Sveriges lantbruksuniversitet
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Klinisk och subklinisk mastit hos mjölkkor och bufflar i Bihar, Indien

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SUMMARY

Bihar, located in north-eastern India, is a state with a growing dairy sector. Many people live under the poverty line and depend on the livestock and dairy production from cattle and buffaloes for their livelihood. Mastitis is known to result in substantial production and economic losses which can be crucial for small-scale dairy farmers. The knowledge about the situation regarding mastitis in Bihar is limited. The objectives of the study were to estimate the prevalence of mastitis in cattle and buffaloes, as well as to identify common udder pathogens and to identify possible risk factors of mastitis in cattle. The study was conducted in rural, peri-urban and urban households in Bihar during September and October 2015. In total, 285 cows and 28 buffaloes were included in the study. General information regarding herd and management factors was collected as well as details of the specific animals. The prevalence of subclinical and clinical mastitis was determined through clinical examination of the udder and by using California mastitis test (CMT) to evaluate somatic cell count in milk samples. Samples with CMT ≥3 were examined for presence of bacteria. Some of the samples were also tested with a rapid test (MastiTest) to evaluate sensitivity and resistance to antimicrobials. In cattle, the prevalence of subclinical and clinical mastitis was 35.4% and 11.6% respectively. The prevalence of subclinical mastitis in buffaloes was 28.6%, no cases of clinical mastitis were found. Out of 145 quarter milk samples from cattle, *Staphylococcus aureus* was the predominant bacteria (28.3%) followed by other *Staphylococcus* species (21.3%) and *Streptococcus* species (17.9%). Out of four quarter milk samples from buffaloes, three were negative for bacterial growth and one was contaminated. Floor type and presence of a drainage system had a significant association with prevalence of subclinical mastitis in cattle. Cows held on concrete floor had a lower prevalence of subclinical mastitis compared to cows kept on earthen or brick floor. Cows held in farms with a drainage system had a lower prevalence of subclinical mastitis. However, parity number, lactation stage and hygiene score had no association with the prevalence of mastitis in cattle. The results from the study indicate that the prevalence of mastitis in dairy cattle and buffaloes is high. Knowledge about preventive measures is essential to control mastitis. As for Bihar, preventive measures should be focused on emphasizing the importance of applying high hygienic standards of housing and milking practices.

SAMMANFATTNING

Bihar är en stat i nordöstra Indien som har en växande mjölksektor. Många människor i området lever under fattigdomsgränsen och är beroende av sina djur och mjölkproduktionen från kor och bufflar för sin överlevnad. Mastit är en välkänd orsak till en betydande produktionsförlust med ekonomiska konsekvenser, vilket kan vara kritiskt för småskaliga mjölkbönder. Det finns begränsat med information kring situationen gällande mastit i Bihar. Syftet med denna studie var att uppskatta förekomsten av mastit hos kor och bufflar, identifiera förekommande patogener samt undersöka möjliga riskfaktorer för mastit hos kor. Studien genomfördes inne i städer, runt städerna samt på landsbygden i Bihar under september och oktober 2015. Totalt 285 kor och 28 bufflar ingick i studien. Allmän information om besättningen och skötselfaktorer samt uppgifter om de specifika djuren samlades in. Förekomsten av subklinisk och klinisk mastit fastställdes genom klinisk undersökning av juvret samt mätning av celltal i mjölkprover med hjälp av California mastitis test (CMT). Prover med CMT ≥3 undersöktes bakteriologiskt. Några av proverna testades även för känslighet mot antibiotika med ett snabbtest (MastiTest). Hos kor var prevalensen av subklinisk och klinisk mastit 35,4 % respektive 11,6 %. Förekomst av subklinisk mastit hos bufflar var 28,6 %, inga fall av klinisk mastit hittades. Av 145 mjölkprover från kor var *Staphylococcus aureus* den vanligaste bakterien (28,3 %) följt av övriga bakterier i genus *Staphylococcus* (21,3 %) och *Streptococcus* (17,9 %). Av fyra mjölkprover från bufflar var tre negativa för bakterieväxt och ett var kontaminerat. Golvtyp och närvaro av ett dräneringssystem hade ett signifikant samband med prevalensen av subklinisk mastit hos kor. Kor som hölls på betong hade en lägre förekomst av subklinisk mastit jämfört med kor som hölls på golv av jord eller tegelstenar. Kor som hölls på gårdar med dräneringssystem hade en lägre förekomst av subklinisk mastit. Det sågs dock inget samband mellan mastitförekomst och laktationsnummer, laktationsstadium eller smutsighetsgrad hos kor. Resultatet i studien visade att mastit är vanlig förekommande hos både mjölkkor och bufflar. Kunskap om förebyggande åtgärder är nödvändigt för att bekämpa sjukdomen. För att minska förekomsten av mastit i Bihar bör vikten av en hög hygienisk standard i kons närmiljö och kring mjölkning belysas.

CONTENT

INTRODUCTION

Mastitis is considered to be one of the most common and substantial production diseases of dairy livestock worldwide (Ruegg & Erskine, 2015). The disease results in decreased production, discarded milk and medical treatments as well as a higher level of premature culling of affected animals. The economic loss due to the disease is considerable and can be crucial, especially for small-scale dairy farmers in developing countries (FAO, 2014). Milk and milk products are considered to contribute to the social and economic development in rural areas where the dairy production from cattle and buffaloes is one of the major sources of income in many households (Singh, 2013). Dairy products also provide essential food and nutrition for people in these areas. After egg products, milk products are the major livestock products in Bihar (Department of Animal Husbandry, Dairying and Fisheries, 2014).

The dairy sector in India is growing and both milk production and the per capita availability of milk have increased (Department of Animal Husbandry, Dairying and Fisheries, 2014). However, India still counts as a developing country with problems with poverty and hunger, especially in rural areas (Gov. of India, 2013). Since many people in the rural areas are depending on livestock and dairy production for their livelihood, development in these sectors is seen as a tool for reducing poverty (Hemme & Otte, 2010).

Bihar is one of the poorest states in India, and with a large part of the population living in rural areas, problems regarding the productivity in livestock is of great concern. In Bihar, mastitis in cattle and buffaloes is estimated to result in a substantial economic loss and is therefore ranked as a high priority disease for research (Singh, 2013). Despite this, there are few available published articles regarding the subject. According to the FAO (2014), awareness of risk factors and pathogens causing mastitis are essential to control the disease in developing countries. Also, preventive measures are important to minimize antimicrobial usage and to avoid development of antimicrobial resistance (OEI, 2003).

OBJECTIVES

The objective of the study was to investigate the prevalence of mastitis in cattle and buffaloes, to identify common pathogens and to identify risk factors that can be controlled to reduce disease in cattle. The long-term aim of research in this area is to contribute knowledge that in the future may lead to an improvement of animal health and production in Bihar.

The study was conducted within the MFS (Minor Field Studies) programme, financed by the Swedish government agency Sida. Therefore, the aim of this project was also to exchange knowledge about development issues as well as contribute to international collaboration between Swedish University of Agricultural Sciences and local institutions in the area.

This master thesis was a part of a larger project, including three master students, regarding reproductive and zoonotic diseases as well as other diseases and management factors that have a negative impact on productivity.

LITERATURE REVIEW

The dairy livestock situation in Bihar, India

Bihar, located in north-eastern India, is the twelfth largest state in the country. Bihar is a densely populated region and almost 90% of the 104 million inhabitants live in rural areas (Census Organization of India, 2011). Although the poverty ratio has declined in recent years, still about 33.7% of the population was estimated to live below the poverty line in the census of 2011-12 (Gov. of India, 2013).

The agricultural and livestock sectors play an important role in the social and economic development in the region, especially in the rural areas whereas people depend on these sectors for their survival. The economy in Bihar is agricultural-based and the contribution of agriculture to the GDP of Bihar was 21.3% 2009-10 (UNDP, 2011). In Bihar, the livestock sector contributes to about 45% of the state agricultural GDP (gross domestic product) (Singh *et al*., 2010).

The dairy sector in Bihar is important and has increased substantially during the past years (Department of Animal Husbandry, Dairying and Fisheries, 2014). The milk production has increased from 5.9 million tons in 2008-09 to 6.8 million tonnes in 2012-13 (Department of Animal Husbandry, Dairying and Fisheries, 2014). Of these, about 3.8 million tons of milk originate from cattle and 2.9 million tonnes from buffaloes. Milk production from goats only contributes with 0.18 million tonnes. The availability of milk per capita has also increased from 172 gram/day in 2008-09 to 188 gram/day in 2012-13, but this is still less than the national availability of 299 gram/day (Department of Animal Husbandry, Dairying and Fisheries, 2014).

