swabs and isolated by boil chill method, was used as template in the quantitative PCR reactions. Of the 200 nasal swabs, 84 were positive for *Streptococcus species*, 33 tested positives for *S. equi* in table (Fig 6.15a). Figure 6.15b provides a summary of the distribution of positive samples for *Streptococcus species* and *S. equi*, and their incidence across northern India.

Out of 200 samples collected from equines across seven northern Indian states, isolates of *Streptococcus species* were identified, including both *S. equi* and its closely related

commensal/pathogenic species, “*S. zooepidemicus*”. Haryana showed the highest number of *S. equi* isolates, with 8 positive cases, accounting for 4% of the total sample set. The overall streptococcal rate (including *S. zooepidemicus*) in the state was 8 samples, indicating a high state wise prevalence of both pathogenic and commensal streptococci. Jammu and Kashmir region reported 8 *S. equi* positive samples (4%) and 15 total streptococcal isolates, making it the second-highest contributor of “*S. equi*” positivity in the study. The data suggest significant *S. equi* circulation in this state. Uttarakhand yielded 6 *S. equi* positives (3% of total), and 10 total streptococcal isolates, implying a high proportion of the *S. equi* type among streptococcal infections. This indicates active strangles cases or carriage in the sampled population. Rajasthan had 5 samples positive for *S. equi* (2.5%), out of 12 streptococcal isolates, suggesting moderate activity of the pathogen.

Despite contributing the largest number of field samples, Uttar Pradesh reported only 2 *S. equi* positive cases, accounting for 1% of total samples and 10% of total streptococcal isolates. Punjab contributed 1 positive *S. equi* case (0.5% of total), out of 5 streptococcal isolates. This suggests low prevalence in the region at the time of sampling. No *S. equi* cases were detected in Himachal Pradesh, although 5 streptococcal isolates were recovered. This suggests either the presence of non-pathogenic *Streptococcus* species in the region. The overall prevalence of *S. equi* was 33 out of 200 samples, corresponding to 16.5% positivity. *S. equi* was most frequently isolated in “Haryana, Jammu & Kashmir, and Uttarakhand”, indicating these regions as higher-risk zones for strangles outbreaks. *S. zooepidemicus* was also present across several states, reflecting its common occurrence as a commensal or opportunistic pathogen in the equine respiratory tract. This geographical variation underscores the need for region-specific control strategies, enhanced surveillance, and routine molecular diagnostics to differentiate pathogenic *S. equi* from other related organisms particularly in endemic regions.

Table 6.5: Represents the detection of *Streptococcus* species and *Streptococcus equi* in nasal swab samples (n=200) collected from equines across seven northern Indian states using multiplex real-time PCR. The column “*Streptococcus*” indicates the total number of *Streptococcus* spp. detected, while the same column “*S. equi*” specifies the subset confirmed as *S. equi*.

States	Real time PCR (200 Samples)
Haryana	<i>Streptococcus</i> – 8, <i>S equi</i> - 8
Rajasthan	<i>Streptococcus</i> – 12, <i>S equi</i> - 5
Punjab	<i>Streptococcus</i> – 5, <i>S equi</i> - 1
Jammu Kashmir	<i>Streptococcus</i> - 15, <i>S. equi</i> - 8
Uttarakhand	<i>Streptococcus</i> – 10, <i>S equi</i> – 6
Uttar Pradesh	<i>Streptococcus</i> – 20, <i>S equi</i> - 2
Himachal Pradesh	<i>Streptococcus</i> – 5, <i>S equi</i> - 0

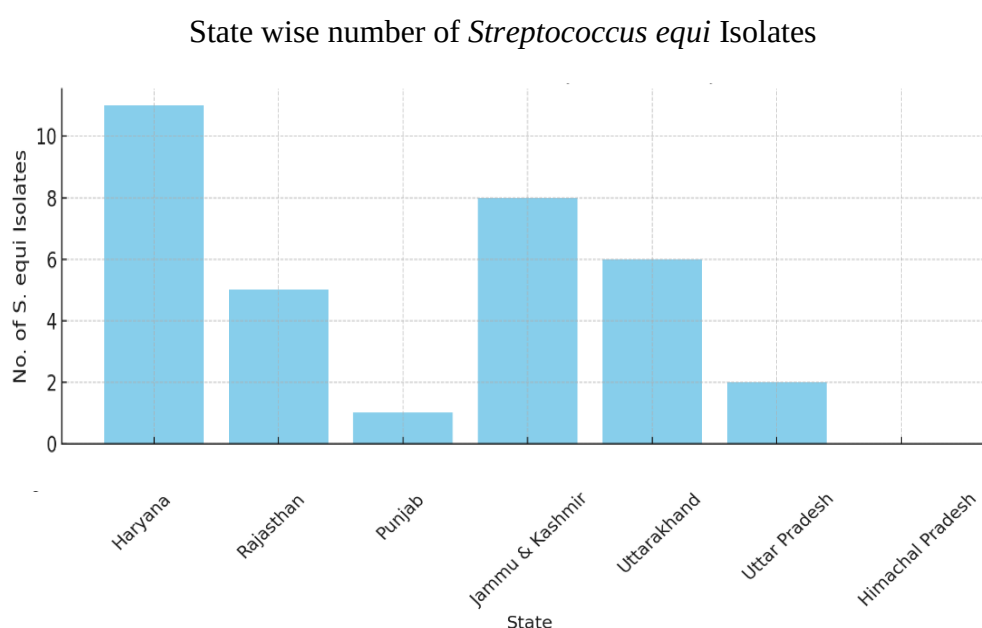


Fig 6.15 A: Graph B depicts the positive cases of *Streptococcus equi* detected from northern states as determined by multiplex real-time PCR

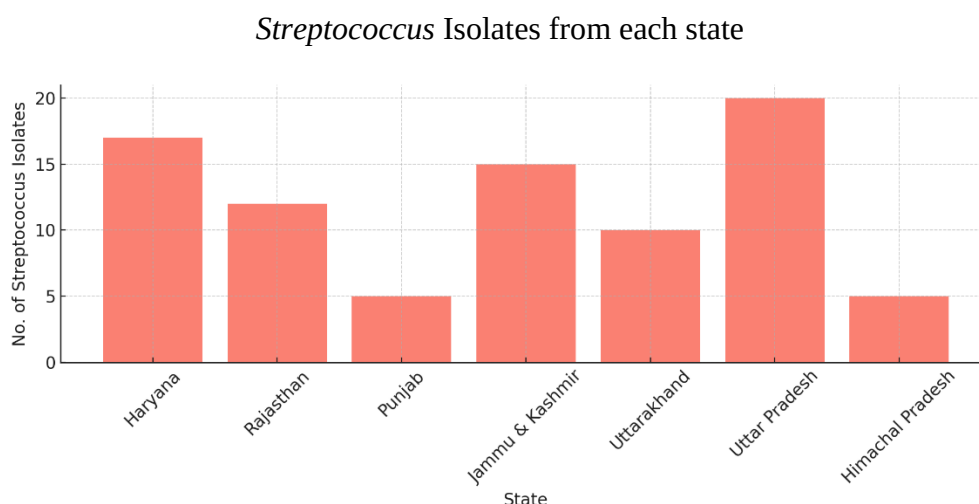


Fig 6.15B: Graph A depicts the positive cases of *Streptococcus* species detected from northern states as determined by multiplex real-time PCR

Detecting *S. equi* can be challenging due to several limitations like poor quality of samples, contamination with other bacteria and non-dominance of *S. equi*. The quality of the sample can significantly impact the accuracy of *S. equi* detection. Degraded during long transportation or improperly collected samples may lead to false negatives. Samples contaminated with other bacterial species can interfere with the detection process. The presence of multiple bacterial species can make it difficult to isolate and identify *S. equi* specifically. Healthy horses may sometimes exhibit a predominance of commensal *Streptococcus* species in their nasal microbiota without showing clinical signs of disease. In addition to *Streptococcus equi* subsp. *equi*—the primary etiological agent of strangles—other *Streptococcus* species commonly detected include *Streptococcus equi* subsp. *zooepidemicus*, a frequent commensal and opportunistic pathogen. Additional species that may be present in equine nasal samples include “*Streptococcus dysgalactiae*, *Streptococcus uberis*, *Streptococcus pneumoniae*, *Streptococcus mitis*, *Streptococcus salivarius*, *Streptococcus pyogenes*”, and various other *Streptococcus* species. In some cases, *S. equi* may not be the dominant organism in the sample, which can complicate its detection. When *S. equi* is present in low abundance relative to other bacteria, its signals may be masked, leading to challenges in accurately identifying its presence. However, we could detect and differentiate *S. equi* from other *Streptococcus* species in multiplex PCR using *eqbE* gene due to its high sensitivity. However, additional data may yield more comprehensive insights.

