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A cross sectional study of prevalence and risk factors associated with subclinical mastitis and intramammary infections, in dairy herds linked to milk collection centers in Rwanda

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ABSTRACT

The objective of this cross-sectional study was to investigate prevalence, causative udder pathogens and their antimicrobial resistance (AMR), as well as cow and herd risk factors associated with subclinical mastitis (SCM = cows with at least one udder quarter with california mastitis test (CMT) score > 2) and intramammary infections (IMI) caused by *Staphylococcus(S.) aureus* or Non aureus staphylococci (NAS) in dairy cows linked to Milk Collection Centers (MCCs) in Rwanda. Screening for SCM with the CMT was done on 572 cows from 404 herds linked to two MCCs in each of four provinces. Milk from udder quarters with a CMT score ≥3 (scale 1–5) was sampled for bacteriological analysis. Antimicrobial resistance was evaluated in 60 selected *S. aureus* isolates. Multivariable mixed effect and ordinary logistic regression analyses were performed to identify cow and herd level risk factors associated with SCM, NAS or *S. aureus* IMI in cows. The prevalence of SCM was 37.3 % at quarter level and 62.0 % at cow level. Bacteria were isolated 73.7 % of the cultured milk samples, whereas 23.3 % were culture-negative and 3.0 % were contaminated. *Staphylococcus aureus* and NAS were the most prevalent pathogens, representing more than half of all bacteriological findings. *Staphylococcus chromogenes* and *S. epidermidis* were the most prevalent NAS species identified. Of the *S. aureus* strains 83.3 % were resistant for penicillin, 100 % for clindamycin and 20 % tetracycline. The risk factor analysis showed that an increased stage of lactation, dirty udder and legs in single cow herds and lack of calf suckling the dam, dirty udder and legs and lack of feeding cow after milking in multiple cow herds were significantly ($P < 0.05$) associated with an increased odds of SCM. Similarly, increasing stage of lactation in single cow herds, and housing cows in individual cattle kraal or on earthen floor and hand washing between cows during milking in multiple cow herds were associated with increased odds for NAS IMI. Poor hygiene of milking area in single cow herds and absence of foremilk stripping in multiple cow herds were significantly ($P < 0.05$) associated with increased odds for *S. aureus* IMI in cows. In conclusion, SCM prevalence was high across MCCs. The majority of identified pathogens were contagious in nature and they exhibited resistance to penicillin. Control of the identified risks factors and improved biosecurity through adoption of best practices, and farmer training could contribute to lowering SCM prevalence in Rwanda.

1. Introduction

Mastitis is one of the most common and costly diseases for dairy industry worldwide. Mastitis is usually caused by microorganisms or occasionally by trauma on the udder and can be clinical where signs of illness and abnormal milk are apparent or subclinical where there are no visible clinical signs (Gruet et al., 2001). Subclinical mastitis (SCM) causes great economic losses mainly due to reduced milk yield (Seegers et al., 2003). Both forms of mastitis result in increased somatic cell counts (SCC) and measurement of SCC directly or indirectly in milk is a widely used method to detect SCM (Östensson et al., 2013). Mastitis poses a great concern of rise of antimicrobial resistance (AMR) because

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it leads to antimicrobial use on dairy farm (Oliveira and Ruegg, 2014), and an inappropriate use of antimicrobials may lead to AMR (Ström et al., 2018).

Pathogens involved in mastitis or intramammary infection (IMI) can roughly be categorized as either contagious or environmental depending on their reservoir and epidemiology characteristics (Dodd et al., 1969). Systematic identification of the prevalent mastitis pathogens is recommended for successful mastitis control (NMC, 2017) because pathogens differ from one region to another and may evolve over time (Myllys et al., 1998). Motaung et al. (2017) reported that variation and dynamics of *Staphylococcus* (*S.) aureus* and other pathogens among regions might originate from abiotic factors, feeding strategies, animal trade and movement. The same authors stipulated that it is important to screen a large sample of cows in order to detect possible variation and provide informative data. Change of etiology over time has happened to *Streptococcus (Str.) agalactiae* which was the dominant pathogen in Europe in the 1950s and 1960s (Stableforth, 1950; Andersen et al., 2003), subsequently, it decreased due to mastitis control programme implemented in many countries, however, evidence of re-emergence have been reported (Mahmmod et al., 2015).

Minimizing both mastitis and IMI in certain regions requires information on animal and herd risks of these infections in those specific regions. Abebe et al. (2016) indicated that cows from farms with larger herd size or farms with no milking order (vs. milking mastitic cows last) were more likely to contract mastitis than other cows in Ethiopia. Furthermore, Mekonnen et al. (2016) and Tolosa et al. (2013) showed that an increasing stage of lactation was associated with higher likelihood of mastitis, indicating continuous exposure of cows to mastitis pathogens throughout lactation. This is contradictory to the established pattern that early lactation is a more susceptible stage due to the periparturient immunosuppression or to infection acquired during the dry period. In the dry period, complex interactions between cow characteristics (host), dry cow management and facilities in general (environment) and the prevalent pathogens (agent) in herds or leads to new infections (Bradley and Green, 2001).Therefore, it is important to determine prevailing risk factors and adjust herd management accordingly. Identification of udder pathogens in infected cows is important for prevention of transmission of these pathogens to healthy cows.

Knowledge about mastitis dynamics in cows and its effect on milk quality is relevant in Rwanda because of recent developments and changes in the dairy industry. Historically, mastitis and udder health has not been sufficiently studied or actively monitored in Rwanda, and a recent study indicates that SCM is common in dairy cows in the peri-urban area of Kigali (Ndahetuye et al., 2019). Moreover, in Rwanda, as in many developing countries, there is low awareness of SCM and lack of application of 5-point mastitis control, a plan that has evolved into a 10-point mastitis plan, which has helped dairy producers in developed countries to minimize both prevalence of mastitis and the presence of contagious udder pathogens (Hillerton and Berry, 2005).