The estimated dairy animal population in Bihar 2012 was 1.6 million exotic/crossbreed cows, 2.9 million indigenous cattle and 3.1 million milking buffaloes (Department of Animal Husbandry, Dairying and Fisheries, 2014). Although the milk production and the population of dairy animals are increasing in Bihar, the average milk yield per animal is low and even decreasing (Department of Animal Husbandry, Dairying and Fisheries, 2014). The milk production in exotic/crossbreed cows has dropped from 6.26 to 6.05 kg/day and in indigenous cows from 2.89 to 2.86 kg/day between 2008-09 and 2012-13. The trend of a decreased productivity is not observed in India in general. However, buffalo milk production in Bihar increased from 3.88 to 3.95 kg/day during the same period.

The development of the livestock and the dairy sector is important to enhance the rural economy and to further decrease poverty. Singh (2013) suggests that the low animal productivity in Bihar is due to several different factors, including poor animal health and insufficient feed and fodder. Research and improvement of these constrains are crucial to obtain a better productivity in the dairy sector.

Mastitis

Mastitis is defined as an inflammation in the mammary gland. Mastitis is commonly caused by a bacterial infection, but other origins, such as yeasts, fungi, algae and trauma may also result in mastitis (Ruegg & Erskine, 2015). The pathogens invade the mammary gland through the teat canal and stimulate an immune response which leads to an inflammatory response in the tissues that can be observed, e.g. as an increase of inflammatory cells in milk. Mastitis can be classified into two main categories, subclinical and clinical. Subclinical mastitis is defined by an increased number of inflammatory cells in the milk without an abnormal appearance of either the milk or the udder (Ruegg & Erskine, 2015). Detection of subclinical mastitis is often based on an increased somatic cell count (SCC) in milk samples. Clinical mastitis is defined by palpable or visible changes in milk and udder. Clinical mastitis can be mild (only abnormalities in the milk), moderate (also clinical inflammatory signs of the udder tissue, such as swelling, redness, hardness or pain) or severe (additional systemic symptoms, such as fever or inappetence). Mastitis can also be classified as chronic or acute depending on the duration of the disease.

Somatic cell count (SCC)

Milk contains somatic cells that primarily consist of leukocytes (macrophages, lymphocytes and polymorph-nuclear cells). A smaller number of epithelial cells can also be found. The somatic cells play an important part in the immune system of the udder. Macrophages are the predominant cell type in milk and of healthy udders (Sordillo *et al*., 1987; Hamed *et al*., 2010). When the udder tissue is inflamed, non-specific inflammatory cells travels from the blood to the udder tissue and to the milk in response to inflammatory mediators. These inflammatory cells primary consist of polymorpho-nuclear leukocytes (neutrophils in particular) (Sordillo *et al*., 1987; Concha *et al*., 1986). The somatic cell count (SCC) in milk significantly increases due to inflammation in the udder and is therefore used as an indicator for mastitis. SCC levels <100 000 cells/mL often indicate a healthy udder, however bacterial infections can occur even at those levels (Schwartz *et al*., 2010). In cattle, a SCC of 200 000 cells/mL is generally considered to be a threshold between healthy and unhealthy udder (Dohoo & Leslie, 1991; Schepers *et al*., 1997), however the reported sensitivity of this threshold varies between different reports. Olde Riekerink *et al*. (2007) estimate that the sensitivity and specificity of SCC as an indicator of intramammary infections (IMI), when using 200 000 cells/mL as a cut-off, is 52-89% and 34-73%, respectively. However, the sensitivity was nearly 100% if only major mastitis pathogens were considered (i.e., *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis* and *Escherichia coli*). With a cut-off value of 500 000 cells/mL the sensitivity was 82% and the specificity 70-91% to detect IMI with a major pathogen. Another study estimated a sensitivity of approximately 75% and a specificity of approximately 90% at a cut-off value of 200 000 cells/mL (Schepers *et al*., 1997). Using a threshold of 205 000 cells/mL, Rodrigues *et al*. (2009) found the sensitivity and specificity to be 91.3 and 96.0% respectively to detect IMI.

Although mastitis is the primary reason for an elevation of SCC, minor variations can occur due to physiological factors such as:

- **Stage of lactation**: In healthy cows, SCC is normally lowest during the middle of lactation. The levels are elevated in early lactation and gradually increase towards the end of lactation (Concha *et al*., 1986; Schepers *et al*., 1997).

- **Parity number/age**: SCC tends to increase with increased parity number (Schepers *et al*., 1997; Nyman *et al*., 2014), primarily due to an increased prevalence of infections (Schepers *et al*., 1997). Older cows are also more likely to have had previous cases of mastitis which may give an elevation in SCC.

- **Milk fraction**: Sarikaya & Bruckmaier (2006) showed a significantly higher SCC level in foremilk and cisternal milk compared to the total SCC concentrations (including alveolar milk). There are also different SCC in different foremilk fractions, where the highest values obtained was in the first fractions.

- **Production level**: Nyman *et al*. (2014) investigated the association between SCC and milk yield and found that SCC decreased with increasing milk yield. This can be explained by the dilution effect (Green *et al*., 2006).

- **Sampling in relation to milking**: SCC is lowest before milking and highest shortly after milking (Olde Reikerink *et al*., 2007). The authors argue that SCC is not reliable if collected after milking and recommend that samples should be collected immediately before milking to make an optimal estimation of SCC.

- **Breed**: SCC variations have been noted between breeds of dairy animals. In high producing Swedish breeds, Holstein has a higher SCC than SRB (Nyman *et al*., 2014). The mean SCC of those breeds was 65 000 cells/mL in primiparous cows (Persson Waller *et al*., 2009). Different healthy Indian indigenous breeds (Tharpaker and Sahiwal) and crossbreeds (Karan Fries and Karan Swiss) had a mean SCC value of 126 000 – 161 000 cells/mL in one study (Singh & Ludri, 2001).

The microscopic method is reference method for the counting of somatic cells (IDF, 2008) but fluoro-opto-electronic method is also used (IDF, 2006). California Mastitis Test (CMT) is an indirect cow-side test that is widely used as an on-farm screening test to estimate SCC from the individual cow and quarter. The CMT reagent composes of a detergent that react with DNA in the milk, some solutions also contain bromcresol purple as an indicator of pH (Ruegg & Erskine, 2015). CMT is subjectively graded using a five-point scale where each score represents an approximate SCC range (Table 1).

Table 1. California Mastitis Test (CMT) scoring system (Scandinavian scale) with interpretation and SCC range according to Schalm & Noorlander (1959): see Quayle (1965)

CMT Score	Interpretation	SCC/mL
1	Negative	0-200 000
2	Trace	150 000- 500 000
3	Weak positive (1+)	400 000 – 1500 000
4	Distinct positive (2+)	800 000 -5 000 000
5	Strong positive (3+)	>5 000 000

In cows, the estimated sensitivity and specificity of CMT to identify quarters with IMI varies between 2.4% - 94.1% and 49.5% - 86.5% respectively (Sargeant *et al*., 2001; Sanford *et al*., 2006; Safi *et al*., 2009; Bhutto *et al*., 2010). The variation partly depends on different cut-off values and different group selection and sample criteria. An increased CMT cut-off results in an increased specificity and a decreased sensitivity (Dingwell *et al*., 2003; Rodrigues *et al*., 2009).

Udder pathogens

Mastitis is a complex disease, involving many different factors. However, a bacterial infection exists in most cases of mastitis. Mastitis causing pathogens are commonly divided into two main groups, based on the most common source of infection, the udder (contagious) or the environment (environmental) (NMC, 2011). Environmental bacteria can be found in the surrounding of the cow, e.g. in manure, bedding or on the ground and is mainly transmitted to the teat via direct contact between milkings. Coliforms (e.g. *Escherichia coli* and *Klebsiella* species) and *Enterobacter* spp. are the most common environmental pathogens (Ruegg & Erskine, 2015). *Streptococcus uberis* and *Streptococcus dysgalactiae* are environmental bacteria, but they can also be contagious. Contagious bacteria are mainly associated with the udder and often transmitted between cows during milking. Contaminated milk can transmit bacteria via hands of milkers, milking machines, or other equipment. After transmission, the bacteria colonize the skin of the teat and spread to the udder through the teat canal. *Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma bovis* are considered to be the most important contagious pathogens (Ruegg & Erskine, 2015).

In India, the most common causative agents of clinical mastitis in cattle are *Staphylococcus* species and *E. coli* (Sumathi *et al*., 2008; Kumar *et al*., 2010; Jeykumar *et al*., 2013) while *S. aureus* and *S. dysgalactiae* cause most of the subclinical cases (Sharma *et al*., 2012) (Table 2). In buffaloes, *Staphylococcus* species is predominant in both clinical and subclinical mastitis followed by *Streptococcus* species in subclinical mastitis and *E. coli* and other pathogens in clinical mastitis (Das & Joseph, 2005; Bulla *et al*., 2006; Sharma & Sindhu, 2007; Pankaj *et al*., 2013; Kaur *et al*., 2015) (Table 3).