6.8.4 Diagnostic comparison of multiplex real time PCR and conventional PCR against bacterial culture for *Streptococcus equi* detection

Streptococcus species cultures were characterized using biochemical assays, conventional PCR, and real-time PCR employing species-specific primer sets. The results obtained were utilized for the standardization of the real-time PCR assay.

Table 6.6: Comparative analysis of bacterial cultures of *Streptococcus* species available in the “ICAR - NRCE” Repository as determined by multiplex real time PCR, Conventional PCR, and Biochemical methods.

Clinical Samples	Multiplex real time PCR			Conventional PCR			Biochemical		
	SEE	SZ	Other isolates	SEE	SZ	Other isolates	SEE	SZ	Other isolates
Nasal swabs n = 36	26 (72.2%)	9 (25%)	1 (2.7)	23 (63%)	7 (19.4%)	6 (16.6%)	26 (72.2%)	8 (22.2%)	2 (100%)
Abscess swabs n = 2	1 (50%)	1 (50%)	0 (0%)	2 (100%)	0 (0%)	0 (0%)	1 (50%)	1 (50%)	0 (0%)
Bacterial Culture n = 2	2 (100%)	0 (0%)	0 (0%)	2 (100%)	0 (0%)	0 (0%)	2 (100%)	0 (50%)	0 (0%)
Lesion swabs n = 5	5 (100%)	0 (0%)	0 (0%)	5 (100%)	0 (0%)	0 (0%)	5 (100%)	0 (0%)	0 (0%)
Total	34	10	1	32	7	6	34	9	2

Among these cultures, thirty-four were accurately identified as *Streptococcus equi* isolates through real-time PCR, with confirmation by both biochemical characterization and conventional PCR via gel electrophoresis (Table 6.6). The remaining eleven isolates were classified as other *Streptococcus* species, including *S. zooepidemicus* (n=10) and one unidentified *Streptococcus* species, based on real-time PCR analysis.

Table 6.7 presents a comparative analysis of multiplex real time PCR and conventional PCR diagnostic methods against the gold standard, bacterial culture, for the detection of *S. equi*. The comparison highlights each method's sensitivity and specificity based on the outcomes.

Table 6.7: Comparative analysis of multiplex real time PCR and conventional PCR diagnostic methods against the gold standard, bacterial culture, for the detection of *S. equi*.

Test Method	True Positives	False Negatives	False Positives	True Negatives
Multiplex real time PCR	34	0	0	9
Conventional PCR	32	2	0	9

Using bacterial culture as the gold standard, multiplex real time PCR yielding a diagnostic sensitivity and specificity were 100% whereas Conventional PCR's Sensitivity was 94.1 with 100 specificities. In our study we have observed in comparison to three methods, real-time PCR successfully distinguished between the subspecies of *S. equi* with other isolates, owing to its enhanced sensitivity and precise detection and quantification of specific gene targets. Multiplex real time PCR using *eqbE* primers is more sensitive than conventional PCR for detecting *S. equi*, making it an excellent diagnostic tool. Specificity is equally high for both tests, indicating low false-positive rates. In clinical decision-making, multiplex real time PCR is preferable, especially in early detection or low bacterial load cases. When compared with conventional PCR, multiplex real time PCR is more sensitive and detected all cases that conventional PCR found, plus two additional cases that conventional PCR missed. It is detecting low-level infections or degraded DNA that conventional PCR fails to amplify, thus may have a diagnostic advantage in early or subclinical infections due to its higher sensitivity.

6.9 Statistical analysis

A chi-square test of independence was performed to assess the diagnostic performance of multiplex real-time PCR in comparison with conventional PCR for the detection of

Streptococcus equi subsp. *equi*. The analysis revealed a significant association between the diagnostic method and detection outcome (χ^2 (1, N = 200) = 169.64, $p < 0.0001$), indicating the superior sensitivity of multiplex real-time PCR. These results support the effectiveness of multiplex real-time PCR as a rapid and reliable diagnostic tool for accurate differentiation of *S. equi* from other *Streptococcus* species, aiding in timely disease control in equine populations.

6.10 Carrier status of equine samples

Carrier status of an equine refers to an animal that is infected with a disease but shows no symptoms and can still transmit the pathogen to others. A total of 200 samples were enrolled for carrier detection analysis. An animal was classified as a confirmed carrier of *S. equi* subsp. *equi* if any of the following were true after recovery from primary infection.

- **Positive bacterial isolation from nasopharyngeal swab**
- **PCR/qPCR-positive detection of bacterial DNA in any collected sample**
- **Persistently elevated *SeM*-specific antibody titres post-recovery, indicative of subclinical infection or chronic carriage.**

6.10.1 Carrier Status Data and Interpretation

A total of 67 animals were found to be serologically positive for anti-*SeM* antibodies by ELISA, indicating prior exposure or possible persistence of *S. equi* infection. However, seropositivity alone was not considered sufficient to confirm the carrier state. Therefore, these seropositive animals were further evaluated using bacteriological culture, conventional PCR, and real-time PCR to detect the presence of viable bacteria or bacterial DNA. Among the 67 seropositive horses, 20 animals yielded positive results by one or more of these confirmatory diagnostic methods. Hence, these 20 animals were identified as true carriers of *S. equi*, as they demonstrated both serological evidence of exposure and direct detection of the pathogen through molecular assays.

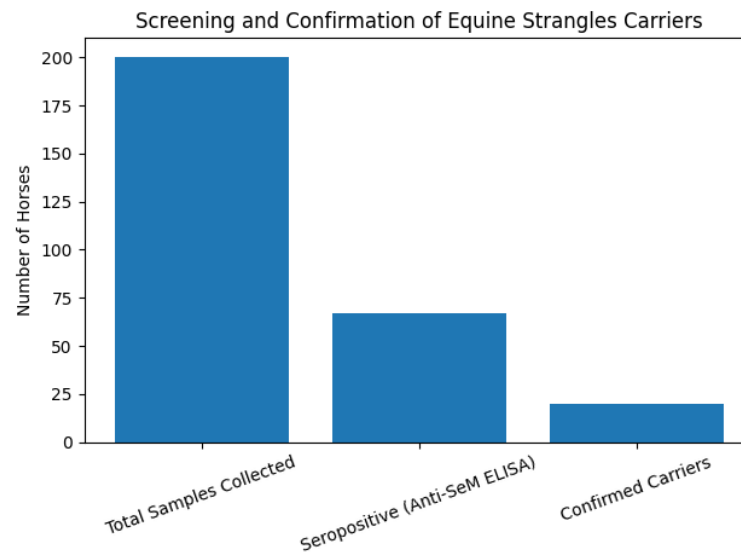


Fig 6.16: Bar graph showing the screening and confirmatory diagnosis of carriers of *Streptococcus equi subsp. equi* associated with Strangles in the equine population. A total of 200 equine samples were collected and screened using Anti-SeM ELISA, of which 67 animals were seropositive. Further confirmation using bacterial culture, conventional PCR, and real-time PCR (qPCR) identified 20 animals as true carriers, resulting in an overall carrier prevalence of 10% in the studied population.