Staphylococcus spp.

Staphylococcus spp. are gram-positive bacteria that are common causes of mastitis. Within the mastitis diagnostic, *Staphylococcus* spp. are often divided into coagulase-negative (CNS) and coagulase-positive (CPS) staphylococci.

S. aureus is a CPS and one of the most common causes of mastitis. This species is contagious and can cause everything from subclinical to severe clinical mastitis (Ruegg & Erskine, 2015). The infection mainly spreads between cows during milking, but *S. aureus* can also be found on skin and skin lesions on the hock as well as in the environment around cows and heifers (Capurro *et al*., 2010; Nyman *et al*., 2010; Anderson et al., 2012), suggesting a environmental source as well. Heifers can also be infected and act as a reservoir for the bacteria (Trinidad *et al*., 1990). Even though *S. aureus* may often be sensitive to penicillin in some countries (SWEDRES-SVARM, 2014), insufficient response to therapy frequently occurs which may result in chronic infected animals (Taponen *et al*., 2003). Because of this, preventive measures are of a great importance to reduce the prevalence of the disease (Petersson-Wolfe *et al*, 2010). The common recommendation is to segregate the infected cows and milk them last. A good hygiene around milking (i.e. good hand hygiene, clean and dry udders and usage of teat disinfection) is essential to avoid spreading the bacteria from infected udders to healthy cows. Culling of infected animals is also recommended.

CNS consist of a large group of different species that commonly cause subclinical or mild clinical mastitis (Ruegg & Erskine, 2015). According to one study in India, *S. hyicus* and *S. epidermidis* was the most common CNS in subclinical mastitis (Sharma *et al*., 2012). Other studies also mention *S. chromogenes*, *S. simulans* and *S. haemolyticus* as important pathogens (Thorberg *et al*., 2009). CNS are often susceptible to penicillin although some strains are resistant due to production of betalactamases (Persson Waller *et al*., 2011). CNS have been associated both with the cows' skin and udder and with the environment (Dufour *et al*., 2012). Preventive measures therefore aim both to avoid transmission during milking and to minimize transmission from the environment to the cows by maintaining a good hygiene in the stall.

Streptococcus spp.

Streptococcus spp. are a genus of gram-positive bacteria where *S. dysgalactiae*, *S. agalactiae* and *S. uberis* are the most important mastitis pathogens. *S. dysgalactiae* are classified as both a contagious and environmental bacteria. The fly *Hydrotaea irritans* has also been shown to transmit *S. dysgalactiae* between udders (Chirico *et al*., 1997). Infection can be prevented by good milking hygiene, post-milking teat disinfectants and a clean and dry environment. The bacteria are sensitive to penicillin, which has a good therapeutic effect (McDougall *et al*., 2014).

S. agalactiae is a highly contagious obligate udder pathogen that can cause subclinical and clinical mastitis (Ruegg & Erskine, 2015). Since the bacteria are strongly associated with the udder, control measures focus on a good biosecurity to avoid introducing the pathogen. In already infected herds, milking hygiene and culling of chronically infected animals are

important measures. *S. agalactiae* is also commonly sensitive to penicillin (Oliver & Murinda, 2012).

S. uberis is primarily classified as an environmental pathogen, although it sometimes also is considered to be contagious (Zadoks *et al*., 2001). The bacteria have been found in water, soil, farm tracks, bedding, hay and faeces (Zadoks *et al*., 2005; Lopez-Benavides *et al*., 2007). The prevalence of *S. uberis* infections has been found to be higher in farms with pasture- based systems (Compton *et al*., 2007). *S. uberis* can cause both subclinical and clinical mastitis. The bacteria are sensitive to penicillin (McDougall *et al*., 2014), but environmental measures are important to control the infection. Clean and dry lying area, regular change of bedding material and clean cows are important to minimize spread. Since there is a risk for contagious transmission between cows, a good milking hygiene is also important.

E. coli and Klebsiella spp.

E. coli and *Klebsiella* spp. are gram-negative bacteria that often cause severe acute clinical mastitis, although development of mild and moderate clinical mastitis is also common (Oliveira *et al*., 2013), and subclinical infections can also occur (Gianneechini *et al*., 2002; Bhatt *et al*., 2012). *E. coli* is a known intestinal species that spread due to faecal contamination. Studies indicate faecal shedding of *Klebsiella* spp. as well (Munoz *et al*., 2006; Zadoks *et al*., 2011). Outbreaks of *Klebsiella* mastitis have also been associated with sawdust bedding (Bengtsson *et al*., 2003) but the bacteria has also been found in both soil and other bedding (Zadoks *et al*., 2011). *E. coli* and *Klebsiella* mastitis generally responds poorly to antimicrobial treatment (Zadoks & Schukken, 2011; Persson *et al*., 2013; Suojala *et al*., 2013). Since *E. coli* and *Klebsiella* spp. are environmental pathogens, infection can be prevented by a good hygiene in the stall and pastures, especially in the calving areas. Clean and dry cows and udders are also important measures to avoid infection.

Table 2. Prevalence of udder pathogens in growth positive milk samples collected from cattle with clinical or subclinical mastitis in India. CPS = Coagulase-positive staphylococci, CNS = Coagulase-negative staphylococci.

Reference	***Jeykumar* et al., *2013.***	***Sumathi* et al., *2008.***	***Kumar* et al., *2010.***	***Sharma* et al., *2012.***
State	*Tamilnadu*	*Bangalore*	*Mathura*	*Haryana*
Sample size and sample information	*74 samples of clinical mastitis*	*60 samples of clinical mastitis*	*50 samples of clinical mastitis*	*145 samples of subclinical mastitis*
Pathogens				
Staphylococcus spp.	44.4%	-	37.0%	29.3% undefined CNS+ 4.1% CPS (other than *S.aureus*)
- *S. aureus*	-	24.0%	-	34.7%
- *S. epidermidis*	-	16.0%	-	-
Streptococcus spp.	5.5%	16.0%	11.1%	-
- *S. dysgalactiae*	-	-	-	22.7%
- *S. agalactiae*	-	-	-	6.7%
- *S. uberis*	-	-	-	2.7%
E. coli	41.7%	20.0%	14.8%	-
Klebsiella spp.	8.3%	10.7%	7.4%	-
Other (e.g. *Bacillus* spp., *Corynebacterium* spp., *Proteus* spp., *Pseudomonas* spp.)	-	13.3%	29.6%	3.3% mixed *Staph*. + *Strept*. spp.

Table 3. Prevalence of udder pathogens in growth positive milk samples collected from buffaloes with clinical or subclinical mastitis in India. CPS = Coagulase-positive staphylococci, CNS = Coagulase-negative staphylococci.

Reference	***Kaur* et al., *2015***	***Pankaj* et al., *2013***	***Bulla* et al., *2006***	***Sharma & Sindhu, 2007***	***Das & Joseph, 2005***
State	*Punjab*	*Haryana*	*Haryana*	*Haryana*	*Madhya Pradesh*
Sample size and sample information	*163 quarter samples of subclinical mastitis*	*38 quarter samples of subclinical mastitis*	*21 quarter samples of subclinical mastitis*	*1070 quarter samples of subclinical mastitis and 1879 quarter samples of clinical mastitis*	*86 quarter samples clinical mastitis*
Pathogens					
Staphylococcus spp.	39.0%	15.9% undifined CPS+ 47.7% undifined CNS	-	38.8%	27.9% undifined CPS+ 16.3% undifined CNS
- *S. aureus*	-	-	30.4%	-	-
- *S. epidermidis*	-	-	39.1%	-	-
Streptococcus spp.	31.0%	-	-	32.4%	-
- *S. dysgalactiae*	-	25.0%	13.0%	-	-
- *S. agalactiae*	-	9.1%	13.0%	-	7.0%
- *S. uberis*	-	2.3%	-	-	-
E. coli	5.0%	-	-	11.8%	17.4%
Klebsiella spp.	-	-	-	2.0%	5.8%
Other (e.g. *Bacillus* spp., *Corynebacterium* spp., *Proteus* spp., *Pseudomonas* spp.)	35.0% (*Corneybacterium* spp)	13.6% mixed *Staph.* + *Strept*. spp.	4.3%	7.6%	25.5%

Treatment and antimicrobial resistance

Clinical mastitis is generally treated with antimicrobial drugs, either through systemic and/or local intramammary administration. Treatment of mastitic cases may vary between countries. For example, in Sweden, diagnosis based on history, clinical examination and bacteriological examination of the milk is recommended prior to antimicrobial treatment. Choice of treatment and antimicrobial substances depends on causative pathogen, antimicrobial susceptibility,

prognosis and availability of the drugs. In Sweden, benzylpenicillin is the most commonly used antimicrobial in cases of clinical mastitis (SWEDRES-SVARM, 2014). In one survey in Gujarat state (India) ampicillin, penicillin, streptomycin and oxytetracycline was frequently used antimicrobial drugs, while gentamicin, enrofloxacin, ciprofloxacin and chloramphenicol were not as common (Bhatt *et al*., 2011). In India, homeopathic therapy and other alternative treatments also occur (Varshney & Naresh, 2005; Subrahmanyeswari & Chander, 2013). Supportive therapy, including fluid therapy, anti-inflammatory drugs and frequent milking is recommended in cases of severe clinical mastitis (Leslie & Petersson-Wolfe, 2012; Ruegg & Erskine, 2015). The outcome of antimicrobial treatment depends on several factors including type of pathogen, appropriate choice of drug, duration of infection (acute or chronic), treatment duration, parity number of the cow, breed and pre-treatment SCC (Owens *et al*., 1997; Sol *et al*., 2000; Deluyker *et al*., 2005; Sandgren *et al*., 2008).

Antimicrobial resistance is considered to be one of the biggest threats to both public and animal health. Antimicrobial resistance can occur naturally, but an increased use and misuse can accelerate the development of resistant bacteria (WHO, 2014). Apart from the risk of therapy failure, mastitis causing resistant bacteria can also be a hazard for human health due to transmission of pathogens through consumption of unpasteurized milk (Oliver & Murinda, 2012).

There are no official records of resistance in mastitis-causing bacteria in India. However, a few studies with *in vitro* antimicrobial sensitivity test of mastitis pathogens from cattle have been conducted. *S. aureus* isolates have shown a high resistance to penicillin (41.4-63.5%), amoxicillin (61.5%) and methicillin (52.9%) (Mubarack *et al*., 2012; Chandrasekaran *et al*., 2015). Vishnupriya *et al*. (2014) found CPS to be most resistant to ampicillin (81.8%), amoxicillin (72.8%) and penicillin (63.6%), the corresponding figures for CNS isolates was 77.3%, 64.9% and 52.7%, respectively. *E. coli* was found to be most resistant to penicillin (63.0%), amoxicillin (52.1%) and oxytetracyclin (47.9%) (Chandrasekaran *et al*., 2015).

Risk factors of mastitis

Mastitis is considered to be a multifactorial disease, in which inflammation is often caused by a disturbed balance between infectious agents and the local immune system. There are several factors on both cow and herd level that are associated with a higher risk of mastitis. On cow level, factors such as age, breed, parity and lactation number and stage of lactation are correlated to mastitis prevalence (Joshi & Gokhale, 2006; Persson Waller *et al*., 2009; Breen *et al*., 2009; Jingar *et al*., 2014; Kurjogi & Kaliwal, 2014; Ramirez *et al*., 2014; Oliveira *et al*., 2015). Udder hygiene is also considered to be an important factor. Sant'Anna & Paranhos da Costa (2011) found a significant association between hygiene of leg, udder, flank and abdomen and SCC. The cleanest cows had a low SCC whereas dirty cows had a higher SCC score. Other studies have also determined the relationship between poor hygiene of the udder and the leg with the occurrence of both subclinical mastitis (Schreiner & Ruegg, 2003) and clinical mastitis (Breen *et al*., 2009).

Milking hygiene and routines is also known to affect the prevalence of mastitis and high SCC.

Udder preparation, milking order, dry period practices and the use of teat disinfectants are associated with the incidence of mastitis (Ramirez *et al*., 2014). Other external variables that are associated with mastitis are season, bedding or floor type and stall hygiene (Joshi & Gokhale, 2006; Rahman *et al*., 2009; Abera *et al*., 2012; Kurjogi & Kaliwal, 2014; Oliveira *et al*., 2015). Inadequate sanitation and poor veterinary service have also been suggested to be predisposing for mastitis (Sinha *et al*., 2014).

Occurrence of clinical and subclinical mastitis in India

Cattle

Reports published the last ten years indicate a high level of subclinical mastitis in cattle throughout the whole country (Table 4a and 4b), however, no studies have been published from Bihar. In a study of 263 cows in Karnataka India, the prevalence of clinical mastitis was 4.7-8%, depending on diagnostic tests (Kurjogi & Kaliwal, 2014). Apart from that, no reliable data of the prevalence of clinical mastitis are present.

Table 4a. Prevalence of subclinical mastitis in cattle in India. HFC = HolsteinFriesian cross, JC = Jersey cross, I = Indigenous breeds

Prevalence of subclinical mastitis	**Definition of mastitis used**	**Sample size and study animals**	**Sampling information**	**State**	**Referance**
39.8% on cow level 64.2% on quarter level	Culturally positive	95 animals (HFC and I), 364 quarters	Organized dairy herds	Haryana	Sharma *et al*., 2012
15.4% on quarter level	SCC>500 000/ ml + culturally positive				
4.7% on quarter level	SCC>500 000/ ml + culturally negative				
24.5% on quarter level	SCC<500 000/ ml + culturally positive				
33.5% on quarter level	Culturally positive	69 animals (HFC and I), 266 quarters	Cows held at a university Livestock Research Station	Rajasthan	Langer *et al*., 2014
45.2 % on quarter level	SCC>500 000/ ml				
57.8% on cow level 30.7% on quarter level	CMT positive + culturally positive	218 HFC, about 872 quarter	Machine milked dairy farms	Punjab	Mir *et al*., 2014

Table 4b. Pooled estimated prevalence of subclinical mastitis in cattle in India.

Prevalence of subclinical mastitis	**Method**	**Sample size and study animals**	**Sampling information**	**State**	**Referance**
46.4% on cow level	Meta-analysis of published literature	28 studies, 6344 cows	Review of many authors (1995–2014)	Punjab, Haryana, Uttar Pradesh, Madhya Pradesh, Maharashtra	Bangar *et al*., 2015
23.3% on quarter level	Meta-analysis of published literature	23 studies, 18 721 quarters	Review of many authors (1995–2014)	Punjab, Haryana, Uttar Pradesh, Madhya Pradesh, Maharashtra	Bangar *et al*., 2015

Table 5. Prevalence of subclinical mastitis in buffaloes in India

Prevalence of subclinical mastitis	**Definition of mastitis used**	**Sample size and study animals**	**Sampling information**	**State**	**Reference**
9.8% on quarter level	SCC>200 000/ ml + culturally positive	200 buffaloes, 800 quarters	Various, undefined, farms	Punjab	Kaur *et al*., 2015
2.8% on quarter level	SCC>200 000/ ml + culturally negative				
7.8% on quarter level	SCC<200 000/ ml + culturally positive				
32.9% on quarter level	Culturally positive	2057 buffaloes, 5707 quarters	Rural and urban dairy farmers	Haryana	Sharma & Sindhu, 2007
30.0% on buffalo level 8.8% on quarter level	Culturally positive	60 buffaloes, 239 quarters	Livestock-farm located at agricultural university	Haryana	Bulla *et al*., 2006
16.7% on buffalo level 6.3% on quarter level	SCC>500 000/ ml				
4.1% on quarter level	SCC>500 000/ ml + culturally positive				

Buffalo

The prevalence of subclinical mastitis varies depending on different criteria and methods used (Table 5). One study reports the prevalence of clinical mastitis on quarter level to be 18.7% (Sharma & Sindhu, 2007).

Economic impact of mastitis

Mastitis, both clinical and subclinical, is known for resulting in a substantial economic loss. Sinha *et al*. (2014) divided the losses into following categories: Milk yield loss, loss from discarded milk, veterinary service, medicine, increased sanitation (both stall and milk hygiene), additional labour and equipment.

Subclinical mastitis in cattle and buffaloes is estimated to result in a loss of 1592.87 Indian rupee (INR) and 892.42 INR per lactation, respectively (Sinha *et al*., 2014). The largest loss was due to milk yield loss and medicine. Singh *et al*. (2014) estimated the economic loss per animal per lactation to be 2182.44 and 1272.36 INR for cattle and buffaloes respectively. Yield loss and treatment costs were the largest expenses also in this study. Another study estimated the direct losses due to clinical mastitis in cows to be 2086.96 INR per clinical case (John Christy, 2014).

Halasa *et al*. (2007) also mention a poorer product quality and culling of diseased animals as factors that affect the economy. However, slaughter of cattle is limited in some states in India, but regulations vary. Slaughter of cows is totally prohibited in Bihar (The Bihar Preservation And Improvement of Animals Act, 1955). Only female buffaloes over the age of 25, or which are permanently unable of breeding or yielding milk can be allowed to be slaughtered.