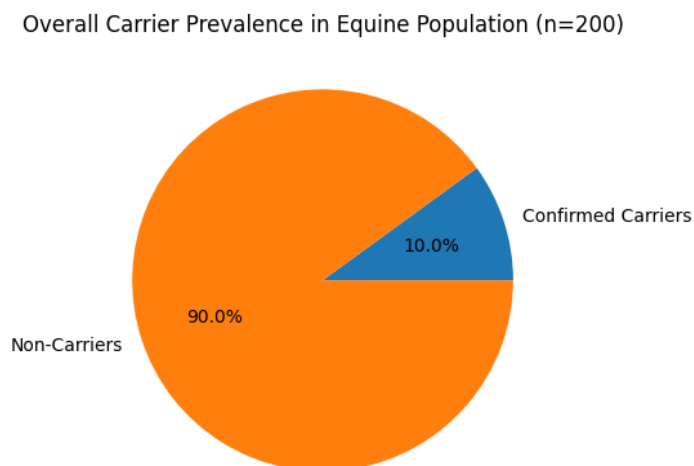


Fig 6.17: Pie chart represented the overall carrier prevalence of *Streptococcus equi subsp. equi* associated with Strangles in the equine population (n = 200). Out of the total animals screened, 20 horses (10%) were confirmed as carriers by bacteriological culture and molecular diagnostic methods, while 180 horses (90%) were classified as non-carriers.

Chapter 7

Discussion

Overview

Strangles remains one of the most contagious bacterial diseases affecting equines worldwide and continues to cause substantial economic losses because of reduced animal performance, prolonged recovery, movement restrictions, and persistent carrier animals. The disease is caused by *Streptococcus equi* subsp. *equi*, an obligate pathogen that primarily infects the upper respiratory tract and associated lymph nodes. Although conventional bacteriological techniques have long been regarded as the standard diagnostic approach, their diagnostic sensitivity is frequently compromised under field conditions because of prior antimicrobial treatment, intermittent bacterial shedding, contamination with commensal microorganisms, and the fastidious nature of the organism.

The present investigation combined conventional microbiology, biochemical characterization, serological surveillance, conventional PCR, and multiplex real-time PCR to evaluate the epidemiology and diagnosis of strangles in equines from northern India. This integrated diagnostic approach enabled assessment of disease prevalence, comparison of diagnostic methods, and identification of carrier animals. The findings demonstrate that although conventional microbiological methods remain useful for preliminary investigation, molecular diagnostic techniques provide greater diagnostic sensitivity and specificity, particularly when bacterial numbers are low or when mixed microbial populations are present in clinical specimens.

Isolation and Characterization of *Streptococcus equi*

The successful revival and characterization of repository isolates confirmed the usefulness of conventional phenotypic and biochemical methods for the preliminary identification of *Streptococcus equi*. Colony morphology, β -haemolysis, Gram-positive cocci arranged in chains, and negative catalase reaction observed in the present study were consistent with the classical descriptions of *S. equi* reported by Timoney (2004) and Newton *et al.*, (2008). These findings demonstrate that routine bacteriological

examination continues to provide valuable preliminary information during laboratory diagnosis.

Biochemical differentiation based on carbohydrate fermentation further supported the identification of *S. equi* and distinguished it from *S. zooepidemicus* and *S. equisimilis*. The observed biochemical profiles largely agreed with previous reports (Quinn *et al.*, 1994; Kumamoto *et al.*, 2001; Silva *et al.*, 2007). Nevertheless, minor variations in fermentation reactions were observed among some isolates, which may reflect strain diversity, environmental adaptation, or differences in metabolic activity. Similar variability has been documented previously, suggesting that biochemical methods alone cannot always provide definitive species identification.

One of the important findings of the present investigation was the inability to obtain pure *S. equi* isolates from field samples. Although several samples produced β -haemolytic colonies suggestive of *S. equi*, pure isolation could not be achieved because of mixed microbial growth. The upper respiratory tract of horses naturally harbors numerous commensal bacteria, particularly *S. zooepidemicus*, which may rapidly overgrow *S. equi* during culture. In addition, previous antimicrobial therapy, delayed sample transportation, intermittent bacterial shedding, and reduced bacterial viability may further decrease culture sensitivity.

This limitation is consistent with previous reports demonstrating that conventional culture frequently underestimates the true prevalence of strangles, especially in chronic infections and asymptomatic carriers (Laus *et al.*, 2016; Boyle *et al.*, 2017). Therefore, the inability to obtain pure isolates should not be interpreted as absence of infection but rather as a recognised limitation of culture-based diagnosis under field conditions. The successful molecular detection of *S. equi* in culture-negative samples further supports this interpretation and highlights the greater diagnostic utility of PCR-based methods.

Molecular Detection of *Streptococcus equi* by Conventional PCR

Conventional PCR targeting the *SeM* gene proved to be a reliable method for confirming the presence of *Streptococcus equi* subsp. *equi* in clinical samples. The *SeM* gene is highly conserved and encodes an important virulence-associated surface protein involved in resistance to phagocytosis, making it a suitable molecular target for specific identification of the organism (Timoney, 2004). In the present study, conventional PCR

successfully confirmed infection in clinically suspected animals and demonstrated greater specificity than conventional bacteriological methods.

Although conventional PCR provided accurate species-level identification, its diagnostic sensitivity was influenced by the quality and quantity of DNA present in clinical specimens. Samples containing low bacterial loads or degraded DNA occasionally produced negative results despite strong clinical suspicion. Similar observations have been reported by Newton *et al.*, (2000) and Boyle *et al.* (2017), who suggested that PCR sensitivity may decrease during the early stage of infection, following antimicrobial therapy, or in carrier animals with intermittent bacterial shedding.

Compared with culture, PCR substantially reduced the time required for laboratory confirmation and minimized errors associated with phenotypic variation among closely related streptococcal species. Nevertheless, PCR remains dependent on appropriate sample collection and efficient DNA extraction. Therefore, while conventional PCR represents a valuable confirmatory tool, more sensitive molecular assays may be preferable for surveillance programmes and carrier detection.

Multiplex Real-Time PCR

Multiplex real-time PCR demonstrated superior diagnostic performance compared with conventional PCR and culture, confirming its value as a rapid and highly sensitive diagnostic tool for strangles. The assay simultaneously detected *Streptococcus equi* and differentiated it from closely related streptococcal species, thereby improving diagnostic accuracy while reducing laboratory turnaround time. These findings agree with previous investigations reporting that real-time PCR offers greater analytical sensitivity because it can detect very small quantities of bacterial DNA that may not be recoverable by culture.

The improved sensitivity observed in the present study is particularly important for identifying early infections and asymptomatic carrier animals, in which bacterial shedding is often intermittent and bacterial numbers are low. Early and accurate diagnosis is essential for implementing quarantine measures, preventing disease transmission, and minimizing economic losses associated with strangles outbreaks.

Furthermore, multiplex real-time PCR reduced the likelihood of false-negative results arising from poor bacterial viability or contamination by mixed microbial flora, both of which frequently affect conventional culture methods. These findings support the incorporation of multiplex real-time PCR into routine diagnostic laboratories and surveillance programmes for rapid confirmation of suspected strangles cases.

Despite its advantages, the widespread application of multiplex real-time PCR may be limited by the requirement for specialized laboratory infrastructure, trained personnel, and relatively higher operational costs. However, considering its superior sensitivity, specificity, and rapid turnaround time, the assay represents an important advancement in the molecular diagnosis of equine strangles.

Sero-surveillance Using SeM ELISA

Serological surveillance using the SeM ELISA demonstrated that exposure to *Streptococcus equi* is present among equine populations in northern India. Detection of antibodies against the SeM protein reflects previous exposure to the pathogen and provides valuable information regarding disease circulation within a population. Similar observations have been reported by Robinson *et al.*, (2013) and Waller (2014), who highlighted the usefulness of SeM-based ELISA in epidemiological investigations and surveillance programmes.

Unlike bacteriological culture and PCR, which primarily detect active infection, ELISA identifies the host immune response following exposure. Consequently, seropositive animals may represent recent infection, previous infection, or vaccinated animals, depending on the epidemiological context. Therefore, serological findings should be interpreted together with clinical history and molecular diagnostic results to distinguish active infection from previous exposure.

The present findings emphasize the importance of serological surveillance in identifying areas where *S. equi* circulates and in monitoring herd immunity. Such information is valuable for designing preventive strategies, implementing biosecurity measures, and planning vaccination programmes in endemic regions.