The economic losses of mastitis should be seen in context with the per capita income. The annual per capita income in Bihar during 2012-13 was estimated to be 30.930 INR (Economic survey 2013-2014).

MATERIAL AND METHODS

Study area

The study was conducted in three different districts (Patna, Nalanda and Vaishali) in the state Bihar in Northeast India during September and October 2015. Bihar is located in a subtropical region and the average temperature during the time of the study was around 29 °C (Sep) and 26.5 °C (Oct) with an average precipitation of 200 mm (Sep) and 70 mm (Oct) (Weatherbase, 2015). The elevation varies between 55-60 meters between the different districts (Weatherbase, 2015).

Study animals

The study animals were lactating dairy cattle and buffaloes with or without signs of mastitis. All animals were hand milked. A total of 285 cows and 28 buffaloes were included in the study. The included animals were of different breed, parity number and lactation stage.

Study design

The data collection was conducted during the period of 8th of September to 17th of October 2015. Each district was divided into two separate strata. The strata were located in rural or urban areas. In Patna, peri-urban areas were also included. In each stratum, four different villages were randomly selected and 8-12 households were selected with the help of a local veterinarian. In total, 226 households were visited and a signed informed consent was obtained from the farmer. Each farmer answered a questionnaire with questions regarding herd size, housing, symptoms of disease, milk production and milking routines. At each farm, up to three lactating cows or buffaloes were examined and sampled. If the farm had more than three eligible animals they were randomly selected. An animal history sheet was used for the individual cow or buffalo to collect information regarding breed, age, lactation number, lactation stage, pregnancy, present treatments and present or previous symptom of diseases. To estimate the degree of dirtiness a hygiene scoring system from 1-5, with one being cleanest, was used (Reneau *et al*., 2005: see Cook & Reinemann, 2007). The hygiene score was based on a combined assessment of the hygiene of tail head, upper rear limb, ventral abdomen, udder and lower rear limb. The udder was examined for teat lesions and signs of clinical mastitis (hard, warm, painful or swollen udder). The milk was examined ocular for the presence of clots, flakes, blood or changes in colour. Also, CMT was performed to assess SCC and changes in pH. The criteria for clinical mastitis were deviation in milk appearance, with or without signs of inflammation in the udder (swollen, hard, warm or painful). If only a positive CMT test (CMT $\geq$3) without other signs of mastitis, the case was categorized as a subclinical mastitis.

CMT screening and sample collection

CMT was conducted on all selected lactating animals. During the first month of the study a CMT solution prepared in a local laboratory was used (5 mg Bromocresol purple, 15 g Sodium hydoroxide, 15 ml Teepol and 1000 ml distilled water). During the second month a ready-to-use CMT (Kruuse, Langeskov) was used.

The first streams of milk were discarded, and after that milk was collected separately from each quarter in a plastic paddle with four wells (approximately 2 ml in each well). An equal amount of CMT reagent was added to the well and gently mixed with the milk by rotating the paddle. The reaction was immediately scored (within 15-30 seconds) using a five point scale where 1 is negative and 5 is strongly positive (Table 1). A CMT score of 3 or higher was considered as a positive result. In those cases, milk was sampled from the affected quarter for bacteriological analysis. Before sampling the udder and teats were brushed or cleaned. In cases of heavily soiled animals the udder and teats were cleaned with water and dried. The tip of the teat was disinfected by a cotton swab with 70-99% alcohol, new swabs was used until no dirt was visible. Contact with the disinfected teats was avoided until the sampling was completed. Milk was collected in sterile plastic tubes. The tubes were held in an angel of approximately 45 degrees to avoid contamination during the collection. After being collected the samples were stored in a cool box with ice packs. Plastic gloves were used during the whole procedure.

Bacterial examination

For cultivation, a three-portioned petri dish with selective media was used (SELMA, produced by the National veterinary institute of Sweden, SVA). The three different media was: Bovine blood agar (with esculine) for growth of aerobic bacteria, MacConkey agar for growth of gram-negative bacteria and Mannitol salt agar on which only *Staphylococcus* spp. and *Enterococcus* spp. can grow. Cultivation of the milk sample was carried out the same day as the samples were collected.

At each field, 10 µl of the milk sample were spread with a sterile plastic loop. The plate was incubated at room temperature (approximately 25 °C) since no laboratory was available. The plates were examined after 24 h and 48 h. Less than 5 colonies on the blood agar were considered as negative growth. The criteria for contamination were the presence of three or more different colonies on the blood agar.

In cases of bacterial growth, the colonies were identified by ocular examination of morphology (colour, shape, size) and haemolytic characteristics. Colonies with morphology similar to staphylococci, which grew on both blood agar and Mannitol salt were considered as *Staphylococcus* spp. If haemolysis (single or double) was present on blood agar and if the Mannitol salt agar turned yellow, the colonies were categorized as *S. aureus*. If no change in colour on the Mannitol salt agar was observed the colonies were categorized as unspecified staphylococci and called *Staphylococcus* spp.

Colonies with an appearance in accordance with streptococci on blood agar and negative growth on Mannitol salt and MacConkey agar was categorized as *Streptococcus* spp. Growth on MacConkey agar but not Mannitol salt agar was considered as gram-negative bacteria and were identified as coliforms. No further analysis to differentiate these was performed.

Antimicrobial sensitivity test

Thirty seven of the milk samples were tested with MastiTest (HiMedia Laboratories, Mumbai), a commercial *in vitro* antimicrobial susceptibility test. The kit is designed to easily determine the choice of antimicrobial treatment of mastitis without previous culturing and bacterial examination.

The test was performed according to the instructions from the producer (HiMedia Laboratories, 2010). The test contained eight test vials containing antimicrobial discs of ampicillin/cloxacillin (AX 128/128 mcg), amoxicillin/cloxacillin (ACX 128/128 mcg), gentamicin (GEN 128 mcg), enrofloxacin (EX 8 mcg), ciprofloxacin (CIP 8 mcg), tetracycline (TE 128 mcg), chloramphenicol (C 8 mcg) and streptomycin/penicillin (SPN 128/128 mcg). 1 ml of a mix between diluent and milk was put in each test vial plus a control vial without antimicrobial discs. The vials were incubated at room temperature (approximately 25 °C) for 16-24 h. A change in colour from blue to light yellow or white indicates bacterial resistance towards the corresponding antimicrobial. A change from blue to

light blue indicates an intermediate susceptibility to the antimicrobial. If no colour change occurred in the vial the bacteria were considered sensitive to the corresponding antimicrobial.

Statistical analyses

Calculation of the study population had been made in a sample size calculator for prevalence studies (Naing *et al*., 2006) using a confidence level of 95%, expected prevalence of 85% (0.85) and precision +/- 0.05. "Infinite sample size" was used because the population size is large but unknown. Sample size without FPC (finite population calculation) became 196.

Statistical analyses were performed in the program Minitab. Prevalence of subclinical mastitis and clinical mastitis on animal and quarter level, respectively, was calculated on the basis of data collected regarding CMT score (≥3) and clinical findings of mastitis. Samples with CMT ≥3 but no ocular changes in milk or signs of inflammation in the udder quarter were classified as subclinical mastitis. If a deviation in milk appearance were present, with or without signs of inflammation in the udder, the case was categorized as a clinical mastitis. Only descriptive statistics were presented regarding buffaloes due to the small sample size.

The results were analysed with a χ^2–tests for individual cow factors and management factors to see if there was a correlation to the prevalence of subclinical and clinical mastitis. If the p-value was below 0.05 the correlation was classified as significant. Fisher's exact test was used if the sample size in a category were too small for a χ^2–test.

The cow factors included were stage of lactation (<30d, 31-120 d, <120 d), parity number (from 1 to ≥5) and hygiene score (1-5). Management factors included were floor type (Concrete/Earthen/Bricks), presence of drainage system (Yes/No), pre-milking cleaning of the udder (Never/Sometimes/Always) and usage of teat disinfection post-milking (Never/Sometimes/Always).

Potential sources of error

Two different CMT reagents were used during the project due to problems with the transportation of the ordered solution. There is a possibility that the two different reagents did not correspond completely, which can have had an effect on the CMT results. The different solutions are not distinguished between in the results.

Initially there was a problem with the cool chain which resulted in that some of the milk samples were stored in temperatures above 4 °C for a couple of hours before cultivation. There is a potential risk that this leads to an overgrowth of contaminating bacteria in the samples.

The cultivation and examination of the bacteria were carried out in field condition without proper laboratory facilities. No other typing or confirmatory testing was therefore performed. This means that the results of the bacterial categorization could be inaccurate, and therefore no distinction was made between different species within the three main groups; *Streptococcus* spp., *Staphylococcus* spp. and gram-negative bacteria (coliforms) (apart from a

tentative definition of likely *S. aureus*). There is also a risk of contamination of the samples during preparation for cultivation as a fume hood was not used.

Due to lack of space, the MastiTest was stored in a cool box with some ice packs, in room temperature (approximately 25 °C) instead of 2-8 °C as recommended. It is not known how this can affect the results. However, the control vial showed valid results in all tests.

Some linguistic confusion may have occurred as the questionnaire was in English and farmers rarely spoke English. This may have caused some miscommunication, which is a possible source of error in the questionnaires.

Also, the assessments regarding cow factors such as hygiene are subjective measures which can affect the result. To avoid differences in the evaluations, all the three different observers used descriptive charts and correlated the assessments on the first cows to standardize the scoring.

RESULTS

A total of 285 lactating cows between 2-16 years old from 212 different households were included in the study. A majority, 279, were cross-breeds (mostly Holstein cross-breeds), one was Jersey and five were indigenous breeds. The average herd size was two milking cows with a range from one to 25 cows. A total of 28 lactating buffaloes from 27 different households were included in the study. All of them, except two cross-breeds, were indigenous breeds. The average herd size was one milking buffalo with a range from one to eight buffaloes.

Prevalence of mastitis

Cattle

CMT was conducted on all the 285 lactating cows. The prevalence of clinical and subclinical mastitis on cow level was 11.6% (n=33) and 35.4% (n=101), respectively. The occurrence of mastitis in different locations (rural, peri-urban and urban) and districts (Patna, Nalanda and Vaishali) is presented in Table 6. The prevalence of subclinical mastitis on cow level was significantly lower in peri-urban areas compared to rural and urban locations ($p<0.001$). Vaishali had a significantly ($P=0.001$) higher prevalence of subclinical mastitis compared to Nalanda and Patna.

Out of 1139 tested quarters, the overall prevalence of mastitis was 23.1% (n=263). The prevalence of clinical and subclinical mastitis on quarter level was 4.5% (n=51) and 18.6% (n=212), respectively. The mean CMT score was 1.8 and the median was 1.

Table 6. Prevalence of mastitis at cow level in different locations and districts in Bihar, India

	No of tested cows	Number of subclinical mastitis (% of cows)	Number of clinical mastitis (% of cows)	Total number of mastitis cases (% of cows)
Location				
Rural	106	39 (36.8)	13 (12.3)	52 (49.1)
Peri-Urban	47	4 (8.5)	4 (8.5)	8 (17.0)
Urban	132	58 (43.9)	16 (12.1)	74 (56.1)
	Total: 285	Total: 101 (35.4)	Total: 33 (11.6)	Total: 134 (47.0)
District				
Patna	161	38 (23.6)	16 (9.9)	54 (33.5)
Nalanda	51	18 (35.3)	6 (11.8)	24 (47.1)
Vaishali	73	45 (61.6)	11 (15.1)	56 (76.7)
	Total: 285	Total: 101 (35.4)	Total: 33 (11.6)	Total: 134 (47.0)

Buffalo

CMT was conducted on 28 lactating buffaloes. The prevalence of subclinical mastitis on buffalo level was 28.6% (n = 8). No cases of clinical mastitis were found. Out of 104 tested quarters, the overall prevalence of subclinical mastitis was 10.6% (n=11). The mean CMT score was 1.5 and the median was 1.

Distribution of udder pathogens

Cattle

A total of 99 milk samples from 212 quarters with subclinical mastitis and 46 milk samples from 51 quarters with clinical mastitis were cultivated for bacterial growth (Table 7). The most common pathogen in total was *S. aureus*. In clinical cases other *Staphylococcus* spp. occurred most frequently, followed by *S. aureus* and *Streptococcus* spp. Most of the cases of subclinical mastitis was caused by *S. aureus*, followed by *Staphylococcus* spp. and *Streptococcus* spp. Among the 31 other *Staphylococcus* spp. isolates that were identified, 16 lacked hemolysis.

Table 7. Results of bacteriological analyses of milk samples from cows with mastitis in three districts in Bihar, India

Analysis result	Subclinical mastitis (%)	Clinical mastitis (%)	Total (%)
S. aureus	31 (31.3)	10 (20.4)	41 (28.3)
S. aureus + other *Staphylococcus* spp.	2 (2.0)	1 (2.2)	3 (2.1)
S. aureus + *Streptococcus* spp.	2 (2.0)	0 (0)	2 (1.4)
Staphylococcus spp. (other than *S.aureus*)	17 (17.2)	14 (30.5)	31 (21.3)
Streptococcus spp.	17 (17.2)	9 (19.6)	26 (17.9)
Streptococcus spp. + *Staphylococcus* spp. (other than *S.aureus*)	3 (3.0)	0 (0)	3 (2.1)
Gram-negative	2 (2.0)	0 (0)	2 (1.4)
Contaminated	11 (11.1)	9 (19.6)	20 (13.8)
Negative	14 (14.1)	3 (6.5)	17 (11.7)
Total	99 (100.0)	46 (100.0)	145 (100.0)

Buffalo

Of the eight CMT positive buffaloes, only four was examined for bacterial growth due to difficulties in making the buffalo let down milk for sampling. Three of these were negative for growth and one sample was classified as contaminated.

MastiTest

MastiTest was performed on 37 milk samples (Table 8), on which bacterial examination was done simultaneously. The results from the bacterial examination of the samples were: *S. aureus* (n=14), *S. aureus* + other *Staphylococcus* spp. (n=1), other *Staphylococcus* spp. (n=7), *Streptococcus* spp. (n=5), *Streptococcus* spp. + other *Staphylococcus* spp. (n=1), contaminated (n=3) and negative growth (n=6).

Among the 14 *S. aureus* isolates, resistance against gentamicin was most common (57.1% of the isolates) followed by ampicillin/cloxacillin (35.7%), amoxicillin/cloxacillin (28.6%), chloramphenicol (28.6%) and streptomycin/penicillin (28.6%). It was less common with resistance against enrofloxacin and ciprofloxacin among the samples.

Table 8. Result from MastiTest: In vitro antimicrobial susceptibility of milk samples with S. aureus, S. aureus + *other* Staphylococcus *spp. (*Staph. *spp.), other* Staphylococcus *spp. (*Staph. *spp.),* Streptococcus *spp. (*Strept. *spp.),* Streptococcus *spp. + other* Staphylococcus *spp. (*Strept.+ Staph. *spp.), contamination and negative growth. S=sensitive, I= Intermediate and R=Resistant*

		S. aureus (n=14)	***S. aureus* + *Staph.* spp.** (n=1)	***Staph.* spp.** (n=7)	***Strept.* spp.** (n=5)	***Strept.*+ *Staph.* spp.** (n=1)	**Negative growth** (n=6)	**Contamination** (n=3)
Ampicillin/ cloxacillin	***S***	2	0	2	2	1	2	0
	I	7	1	4	3	0	4	2
	R	5	0	1	0	0	0	1
Amoxicillin/ cloxacillin	***S***	1	0	2	1	0	2	1
	I	9	1	4	3	1	2	1
	R	4	0	1	1	0	2	1
Gentamicin	***S***	4	1	1	2	0	2	0
	I	2	0	3	2	1	2	0
	R	8	0	3	1	0	2	3
Enrofloxacin	***S***	14	1	7	5	1	6	3
	I	0	0	0	0	0	0	0
	R	0	0	0	0	0	0	0
Ciprofloxacin	***S***	13	1	6	5	1	5	3
	I	1	0	1	0	0	0	0
	R	0	0	0	0	0	1	0
Tetracycline	***S***	7	0	4	5	1	4	2
	I	6	1	2	0	0	2	0
	R	1	0	1	0	0	0	1
Chlor-amphenicol	***S***	2	1	4	2	0	0	0
	I	8	0	2	3	1	5	2
	R	4	0	1	0	0	1	1
Streptomycin/ penicillin	***S***	2	0	5	4	1	2	3
	I	8	1	1	1	0	4	0
	R	4	0	1	0	0	0	0

Occurrence of mastitis in cattle on the basis of different cow and management factors

Cow factors

The prevalence of mastitis depending on parity number, stage of lactation and hygiene score was evaluated (Table 9). There was no significant association between these factors and the occurrence of clinical or subclinical mastitis.