Carrier Detection and Epidemiological Significance

Identification of carrier animals represents one of the most important components of strangles control because clinically recovered horses may continue to harbour

Streptococcus equi within the guttural pouch and intermittently shed the organism without exhibiting obvious clinical signs. These asymptomatic carriers act as a persistent source of infection and contribute to the maintenance and spread of strangles within equine populations.

The present study demonstrated that combining serological screening with molecular confirmation improved the identification of potential carrier animals. ELISA served as an effective screening tool for identifying animals previously exposed to *S. equi*, whereas PCR-based assays confirmed the presence of bacterial DNA in selected animals. This combined diagnostic approach reduced the limitations associated with the use of a single diagnostic method and increased overall diagnostic confidence.

The findings support previous reports recommending integrated diagnostic strategies for surveillance and outbreak investigations. Identification and management of carrier animals through appropriate isolation, treatment, and follow-up testing remain essential components of successful strangles control programmes.

Comparison of Diagnostic Techniques

The comparative evaluation of diagnostic methods demonstrated that each technique possesses distinct advantages and limitations. Conventional bacteriological culture remains valuable because it permits phenotypic characterization and antimicrobial susceptibility testing; however, its sensitivity is frequently reduced by mixed bacterial growth, prior antimicrobial therapy, and poor bacterial viability. Conventional PCR improves diagnostic specificity by confirming the presence of *S. equi* DNA but may fail when bacterial DNA concentrations are very low.

Among all the methods evaluated, multiplex real-time PCR exhibited the greatest diagnostic performance owing to its high analytical sensitivity, rapid turnaround time, and ability to differentiate closely related streptococcal species simultaneously. In contrast, SeM ELISA was particularly useful for serological surveillance and identifying animals previously exposed to infection but could not distinguish active infection from past exposure. Consequently, no single diagnostic method was sufficient for all clinical situations.

The present study therefore supports the use of an integrated diagnostic approach in which serology, bacteriology, and molecular assays are used complementarily. Such an

approach improves diagnostic accuracy, enhances surveillance programmes, and facilitates effective disease control in equine populations.

Limitations of the Present Study

The present investigation had certain limitations that should be considered while interpreting the findings. Firstly, pure isolation of *Streptococcus equi* from field samples could not be achieved because of mixed microbial growth, the fastidious nature of the organism, possible prior antimicrobial treatment, and intermittent bacterial shedding by infected animals. Consequently, culture-based confirmation was limited, emphasizing the superior diagnostic utility of molecular methods under field conditions.

Secondly, although molecular detection confirmed the presence of *S. equi*, DNA sequencing and allele typing of the detected isolates were not performed. As a result, the genetic diversity, strain variation, phylogenetic relationships, and molecular epidemiology of circulating *S. equi* strains in northern India could not be determined. Such analyses would have provided valuable information regarding strain evolution, geographical distribution, and possible transmission pathways.

Future Scope

Future investigations should include whole-genome sequencing or targeted DNA sequencing of *Streptococcus equi* isolates to determine genetic diversity, identify circulating alleles, and establish phylogenetic relationships among strains from different geographical regions. Such molecular epidemiological studies would improve understanding of pathogen evolution and transmission dynamics and would facilitate comparison of Indian isolates with international strains.

Longitudinal surveillance involving larger equine populations across different regions of India should also be undertaken to monitor disease prevalence, identify persistent carrier animals, and evaluate seasonal patterns of infection. Incorporation of guttural pouch endoscopy and lavage sampling, together with highly sensitive molecular diagnostic techniques, would substantially improve carrier detection. In addition, studies evaluating antimicrobial resistance, and genomic variation would contribute to the development of more effective prevention and control strategies for strangles in India.

Chapter-8

Conclusion

This research represented the first integrated and comprehensive investigation into *Streptococcus equi* subsp. *equi* (*S. equi*) in northern India, encompassing isolation, phenotypic and biochemical characterization, molecular confirmation with qPCR, sero-surveillance, and advanced diagnostic evaluation. Strangles remains a disease of significant economic and welfare concern for the equine industry due to its high contagiousness, potential for long-term carriers, and the diagnostic challenges posed by its close genetic relationship with *S. zooepidemicus* and other β -hemolytic *streptococci*. This study was designed to address these gaps systematically, using a multi-objective approach.

The characterization of *S. equi* isolates from clinical and field samples demonstrated a predominance of this pathogen among collected specimens. Phenotypically, the isolates exhibited the typical mucoid colony morphology and β -hemolysis on sheep blood agar, while biochemical characterization revealed a distinctive fermentation profile that aided differentiation from other streptococcal species. Molecular confirmation through *SeM* gene-specific PCR further validated these identifications. The integration of these complementary methods ensured a high level of diagnostic accuracy and reinforced the continued importance of phenotypic and biochemical characterization, particularly in laboratories with limited access to advanced molecular facilities.

The sero-surveillance component of the study provided critical epidemiological insights. For the first time in India, seroprevalence data were generated for strangles in equines, revealing a substantially higher prevalence in field animals (35%) compared to those from organized farm settings (5.6%). This disparity underscores the profound impact of management practices, biosecurity measures, and population density on disease transmission. Importantly, the detection of antibodies in apparently healthy equines confirmed the presence of subclinical carriers—an epidemiologically important reservoir that can silently sustain and propagate outbreaks. The results emphasise the necessity of incorporating carrier detection into disease control strategies and implementing regular serological monitoring to detect the exposure to *S. equi*.

In evaluating diagnostic methodologies, multiplex real-time PCR emerged as a rapid, reliable, and highly sensitive tool for the detection and differentiation of *S. equi* from closely related species. Of the 200 total samples tested multiplex real-time PCR demonstrated 100% sensitivity and specificity in identifying culture-positive cases, outperforming conventional PCR, which, while maintaining high specificity, displayed slightly lower sensitivity (94.1%). Crucially, multiplex real-time PCR identified 14 additional positive cases (representing 43% more detections) missed by conventional PCR—many of which likely represented early or low-bacterial-load infections. This finding validates the incorporation of real-time PCR into routine diagnostic workflows, particularly in outbreak investigations where early detection is vital for effective control.

Geographical analysis of nasal swab results revealed notable variations: the highest detection rates occurred in Haryana, Uttarakhand, and Jammu & Kashmir, whereas no positives were detected in Himachal Pradesh despite a substantial equine population. This may reflect true differences in disease burden, seasonal influences, or the success of local prevention and control measures. Such regional variation highlights the need for geographically targeted surveillance and tailored intervention strategies.

Collectively, the findings from all objectives build a robust diagnostic and epidemiological framework for the effective management of strangles in India. By integrating conventional and advanced diagnostic approaches, this study provides evidence-based strategies for identifying and differentiating *S. equi*, monitoring disease prevalence, and improving outbreak response. The results stress the importance of continued surveillance, carrier detection and management, targeted vaccination programs, and improved farm hygiene practices to break the chain of transmission. In conclusion, this study significantly advances the understanding of *S. equi* epidemiology, diagnostics, and control in India. It provides the first sero-prevalence data for the country, validates multiplex real-time PCR as a superior diagnostic tool, and underscores the role of management practices and surveillance in disease mitigation. These outcomes form a strong foundation for improved national control programs, ultimately aiming to safeguard equine health, welfare, and the economic viability of India's equine sector.

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Strangles in equines: An overview

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ABSTRACT

Strangles, caused by *Streptococcus equi* subspecies *equi*, is a highly infectious respiratory disease affecting horses and other equines. The disease is economically important and compromises the productivity of equine farm significantly. The disease is characterized by pyrexia, mucopurulent nasal discharge, and abscess formation in the lymph nodes of the head and neck of horses. The disease transmission occurs either directly by coming in contact with infectious exudates or indirectly via fomite transmission. Besides this, carrier animals are the primary and most problematic source of disease infection. The organism not only initiates outbreaks but also makes the control and prevention of the disease difficult. The diagnosis of strangles is best done by isolating and characterizing the bacteria from nasal discharge, pus from abscesses, and lymphoid tissues or by using PCR. ELISA can also be used to detect serum protein M (SeM) antibodies for diagnosis. The most popular treatment for strangles is with penicillin; however, the treatment is affected by the stage, feature and severity of the disease. Prevention and control of strangles can be achieved through vaccination and good hygiene practices. Basically, this review describes the global prevalence of *S. equi*, as well as general aspects of the disease, like pathogenesis, diagnosis, treatment, prevention, control and management of the disease.