Management factors

All the animals were milked by hand. The prevalence of mastitis depending on floor type, presence of drainage system, pre-milking cleaning of the udder and usage of teat disinfection post-milking was evaluated (Table 9). Of the three different floor types that occurred, concrete was associated with a significantly (P=0.002) lower prevalence of subclinical mastitis compared to earthen and brick floor. A difference between floor types was not seen between cows with clinical mastitis. The prevalence of subclinical mastitis was significantly (P=0.01) higher on farms without a drainage system than in farms with a drainage system. The occurrence of clinical mastitis was not associated with drainage. Concrete floor and drainage system was most common in peri-urban areas, significantly more than in rural areas (p=0.007, and p=0.11 respectively). In addition, concrete floor (p<0.001) and drainage system (p<0.001) was less common in Vaishali compared to the other districts.

A majority of the farmers cleaned the udder before milking, but the usage of teat disinfection post-milking was uncommon. No significant correlation between teat disinfection or cleaning of the udder and prevalence of mastitis could be seen.

Table 9. Prevalence of mastitis (subclinical and clinical) at cow level in Bihar (India) on the basis of possible risk factors at cow and management level.

Factors	Type	No of tested cows	Total number of mastitis cases (% of cows)		Number of subclinical mastitis (% of cows)		Number of clinical mastitis (% of cows)	
Parity number	1	82	32	(39.0)	25	(30.5)	7	(8.5)
	2	78	35	(44.9)	27	(34.6)	8	(10.3)
	3	70	36	(51.4)	28	(40.0)	8	(11.4)
	4	34	20	(58.8)	12	(35.3)	8	(23.5)
	≥5	19	10	(52.6)	8	(42.1)	2	(10.5)
	Total:	283	133	(47.0)	100	(35.3)	33	(11.7)
Stage of lactation	<30 d	44	19	(43.2)	12	(27.3)	7	(15.9)
	31-120 d	106	48	(45.3)	38	(35.8)	10	(9.4)
	>121 d	135	67	(49.6)	51	(37.8)	16	(11.9)
	Total:	285	134	(47.0)	101	(35.4)	33	(11.6)
Hygien score	1	60	28	(46.7)	20	(33.3)	8	(13.3)
	2	107	49	(45.8)	35	(32.7)	14	(13.1)
	3	59	26	(44.1)	23	(39.0)	3	(5.1)

	4	43	21	(48.8)	16	(37.2)	5	(11.6)
	5	10	5	(50)	2	(20)	3	(30)
	Total:	279	129	(46.2)	96	(34.4)	33	(11.8)
Floor type	Concrete	117	40	(34.2)	27	(23.1)	13	(11.1)
	Earthen	29	15	(51.7)	12	(41.4)	3	(10.3)
	Bricks	136	76	(55.9)	60	(44.1)	16	(11.8)
	Total:	282	131	(46.5)	99	(35.1)	32	(11.3)
Drainage system	Yes	98	36	(36.7)	25	(25.5)	11	(11.2)
	No	186	98	(52.7)	76	(40.9)	22	(11.8)
	Total:	284	134	(47.2)	101	(35.6)	33	(11.6)
Pre-milking cleaning of the udder	Never	16	6	(37.5)	2	(12.5)	4	(25.0)
	Sometimes	3	2	(66.7)	2	(66.7)	0	(0.0)
	Always	264	126	(47.7)	97	(36.7)	29	(11.0)
	Total:	283	134	(47.3)	101	(35.7)	33	(11.7)
Usage of teat disinfection post-milking	Never	257	123	(47.9)	93	(36.2)	30	(11.7)
	Sometimes	8	4	(50.0)	2	(25.0)	2	(25.0)
	Always	18	7	(38.9)	6	(33.3)	1	(5.6)
	Total:	283	134	(47.3)	101	(35.7)	33	(11.7)

DISCUSSION

Prevalence

This study investigates mastitis in Bihar, a state from which little information about prevalence of mastitis is available. The prevalence of subclinical mastitis on cow basis was 35.4%. Mir *et al* (2014) found a higher prevalence of subclinical mastitis (57.8%) when following the criteria of both CMT and culturally positive samples. However, these results were obtained from farms with machine milked cows. Sharma *et al.* (2012) and Bangar *et al*. (2015) also reported higher prevalence, 39.8% and 46.4% respectively. The prevalence of subclinical mastitis on quarter basis was 18.6%. Earlier studies show prevalences from 4.7% up to 64.2%, depending on the criteria used (Table 4). Sharma *et al*. (2012) reported a similar prevalence (20.1%) when using similar criteria as the present study. The prevalence of clinical mastitis on cow basis (11.6%) was higher than the 4.7-8% that Kurjogi & Kaliwal (2014) found. An incidence of clinical mastitis on national Indian level between 1-10% was reported by Joshi & Gokale (2006), however, it is not clear how they have obtained their results.

Mastitis in cattle was more common in Vaishali district and less common in peri-urban areas than in other districts and urban areas, respectively. This might be due to the difference in floor type and presence of drainage system. It was uncommon that farms in the Vaishali district had concrete floor and drainage system while it was more common on farms in peri-urban areas of Patna. It is also possible that factors such as economic situation and knowledge of the farmers, as well as the infrastructure of veterinary services, may have affected the outcome. However, these factors were not studied in the present study.

The prevalence of subclinical mastitis in buffaloes was 28.6% and 10.6% of animal and quarter level, respectively. No clinical cases were detected. The prevalence of subclinical mastitis was higher compared to the findings of Bulla *et al*. (2006) and Kaur *et al*. (2015) but was lower compared to a study by Sharma & Sindhu (2007). However, Sharma & Sindhu (2007) used culturally positive samples as the criteria of subclinical mastitis which differ from the present study. In general, the prevalence of mastitis in buffaloes is lower compared to cattle, both in previously published papers (Bulla *et al*., 2006; Sharma & Sindhu, 2007; Sharma *et al*., 2012; Langer *et al*., 2014; Mir *et al*., 2014; Kaur *et al*., 2015) and the present investigation. However, it is difficult to draw any conclusions given the low number of buffaloes that was included in the present study.

The variation in prevalence and incidence of mastitis between the different studies might be partly due to different types of diagnostic tests, sampling procedures and criteria for mastitis as well as factors such as stage of lactation, parity number and breed of the animals included in the studies.

Udder pathogens

S. aureus was common in both clinical and subclinical cases and was found in 20.4% and 31.3% of the samples, respectively. This is consistent with previous findings in India of 24% in clinical mastitis (Sumathi *et al*., 2008) and 34.7% in subclinical mastitis (Sharma *et al*., 2012). The prevalence of *Streptococcus* spp. was 19.6% and 17.2% for clinical and subclinical mastitis, respectively. Earlier studies have found a prevalence of *Streptococcus* spp. ranging from 5.5% (Jeykumar *et al*., 2013) to 16% (Sumathi *et al*., 2008) in clinical mastitis and 31.9% in subclinical mastitis (Sharma *et al*., 2012). Other *Staphylococcus* spp. was the most common cause of clinical mastitis (30.5%) and also occurred frequently in subclinical cases (17.2%) in the present study. The study by Sumathi *et al*. (2008) differed slightly with 16% *S. epidermidis* (CNS) in samples from clinical mastitis. Also, the prevalence of *Staphylococcus* spp. among subclinical cases in the present study was lower than the 29.3% of CNS reported by Sharma *et al*. (2012).

The variation in distribution of udder pathogens between different studies might be partly due to different types of classification or typing of the bacterial cultures. Also, the previous studies of subclinical mastitis in India only based the result on samples with bacterial growth, which resulted in a higher proportion of the occurring udder pathogens compared to the present study where negative samples were included.

Gram-negative bacteria could only be detected in two of the samples (1.4%) which is considerably lower compared to previous studies where a high prevalence of both *E. coli* (14.8-41.7%) and *Klebisella* spp. (7.4-10.7%) was found in cases of clinical mastitis (Sumathi *et al*., 2008; Kumar *et al*., 2010; Jeykumar *et al*., 2013). However, Sharma *et al*. (2012) found no cases of subclinical mastitis caused by gram-negative bacteria.

A further classification or typing of the bacteria had been desirable, but was not possible due to constraints in time, equipment and laboratory facilities.

The occurrence of contamination was relatively high (13.8%) compared to previous studies in India which do not report any contamination (Sumathi *et al*., 2008; Kumar *et al*., 2010; Sharma *et al*., 2012; Jeykumar *et al*., 2013). The high presence of contamination may be due to problems with the cold chain that existed during the field work, suboptimal culture conditions or contamination during sampling.

Seventeen samples (11.7%) were negative, which indicates that these quarters did not shed bacteria in a sufficient amount or that the infection had been eliminated. However, mastitis causing bacteria can occur in substantial quantities also in growth-negative milk samples (Taponen *et al*., 2009; Kuehn *et al*., 2013) meaning that an infection cannot be excluded even with a negative sample. Studies also show that SCC can remain elevated for some time after an infection, especially if the infection was caused by *S. aureus, S. uberis* or *S. dysgalactiae* (de Haas *et al*., 2004). Some variations in SCC can also be due to physiological factors, but, it is not likely that they alone can result in a considerable elevation of SCC. It is also possible that the fact that the samples were incubated at room temperature instead of 37 °C, which is the recommended temperature, may have affected the result.