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Phenotypic, Biochemical and Molecular Characterization of *Streptococcus equi* Isolates in Northern India

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Abstract

Streptococcus equi subsp. *equi* (*S. equi*) poses a significant threat to equine health globally. The bacteria causes strangles, a respiratory disease characterized by enlarged, abscessed lymph nodes, leading to airway obstruction. The bacterium, a Gram-positive, chain-forming, Lancefield group C β -hemolytic *Streptococcus*, is closely related to *S. equi* subsp. *zooepidemicus* and distinction between these species is challenging. The present study focused on characterization of *S. equi* isolates from horses in northern India, aiming at elucidation of their sugar fermentation patterns. Nasal and nasopharyngeal swabs collected from different geographical regions of northern India, yielding *S. equi* isolates were characterized phenotypically, biochemically and at molecular level. Phenotypic identification revealed typical characteristics of *S. equi*, including beta-hemolysis on blood agar and majority of them depicted typical mucoid colony characteristic. Biochemical characterization confirmed the identity of the isolates, with most exhibiting fermentation of maltose, lactose, sorbitol and trehalose. PCR analysis targeting the *SeM* gene provided additional confirmation, displaying a 541 bp product, indicative of *S. equi*. The study underscores the prevalence of *S. equi* in equines in India and emphasizes the importance of accurate diagnosis for timely intervention and disease management. The findings contribute to the understanding of *S. equi* epidemiology and aid in the development of effective control measures to mitigate the impact of strangles outbreaks in the region.

Keywords *Streptococcus* · *S. equi* · Strangles · *S. zooepidemicus* · PCR · Biochemical characterization

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Streptococcus equi M-like Protein Gene Detection from Clinical Cases of Strangles and Apparently Healthy Indian Equines

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ABSTRACT

Strangles is a highly contagious bacterial infection affecting the upper respiratory tract of equids, caused by *Streptococcus equi* subsp. *equi*, a β -hemolytic, gram-positive bacterium classified under Lancefield group C. Early and precise identification of the pathogen is crucial for effective disease control. This study focused on the molecular detection of *Streptococcus equi* M like Protein (*SeM*) gene, a surface protein from clinical cases of Strangles in Indian equines. A total of 45 isolates obtained from abscess material and nasal swabs of clinically diagnosed and apparently healthy Indian equines were analyzed. DNA was extracted and amplified using gene-specific primers targeting a 541 bp region of the *SeM* gene. PCR results confirmed the presence of the *SeM* gene in the isolates, demonstrating the effectiveness of this molecular approach for the detection and identification of *S. equi* and serving as a highly specific molecular marker. PCR was also employed to direct nasal swabs by boil and chill method with successful amplification of *SeM* gene in positive cases (16.7%). The study highlighted the effectiveness of PCR-based diagnostics in enhancing the detection of strangles and advocating the broader implementation of molecular techniques in routine diagnostics, surveillance and epidemiological investigations throughout India.

Key words: Strangles, *Streptococcus equi*, *SeM* gene, PCR, *Streptococcus* species

INTRODUCTION

Strangles is an important bacterial infection affecting the upper respiratory system of

preserved specimens. In contrast, molecular methods such as PCR have gained prominence for their speed, specificity and sensitivity.

The *SeM* gene which encodes the M-like

Multiplex Real Time PCR Assay Targeting the *eqbE* for Rapid Detection and Differentiation of *Streptococcus equi* from Other *Streptococcus* Species in Indian Equines

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Abstract

Strangles is a tremendously contagious and chronic infectious disease caused by *Streptococcus equi* subspecies *equi*. The disease compromises the respiratory health of horses and other equines, leading to decreased productivity. The time-consuming culture methods and similarity between *Streptococcus equi* subspecies *equi* and *Streptococcus equi* subspecies *zooepidemicus*, which can cause opportunistic infections in horses and severe infections in humans, complicate its diagnosis. In this study, we applied a multiplex real-time PCR assay on 45 *Streptococcus* isolates from India, aiming to detect *Streptococcus equi* subspecies *equi* and distinguish it from other *Streptococcus* species, including *Streptococcus equi* subspecies *zooepidemicus*. Multiplex real-time PCR using *eqbE* gene of *Streptococcus equi* subspecies *equi* accurately identified all culture-positive samples, yielding 100% sensitivity and specificity. In contrast, conventional PCR revealed a lower sensitivity of 94.1%. Both tests showed no false positives, indicating high specificity. The ability to swiftly identify and differentiate *Streptococcus equi* subspecies *equi* from other *Streptococcus* species facilitates targeted intervention strategies, ultimately aiding in the prevention and mitigation of this debilitating disease. The distribution underscores the necessity of strengthening surveillance, implementing early diagnosis protocols, and adopting targeted control strategies in the affected regions. This is the first report in India of a multiplex real-time PCR for comparative analysis of molecular diagnostic assays for the detection and differentiation of *Streptococcus equi* subspecies *equi* in Indian equines. It established a valuable precedent for future epidemiological surveillance and molecular diagnosis.

Keywords Strangles · *Streptococcus equi* · Multiplex Real-time PCR · *Streptococcus* species

Abbreviations

PCR Polymerase Chain Reaction
RT PCR—Real-time PCR
NTC Non-template control
NFW Nuclease-free water

Introduction

Strangles is an important bacterial infection affecting the upper respiratory system of equids. The etiology of the disease is a spherical, gram positive, beta hemolytic and pus forming bacterium *Streptococcus equi* subspecies *equi* (*S. equi* subsp. *equi*) belonging to "Lancefield group C". It is a widely reported disease throughout the world. Affected equids typically exhibit pyrexia, muco-purulent rhinorrhea, and abscess formation within the head and neck lymph nodes [1–3]. *S. equi* subsp. *equi* primarily infects the upper respiratory tract and can lead to serious complications, including metastatic abscesses (bastard strangles), purpura hemorrhagica, formation of chondroids and persistent carrier states [1, 4, 5]. The major challenge in control of strangles is the shedding of bacteria for several weeks after complete recovery from the disease due to rupture of these abscesses into the guttural pouches and affecting naive horses [6–8].

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Appendix

Table Epidemiological Data of collected samples from Northern India.

Owners Details			Particular of Animal						
Name	Address	Date of Collection	Name/No. of animal	Age (years)	Sex M/F	Breed	Color	Symptoms	Previous Disease history of Animal.
1	Haryana		H - 216	21	F	Marwari		No symptoms	Pregnant
2.			H - 225	21	F	Marwari		No symptoms	No symptoms
3.	NRCE		H - 228	20	F	Marwari		No symptoms	No symptoms
4.	NRCE		H - 256	12	M	Marwari		No symptoms	No symptoms
5.	NRCE		H - 263	9	F	Marwari		Pregnant	Pregnant
6.	NRCE		H - 275	5	F	Marwari		No symptoms	No symptoms
7.	NRCE		H -287 Foal of 225)	1	F	Marwari		No symptoms	No symptoms
8.	NRCE		96		F	Poitou	White	No symptoms	No symptoms
9.	NRCE		74		F	Poitou	White	Nasal Discharge	Nasal Discharge
10.	NRCE		62		F	Poitou	White	No symptoms	No symptoms
11.	NRCE		80		F	Poitou	White	No symptoms	No symptoms
12.	NRCE		26			Pony	White	No symptoms	No symptoms
13	NRCE		24			Pony	White	No symptoms	No symptoms
14	NRCE		19			Pony	White	No symptoms	No symptoms
15	NRCE		17			Pony	White	No symptoms	No symptoms
Pramod 16	Luvas (Jhunjhunu)		1871	7 yrs	Female Mare			No symptoms	No symptoms
Vikram 17	Luvas		11152	8 months	Female	Mare		No symptoms	No symptoms
Haryana Police 18	Bhiwani		49	5 years	Female	Mare	Black	No symptoms	No symptoms
Haryana Police 19	Bhiwani		45	5 years	Female	Mare	Black	No symptoms	No symptoms
Haryana Police 20	Bhiwani		40	5 years	Female		Black	No symptoms	No symptoms
animal health camp 21	Pabra		Basanti		female	Mare			
10 th march 2023									
TARC	Equestrian farm		Copetto	13years	M	Gelding	Brown	No symptoms	

TARC	Equestrian farm		Zua		horse	Gelding	Brown	No symptoms	
23	LUVAS		14227		Mare		Brown	No symptoms	
24	LUVAS		14228		Mare		Brown	No symptoms	
25	LUVAS		14229		Mare		White	No symptoms	
26	LUVAS		14230		Mare		White	No symptoms	

Sample Performa Data of Punjab

Sr No.	Owners Details			Particular of Animal					
	Name	Address	Mobile no.	Name	Age	Sex M/F	Breed	Colour	Symptoms
1.	Mankaran Sandhu	Ludhiana	GADVASU	5985	6.5yrs	Female	Marwari	Dark brown	constipation
2.	Balvir singh	Moga	GADVASU	6049	5 months	Female	Marwari		anaemic
3.	Balvinder Singh	Bathinda	GADVASU	6043	2.5yrs	Male	Nukra	White	leukopenia
4.	Sample Lovely	Jalandhar		Sample Lovely	6 yrs	Female	Mare	Dark bay	No symptoms
5.	Noor Garh stud farm	Moga	9914740969	Katu	8 yrs	Female	Mare	White	No symptoms
6.	Noor Garh stud farm	Moga	9914740969	Mohini	14yrs	Female	Mare	Dark bay	Pregnant No symptoms
7.	Noor Garh stud farm	Moga	9914740969	Roshni	13yrs	Female	Mare	Dark bay	No symptoms
8.	Noor Garh stud farm	Moga	9914740969	Morni	14yrs	Female	Mare	Dark bay	No symptoms
9.	Noor Garh stud farm	Moga	9914740969	Dilbar	19yrs	Male	Horse	Dark bay	No symptoms
10.	Noor Garh stud farm	Moga	9914740969	Parwat	19yrs	Male	Horse	Dark bay	No symptoms

Sample Performa Data of Rajasthan

Owners Details			Particular of Animal						
Name	Address	Mobile no.	Name	Age	Sex M/F	Breed	Color	Symptoms	Previous Disease history of Animal.
EPC NRCE	Bikaner	National Institute Contact no.	94 Karishma	12 Yrs	F	Marwari	Pie Bald	Non-Pregnant	-
EPC NRCE	Bikaner	National Institute Contact no.	97 Babli	12 Yrs	F	Marwari	Skew Bald	Pregnant	-
EPC NRCE	Bikaner	National Institute Contact no.	138 Himmi	8yrs	F	Marwari	Skew Bald	Pregnant	-
EPC NRCE	Bikaner	National Institute Contact no.	147 Madi	7 Yrs	F	Marwari	Roan	Non-Pregnant	-
EPC NRCE	Bikaner	National Institute Contact no.	148 Mangli	7yrs	F	Marwari	Black	Non-Pregnant	-
EPC NRCE	Bikaner	National Institute Contact no.	149 Kanwal	7 Yrs	F	Marwari	Black	Non-Pregnant	-
EPC NRCE	Bikaner	National Institute Contact no.	169 Bobby	5 yrs	F	Marwari	Skew Bald	Pregnant	-
EPC NRCE	Bikaner	National Institute Contact no.	173 Renu	5 yrs	F	Marwari	Bay	Non-Pregnant	-
EPC NRCE	Bikaner	National Institute Contact no.	247	12 yrs	F	Marwari	Roan	Non-Pregnant	-
EPC NRCE	Bikaner	National Institute Contact no.	K- Dev madi	11 yrs	F	Kathiawar	chestnut	Non-Pregnant	-
EPC NRCE	Bikaner	National Institute Contact no.	F- 36	12 yrs	F	Poitou	White	Pregnant	-
EPC NRCE	Bikaner	National Institute Contact no.	F – 47	8 yrs	F	Poitou	White	Pregnant	-
EPC NRCE	Bikaner	National Institute Contact no.	F – 51	6 yrs	F	Poitou	White	Pregnant	-
EPC NRCE	Bikaner	National Institute Contact no.	F – 52	5yrs	F	Poitou	White	Pregnant	-
EPC NRCE	Bikaner	National Institute Contact no.	F – 53	4 yrs	F	Poitou	White	Pregnant	-
	JODHPUR SAMPLES								-
Sh. Akshay Jain	Balotra Rajasthan	Mob: 7568066003		8yrs	Mare		(Pie Bald)	No symptoms	-

Akshay Jain	Balotra	Mob: 7568066003		8yrs	Mare			No symptoms	-
Sh. Akshay Jain	Balotra, Rajasthan	Mob: 7568066003		7 yrs	Mare	Nukra		No symptoms	-
Sh. Akshay Jain	Balotra, Rajasthan	7568066003	Bholi	8 yrs	Mare			No symptoms	-
Sh. Akshay Jain	Balotra, Rajasthan	7568066003	Lakshmi	8 yrs.	Mare	Marwari		No symptoms	-
Sh. Akshay Jain	Balotra, Rajasthan	7568066003	(Radhika)	8 yrs.	Mare	Marwari Nukra		No symptoms	-
Akshay Jain	Balotra, Rajasthan	7568066003						No symptoms	-
Sh. Rawal Mallinath trust Mandir	Tilwara	9928557105	Mohan	22 yrs.		stallion	Bay Color	No symptoms	-
Sh. Rawal Mallinath trust Mandir	Tilwara	9928557105	Maya	25 yrs.	Mare		Chest Nut white strip,	No symptoms	-
Babu Lal	Udaipur	9928557105	-	4yrs	Mare		Bay	No symptoms	-
Bilal Khan	Barmer	9928557105	-	5yrs	Mare		Bay	No symptoms	-
Sh. Jalam Singh	Barmer	8529723690	-			Stallion	Chestnut	No symptoms	-
Kalu Ram	Chittore		-	2 ½ Yrs	Nukra	Stallion		No symptoms	-
Sh. Sanjay Kumar	Udaipur	88242166	Sonu	6yrs	Mare,	Panch Kalyani	Bay Color	No symptoms	-

Sample Performa data of Uttarakhand

Sr No.	Owners Details			Particular of Animal					
	Name	Address	Mobile no.	Name	Age	Sex M/F	Breed	Colour	Symptoms
1.	Rakesh Kumar	Uttarakhand	-	986	3yrs	M	Mule		
2.	Amit Kumar	Uttarakhand	-	1255 RS 44	5 yrs	M	Mule		No symptoms
3.	Satya Prakash	Uttarakhand	-	1259 RS 50	4 yrs	M	Mule		No symptoms
4.	Sunil	Uttarakhand	-	1262 RS 54	6 yrs	M	Mule		No symptoms
5.	Gauri Shankar	Uttarakhand	-	1267 RS 61	6 yrs	M	Mule		No symptoms
6.	Sanjay prasad	Uttarakhand	-	1268 RS 62	5 yrs	M	Mule		No symptoms
7.	Gauri Shankar	Uttarakhand	-	1269 RS 61	6yrs	M	Mule		No symptoms
8.	Sanjay prasad	Uttarakhand	-	1271 RS 63	5yrs	M	Mule		No symptoms
9.	Kailash chander	Uttarakhand	-	1275 RS 67	5yrs	M	Mule		No symptoms
10.	Mukesh	Uttarakhand	-	1279 RS 71	5yrs	M	Mule		No symptoms
11	Akhilesh	Uttarakhand	-	1280 RS - 72	5yrs	M	Mule		No symptoms
12	Vishnu	Uttarakhand	-	1285 RS - 79	5yrs	M	Mule		No symptoms
13	Ram chander Singh	Uttarakhand	-	1287 RS - 82	4yrs	M	Mule		No symptoms
14	Anoop	Uttarakhand	-	1292 RS - 88	5yrs	M	Mule		No symptoms
15	Kishan Singh	Uttarakhand	-	1295 RS - 91	4yrs	M	Mule		No symptoms
16	Prakash goswami	Uttarakhand	-	1299 RS - 97	6 yrs	M	Mule		No symptoms
17	Vishnu	Uttarakhand	-	1323 RS - 129	6yrs	M	Mule		No symptoms
18	Mohammad rafiq	Uttarakhand	9389487725	1325 K - 68	3yrs		Mule		No symptoms
19.	RAHUL	Uttarakhand CHAMOLI	8433118434	1224 DJ -1	4yrs	M	Horse		No symptoms
20.	PREM LAL	Uttarakhand	6397268951	1223 P -9	6yrs		Mule		No symptoms
21.	RAHUL	Uttarakhand	8433118434	1225 DJ2		M	Horse		No symptoms
22.	SANJAY SINGH	Uttarakhand	7078310915	1217 P3	3yrs	F	Mare		No symptoms