MastiTest and resistance

It is difficult to draw any conclusions regarding the antimicrobial resistance profile in the area due to the small amount of samples that were tested with MastiTest. The test is also a commercial "ready to use kit" and not a golden standard laboratory technique which should be considered when evaluating the results. Nevertheless, the results indicate that resistance is a concern which also has been shown in previous studies (Mubarack *et al*., 2012; Vishnupriya *et al*., 2014; Chandrasekaran *et al*., 2015). However, it is difficult to make any more comparisons between the different studies given the use of different combinations of antimicrobial types, different kinds of sensibility tests and occurrence of contamination or mixed flora.

The purpose of MastiTest is to quickly and without culturing give information regarding which antimicrobial substance is most suitable to use for treatment of the diseased animal (HiMedia Laboratories, 2010). Results from the cultivation showed a large portion of negative growth, contamination and combination between *Staphylococcus* spp. and *Streptococcus* spp. It is possible that the milk used in the MastiTest is contaminated with environmental or skin bacteria which might give a wrong assessment of the test and thereby lead to a less suitable

choice of treatment. A milk sample containing no bacteria would indicate sensitivity towards all, and contamination with different bacteria can indicate resistance among environmental bacteria, and the farmer would not know which pathogen had actually caused the mastitis. The suitability of the test can therefore be questioned, and it should not be recommended as a sole method for determining treatments.

According to Bhatt *et al*. (2011) ampicillin and penicillin is commonly used to treat mastitis in India. Resistant bacteria can then spread between animals and to humans partly due to inadequate hygiene routines. It is not impossible that a continued high usage will increase the problem of resistance development even more. Since the dairy sector in Bihar and India is increasing, an increased amount of mastitis cases and thereby an increased consumption of antimicrobial substances can be predicted. Preventive measures to reduce mastitis are therefore essential.

Risk factors

Cow level

There were no significant differences in the prevalence of mastitis on the basis of parity number and stage of lactation, which is inconsistent with studies in the topic from other countries. Breen *et al* (2009) observed a significantly increased risk of clinical mastitis with increasing parity number and decreasing month of lactation. This result is consistent with studies by Oliveira *et al* (2015) that associate the first month of lactation with clinical mastitis in both multiparous and primiparous cows. Cows with parity number 3 and above was also more likely to have clinical mastitis. Persson Waller *et al* (2009) observed the same pattern in primiparous cows where the cases of veterinary treated clinical mastitis were lower compared to multiparous. In that study, the incidence of veterinary treated clinical mastitis was highest during the first month of lactation in primiparous cows. This is in line with studies on indigenous cows and buffaloes in India that showed a significant correlation between early lactation (up to 90 days) and incidence of clinical mastitis (Jingar *et al*., 2014). However, in that study, the incidence of clinical mastitis in crossbreeds was higher in mid lactation (91 to 180 days).

A difference in the prevalence of mastitis between different hygiene score could not be proven in the present study. Here, a hygiene score (1-5) of a combined assessment of the hygiene of tail head, upper rear limb, ventral abdomen, udder and lower rear limb was used. Reneau *et al*. (2003) concluded that only hygiene score (1-5) of udders and lower rear legs have a significant correlation to SCC. Significant associations between SCC and hygiene scores of the tail head, flank and abdomen were not found in their study suggesting that only udder and lower rear legs should be evaluated. On the other hand, Sant'Anna & Paranhos da Costa (2011) found that hygiene scores (1-4) for flank, leg, abdomen and udder each had a significant association with SCC where dirty animals had an increased SCC compared to clean animals. Schreiner & Ruegg (2003) investigated the relationship of SCC, intramammary pathogens and udder and leg hygiene score (1-4). There was a significant association between udder hygiene and SCC, as well as between udder hygiene and presence of environmental intramammary pathogens. However, the SCC only differed significantly between leg hygiene

score 2 to 4 and no association between leg hygiene score and the presence of intramammary pathogens was observed (Schreiner & Ruegg, 2003). These results may indicate that a separate udder hygiene score should be used for these assessments in the future.

Management factors

Floor type and presence of a drainage system was shown to have a significant effect on the prevalence of subclinical mastitis. Cows kept on concrete floor had a significantly lower prevalence of subclinical mastitis compared to cows on brick and soil floor. Cows on bricks had a higher prevalence of subclinical mastitis compared to cows on soil floor; however this difference was not significant. These findings are consistent with earlier comparisons between concrete floor and soil floor (Abera *et al*., 2010; Abera *et al*., 2012) and soil and brick floor (Rahman *et al*., 2009). Previous research also shows that poor condition of the floor (wet, soiled or cracked floor) has a significant impact on the mastitis prevalence (Rahman *et al*., 2009; Mekibib *et al*., 2010), however, these factors were not investigated in this present study. The effect of the floor type might be explained by the fact that environmental mastitis pathogens can be harboured in the soil, manure and bedding and that bacterial growth is promoted by moist surroundings (Zadoks *et al*., 2005; Lopez-Benavides *et al*., 2007; Zadoks *et al*., 2011). It is likely that the space between bricks is hard to clean and preserves damp better than a flat soil or concrete floor that might dry faster. A whole concrete floor is probably easiest to clean and dries fast which might be the reason for the lower prevalence of mastitis cases. *S. aureus*, a primary contagious pathogen, were predominant in the present study. However, possible environmental bacteria, such as other *Staphylococcus* species and *Streptococcus* species were common as well, which might support the reasoning above.

A majority of the cattle in the study were held in an environment without drainage system. However, cows held in farms with a drainage system had a lower prevalence of subclinical mastitis. This could indicate that such housing promotes a cleaner floor and thus a lower prevalence of environmental bacteria as discussed above.

A majority of the farmers cleaned the udder before milking, but there was no significant association with the prevalence of mastitis. During the sampling, it was observed that the farmers often wash the udder with water but rarely let it dry before milking. Uncleaned udders before milking are associated with a higher prevalence of subclinical mastitis caused by *S. agalactiae* (Ramirez *et al*., 2014) and incidence of clinical mastitis (Peeler *et al*., 2000). However, it is considered to be of great importance to dry the udder after cleaning to reduce bacteria from the teats and thereby reduce the risk of new IMI as well as to avoid bacterial contamination from the udder to the milk (Galton *et al*., 1986).

Most of the farmers did not use post-milking teat disinfection (PMTD). No association could be detected between the usage of teat disinfection and the prevalence of mastitis. PMTD is considered to be an important management strategy to reduce new IMI, especially those caused by contagious pathogens (Nickerson, 2001). *S. aureus* (a primary contagious bacterium) was predominant in the present study. It is possible that the high prevalence of *S. aureus* is due to the fact that PMTD is rarely practiced. However, studies regarding the effect

of PMTD are somewhat inconsistent. Eberhart *et al*. (1983) showed that PMTD significantly reduced new cases of IMI and clinical mastitis caused by *S. aureus*, *Streptococcus* spp., CNS and *Corynebacterium bovis*. Ramirez *et al*. (2014) reached the same conclusion regarding clinical mastitis caused by *S. agalactiae*. Also, Quirk *et al*. (2012) saw that PMTD had a protective effect for IMI caused by some CNS species, but, other CNS species were unaffected. However, other studies associate PMTD with an increased risk of clinical mastitis in herds with a low bulk SCC (Barkema *et al*., 1999; Peeler *et al*., 2000). The reason for the different results is unknown, but one theory is that PMTD reduce the infections of minor pathogens and thereby increasing the risk of infections with major pathogens which are more likely to result in clinical cases (Barkema *et al*., 1999). Different efficacy of teat dips products, methods of application (spray or dipping), contact time and hygiene of the solution may also affect the outcome.

CONCLUSIONS

The results from the present study indicate that the prevalence of both clinical and subclinical mastitis in dairy cattle is high. The most common udder pathogen was *S. aureus*, which is considered to be a contagious pathogen that mainly spreads between cows during milking. No association between cow factors such as parity number, stage of lactation or hygiene score could be found, but floor type and drainage system were significantly associated with the prevalence of mastitis. To reduce the occurrence of mastitis, knowledge about transmission and preventive measures are essential. Hygiene training programs for the farmers in adjacent areas resulted in positive effects such as increased milk production (Melin, 2015). Similar education projects could be beneficial in Bihar. With support from the results in the present study, focus should be to emphasize the importance of good hygiene around milking and maintaining good hygienic standards in the herd. Prevention of mastitis is also important to reduce the usage of antimicrobial substances and thereby reduce the risk of development of drug resistance.

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