Sample collection Performa of Himachal Pradesh

Sr No.	Owners Details			Particular of Animal					
	Name	Address	Mobile no.	Name	Age	Sex M/F	Breed	Colour	Symptoms
1.	Sh. Hari Singh	V.P.O Dhanag	7559627865	E1	15years	Female (mule)	Non-Descript	Brown	NAD
2.	Sh. Hari Singh	V.P.O Dhanag	7559627865	E2	25years	Female (mule)	Non-Descript	Bay	NAD
3.	Sh. Hari Singh	V.P.O Dhanag	7559627865	E3	20years	Female (Mule)	Non-Descript	Brown	NAD
4.	Sh. Hari Singh	V.P.O Dhanag	7559627865	E4	15years	Female (Mule)	Non-descript	Brown	NAD
5.	Chet Ram	V.P.O Dhanag	8627839897	E5	19Years	Female (Mule)	Non-descript	Bay	NAD
6.	Chet Ram	V.P.O Dhanag	8627839897	E6	12 years	Female (Mule)	Non-Descript	Brown	NAD
7.	Chet Ram	V.P.O Dhanag	8627839897	E7	18years	Female (Mule)	Non-descript	Black	NAD
8.	Sh. Joginder Ram	Vill Baraun P.O Dhanag	8894387516	E8	18years	Female (Mule)	Non-descript	Brown	NAD
9.	Sh. Joginder Ram	Vill Baraun P.O Dhanag	8894387516	E9	12years	Female (Mule)	Non-descript	Bay	NAD
10.	Sh. Joginder Ram	Vill Baraun P.O Dhanag	8894387516	E10	14years	Female (Mule)	Non-descript	Brown	NAD
11	Sh. Madan lal	Vill Baraun P.O Dhanag	8894347910	E11	17years	Female (Mule)	Non-descript	Black	NAD
12	Sh. Madan lal	Vill Baraun P.O	8894347910	E12	12years	Female (Mule)	Non-Descript	Brown	NAD
13	Sh. Madan lal	Vill P.O Baraun	8894347910	E13	16years	Female (Mule)	Non-Descript	Brown	NAD
14	Sh. Bairagi Ram	Vill P.O Baraun	9805351279	E14	15years	Female (Mule)	Non-Descript	Black	NAD

15.	Sh. bairagi Ram	Vill Baraun P.O Dhanag	9805351279	E15	18years	Female (Mule)	Non-descript	Brown	NAD
16.	Karam-das	HP	-	32 - 25613 32 - 25369	7yrs	Female	Mule	Brown	
17.	Biri Singh	HP	-	53 - 25614 25370	7yrs	Female	Mule	Brown	
18.	Sanjeev Kumar	HP	-	BHJ - 13	5yrs	Female	Mare	Brown	
19.	7 October	HP	-	Nasal serum negative	3yrs	Female	Mare	Blackish brown	
20	Budh ram	Dharam Pur	-	DMP E-2	11yrs	Female	Mule	Chestnut	No symptoms
21	Thakur das	Dharm Pur	-	DMP E-5	7yrs	Female	Mule	Chestnut	No symptoms

Performa of Sample collection of Jammu & Kashmir

Sr No.	Owners Details			Particular of Animal					
	Name	Address	Mobile no.	Name	Age	Sex M/F	Breed	Colour	Symptoms
1.	Mohd Afzal 45	Amarnath (J & K)	-	1550	BT 4	5yrs	Mule	Grey color	No symptoms
2.	NS – BT 1	Amarnath (J & K)	-	1547	NS – BT 1			Grey color	No symptoms
3.	CW – 3	Amarnath (J & K)	-	CW – 3		Pony		Black	No symptoms
4.	5- BT -2	Amarnath (J & K)	-	5- BT -2				Grey color	No symptoms
5.	BT- 3	Amarnath (J & K)	-	BT- 3				Grey color	No symptoms
6.	CW- 1	Amarnath (J & K)	-	CW- 1 1600		Pony		Grey color	No symptoms
7.	CW- 2	Amarnath (J & K)	-	CW- 2 1601		Pony		White color	No symptoms
8.	NS- BT 2	Amarnath (J & K)	-	1602					No symptoms
9.	Basir ahmed hajam	Baltal	-	1548	7yrs		Mule	White	No symptoms
10.	Mohammad Afzal	Baltal	-	1549	6yrs		Mule	Grey color	No symptoms
11.	Mohammad Afzal	Baltal	-	1551	5yrs		Mule	Grey color	No symptoms
12.	Pony raja	Jammu and Kashmir	-	-	4 yrs		Mule	Grey color	No symptoms
13.	Lakshmi	Jammu and Kashmir	-	-	4yrs		Mule	Grey color	No symptoms
14.	Pony Shahan shah	Jammu and Kashmir	-	-	7 yrs		Mule	Grey color	No symptoms
15.	Firdaus horse	Jammu and kashmir	-	-	6yrs		Mule	Grey color	No symptoms
16.	Sufi	Jammu and	-	-	4 yrs		Mule	Grey color	No symptoms

		kashmir							
17.	Darshan	Jammu and kashmir	-	-	7yrs		Mule	Grey color	No symptoms
18.	Pony chib	Jammu and kashmir	-	-	4yrs		Mule		No symptoms
19.	Lone Sazad Ah Mir	Jammu and kashmir	-	-	5yrs		Pony	Grey color	No symptoms
21.	Bilal Ahmed	Anantnag 1586	-	-	6yrs		Pony	Grey color	No symptoms
22.	Bashir	Gade shar 1585	-	-	5 yrs		pony	Grey color	No symptoms
23.	Ishrad Ahmad shah	Chandan wadi 1631	-	-	4yrs		Pony	Grey black	No symptoms
24.	Ishrad ahmad shah	Chandan wadi 1632	-	-	7yrs		Mule	Black	No symptoms
25.	Nadeer		-	-	8yrs		Donkey	Grey and brown	No symptoms
26	Nawaz Ahmed 1572	Anantnag	-	-	4yrs		Pony	Grey black	No symptoms
27.	Mulehtar 1573	Anantnag	-	-	4yrs		Mule	Grey black	No symptoms
28.	Rahat Ahmad 1574	Anantnag	-	-	5yrs		Mule	Grey black	No symptoms
29.	Qadir	Anantnag	-	-	6yrs		Pony	Grey black	No symptoms
30.	Bilal Ahmed	Anantnag	-	-	4yrs		Pony	Black	No symptoms
31.	Gull Mohammad 1620	Anantnag	-	-	4yrs		PONY	Blackish	No symptoms
32	Fayaz	Anantnag	-	-	3 yrs		Pony	Blackish	No symptoms
33	Qammar zaman	Rajouri	-	-	4 yrs		Horse	Blackish	No symptoms
34	Mohammad saqib	Rajouri	-	-	7yrs		Horse	Blackish	No symptoms

35	Mohammad zabir	Rajouri	-	-	4 yrs		Horse	Blackish	No symptoms
36	Layakit	Rajouri	-	-	5 yrs		Horse	Blackish	No symptoms
37	Gulam	Rajouri	-	-	7yrs		Horse	Blackish	No symptoms
38	Mumtaz	Rajouri	-	-	6yrs		Horse	Blackish	No symptoms
39	Shabir AHMAD	Rajouri	9797329664	-	6yrs		Horse	Blackish	No symptoms
40	Mohammad ARIF	Rajouri	9797413466	-	4yrs		Horse	Blackish	No symptoms
41	Rashid khan	Rajouri	7006597079	-	7 yrs		Horse	Blackish	No symptoms
42	Aftab Ahmad	Rajouri	9903608840	-	6 yrs		Horse	Blackish	No symptoms
43	Mohammad BASSHIR	Rajouri	9797404161	-	5 yrs		Horse	Blackish	No symptoms
44	Sahil Khawaja	Rajouri	9682301446	-	4 yrs		Horse	Blackish	No symptoms
45	Mir Kasam	Rajouri	9797404161	-	5yrs		Horse	Blackish	No symptoms
46	Asif khan	Rajouri	9623392594	-	6 yrs		Horse	Blackish	No symptoms
47	Mohammad Tobeeb	Rajouri	7889415860	-	7yrs		Mare	Blackish	No symptoms
48	Mohd Kashan	Rajouri	9596095310	-	6 yrs		Horse	Blackish	No symptoms
49	Mukhtar Ahmad	Rajouri	9622555962	-	5 yrs		Horse	Blackish	No symptoms
50	Arshid Khan	Rajouri	6006143804	-	4yrs		Horse	Blackish	No symptoms
51	Naseer Ahmad	Rajouri	9797329664	-	3 yrs		Horse	Blackish	No symptoms
52	Mohammad Maroof	Rajouri	9596677259	-	7yrs		Horse	Blackish	No symptoms
53	Ali Jan	Rajouri	9596677259	-	7yrs		Horse	Blackish	No symptoms
54	Bashir Ahmad	Rajouri	7051059310	-	4 yrs		Mare	Blackish	No symptoms
55	Bilal Ahmad	Rajouri	7051059310	-	3 yrs		Mare	Blackish	No symptoms
56	Mohd Sjid	Rajouri	9622147207	-	4 yrs		Horse	Blackish	No symptoms
57	Sofian Ahmad	Rajouri	8492056504	-	7 yrs		Horse	Blackish	No symptoms
58.	Kauser Hussain	Rajouri	8492955653	-	7 yrs		Horse	Blackish	No symptoms

Performa of Sample collection of Uttar Pradesh

Sr No.	Owners Details			Particular of Animal					
	Name	Address	Mobile no.	Name	Age	Sex M/F	Breed	Color	Symptoms
	Israr	Akhtiyar-pur	9917249591	Basanti	5years	F	Mare	BAY	No Symptoms
	Ram Nath	Dhingra	7466877930		9 years	F	Mare	BAY	No Symptoms
	Ram Nath	Dhingra		RANI	8 years	F	Mare	BAY	No Symptoms
	Kalua	Dhingra	8126247664	GHODI	8 years	F	Mare	BAY	No Symptoms
	Kailash	Dhingra	8449057411	RANI	3 years	F	Mare	BAY	No Symptoms
	Joginder	Dhingra	9690014084	PINKI	3 years	F	Mare	BAY	No Symptoms
	Naube	Dhingra	9756399749	B GHODI	5 years	F	Mare	BAY	No Symptoms
	Ram Avtar	Dhingra	8285392209		9 years	F	Mare	BAY	No Symptoms
	Tiku	Dhingra	7055954381	Muskan	5 years	F	Mare	BLACK BAY	No Symptoms
	Narender	Dhingra	7983468660	rani	6 years	F	Mare	BALD	No Symptoms
	Shamim	PELLI	9719373598	Ghoda	6 years	F	Mare	BAY	DEPRSEESD
	Shamim	PELLI	9719373598	Ghoda	6 years	F	Mare	BAY	EYE WOUND on DEPRSEESD
	Tahir	Muzaffarabad	-	-	4 years	M	Horse	White Brown	No Symptoms
	8032	Meerut	-	-	6 years	F	Mare	Black	No Symptoms
	8033	Meerut	-	-	8 years	F	Mare	Black	No Symptoms
	8034	Meerut	-	-	10 years	F	Mare	Black	No Symptoms
	8035	Meerut	-	-	9 years	F	Mare	Black	No Symptoms
	8036	Meerut	-	-	6 years	F	Mare	Black	No Symptoms
	Mahendra Kumar	Agra	-	-	8 years	Mare	Kathia wari	Black	No Symptoms
	Mahendra Kumar		-	-	5 years	Female	Mare Kathia wari	Black	No Symptoms
	Mahendra Kumar	Mathura	-	-	1 years	F++	Kathia wari	Black	No Symptoms
	Sunil Kumar	Varanasi	-	-	4 years	F	Mare	Black	No Symptoms
	Sample testing	Lucknow	-	-	7 years	F	Mare		No Symptoms
	Sample testing	1430	-	-	7 years	F	Mare	BAY	No Symptoms
	Sample testing	1431	-	-	7 years	F	Mare	BAY	No Symptoms
	Sample testing	1432	-	-	7 years	F	Mare	BAY	No Symptoms

	Sample testing	Allahabad	-	-	7 years	F	Mare	BAY	No Symptoms
	Som pal	Bareilly	-	-	7 years	F	Mare	BAY	No Symptoms
	Som pal	Bareilly	-	-	7 years	F	Mare	BAY	No Symptoms
	Prem Singh	Bareilly	-	-	8 months	F	Pony	BAY	No Symptoms
	Satpal	Bareilly	-	-	8 years	F	Mare	BAY	No Symptoms
	Monu	Bareilly	-	-	7 years		Pony	BAY	No Symptoms
	Irfan	Bareilly	-	-	4 years	F	Mare	BAY	No Symptoms
	gufran	Bareilly	-	-	6 years	F	Pony	BAY	No Symptoms
	Tahir	Bareilly	-	-	4 years	F	Mare	BAY	No Symptoms
	Mahavir	Bareilly	-	-	8 years	F	Mare	BAY	No Symptoms
	Mahavir	Bareilly	-	-	8 years	F	Mare	BAY	No Symptoms
	Ram Chandran	Bareilly	-	-	7 years	F	Mare	BAY	No Symptoms
39.	Virendra	Muzaffarabad	-	-	6yrs	F	Mule	BAY	No Symptoms
40.	Mahender	Muzaffarabad	-	-	8 years	F	Mule	BAY	No Symptoms
41.	Bhagat	Muzaffarabad	-	-	10 years	F	Mule	BAY	No Symptoms
42.	Bhagat	Muzaffarabad	-	-	9 years	F	Mule	BAY	No Symptoms
43.	Mehar-van	Muzaffarabad	-	-	6years	F	Mule	BAY	No Symptoms
44.	Sukhdev	Muzaffarabad	-	-	8years	F	Mule	BAY	No Symptoms
45.	Mohammad Ali	Muzaffarabad	-	GHK 1	5yrs	Female	Mule	BAY	No Symptoms
46	Nassim Ahmad	Muzaffarabad	-	-	4yrs	Female	Mare	BAY	No Symptoms
47.	Mastan	Muzaffarabad	-	-	6yrs	Female	Mare	BAY	No Symptoms
48	Irshad	Muzaffarabad	-	-	8yrs	Female	Mare		No Symptoms
49	Jai	Muzaffarabad	-	-	6yrs	Female	Mare		No Symptoms
50	Mohammad Ali	Muzaffarabad	-	-	4yrs	Female	Mare		No Symptoms
51	Salim	Muzaffarabad	-	-	3yrs	Female	Mare		No Symptoms
52	Kayyum	Muzaffarabad	-	-	2yrs	Female	Mare		No Symptoms

Table 6.3

Tests	1	2	3	4	5
Voges Proskauer's	Not detected	-ve	Variable reaction	-ve	+ve
Esculin Hydrolysis	Not detected	-ve	+ve	Variable reaction	-ve
PYR	+ve	+ve	+ve	-ve	+ve
ONPG	+ve	Variable reaction	+ve	+ve	-ve
Arginine Utilization	+ve	-ve	+ve	+ve	-ve
Glucose	+ve	+ve	Not detected	Variable reaction	+ve
Lactose	Not clear	+ve	Not detected	Variable reaction	+ve
Arabinose	Not clear	+ve	+ve	Not clear	+ve
Sucrose	+ve	+ve	-ve	-ve	+ve
Sorbitol	-ve	+ve	+ve	-ve	+ve
Mannitol	+ve	+ve	-ve	-ve	+ve
Raffinose	+ve	Variable reaction	Variable reaction	Variable reaction	Variable reaction