Mastitis is often mentioned as a reason for milk quality problems that lead to milk rejections at the milk collection centers (MCC) in Rwanda (Miklyaev, 2017). Milk collection centers serve as centralized cooling and storage aggregators for milk from many producers before forwarding the milk to fresh milk selling kiosks or to factories for processing (Miklyaev, 2017). Current regulation in Rwanda requires milk intended for sale to be collected at MCCs (Ministerial order, 2016). Therefore, to improve milk quality at MCCs depends on good udder health of the dairy cows providing the milk for that MCC. The objective of this research was to investigate the prevalence of SCM and identify cow and herd risk factors that were associated with SCM, non-aureus staphylococci (NAS) and *S. aureus* IMI in dairy cows from farmers that supply milk to MCCs in Rwanda. Furthermore, the study aimed to evaluate the distribution of causative pathogens and their AMR pattern.

2. Materials and methods

2.1. Study area

This cross-sectional study was conducted in dairy herds linked to eight MCCs among 100 MCCs functioning in Rwanda (IFAD, 2016). The MCCs included were selected from four provinces that should cover possible differences in agro-ecology conditions and milk production systems commonly known as milk sheds in Rwanda (TechnoServe, 2008). These eight MCCs were located in the following sites: MCC1 and MCC2 were located in Rwamagana and Nyagatare in the eastern province; MCC3 and MCC4 in Nyankenke and Rubaya in the northern province; MCC5 and MCC6 in Mudende and Rubengera in the western province and lastly MCC7 and MCC8 in Rugobagoba and Muyira in the southern province. The majority of herds in the eastern province are large and practice open grazing as grazing land is abundant. However, there are some small herds with zero grazing present at the periphery of the province such as in the Rwamagana region. Previously, dairy farmers in the northern and southern province milk shed practiced semi-grazing, but currently zero-grazing and smaller herds size (one to two lactating cows) with cows of mixed breeds prevail. In the western provinces the herds are small, with limited land area, semi-grazing systems dominate except in the Gishwati where dairy farmers practice open range with large herd size with pure exotic breeds (TechnoServe, 2008).

2.2. Sample selection

The study was approved and performed in accordance with the ethical guidelines of the Research Screening and Ethics Clearance Committee (RSEC-C) of the College of Agriculture Animal Sciences and Veterinary Medicine, University of Rwanda (UR-CAVM). Inclusion of each MCC was based on its capacity to receive 4,000 L of milk per day. Subsequently, sample size was estimated from a population of dairy cows linked to each MCC (400 lactating cows, which yield 4,000 L that MCC receives per day, considering a milk production in Rwanda of 10 L per cow per day and from expected regional SCM prevalence of 52 % (Iraguha et al., 2015) and a high SCM prevalence of 86.2 % (Abrahmsén et al., 2013). Final sample size of dairy cows per MCC was 69 lactating cows (confidence interval of 95 % and a precision of 10 %). The trained research team and local MCC technician used the modified snowball-sampling method (Faugier and Sargeant, 1997) to locate dairy herds willing to participate in the study. This method was used because there were no systematic registration of dairy farmers or cows at the MCC level. Small herds of one or two lactating cows dominated in all MCCs except Nyagatare, and the research team included all herds sequentially until reaching a minimum of 69 cows per MCC. Since herd size is large in only eastern province where a farmer can have up to 100 cows (TechnoServe, 2008) a limit of maximum 5 randomly selected lactating cows were selected per herd in all possible sub-regions in each MCC of Rwamagana and nyagatare with exception one herd in Rwamagana where14 lactating cows were screened in order to fulfil required cows per MCC. In total, 572 cows from 404 dairy herds were included in the study and the herds were visited once from April to September 2017.

2.3. Cow and herd data collection

Questionnaires were used to collect cow and herd information on potential risk factors for SCM as well as for NAS and *S. aureus* IMI through interviewing herd owners or workers and by observations during the visit. Cow level information included parity (1, 2–3, 4–5, ≥6), if restraint measures (which means to tie the cow with a rope in order to stabilize it) were used during milking (yes versus no), average daily milk production per cow (litres), if the dam was suckled by the calf (yes versus no), stage of lactation (≤3 months, 4–7 months, ≥ 8 months), age (≤ 5 years versus > 5 years) and udder and leg hygiene

Table 1
Herd variables related to subclinical mastitis prevalence in milk sheds in Rwanda included in the questionnaire administered to 404 farms across four provinces in Rwanda.

Variable	Categories
Herd size	One lactating cow /multiple lactating cows
Type of cattle kraal	Individual kraal/ grouped kraal/ no kraal[a]
Grazing type	Zero grazing/ semi grazing/ free grazing
Separate calving area; separate milking area	Yes/no
Type of milking	Hand vs machine
Technique of milking	Stripping/full hand
Milking frequency	Once / twice
Who milks the cow	Owner/worker/child
Hand washing before milking	With water only/with water and soap/no hand washing
Hand washing between cows, Teat and udder washing before milking; Teat and udder drying; Use of clean individual towel for drying; Pre milking teat dipping; Foremilk stripping; Post milking teat dipping; Milking mastitis cows last; Feed cows after milking;	Yes/no
Do you know what subclinical mastitis is?; Do you know what clinical mastitis is?; Performing CMT once a month; Dry cow therapy; Culling of chronically infected cows	Yes/no
Farm hygiene	Good/poor
Type of bedding materials	Sawdust/grass/none
Frequency of bedding material replacement	Once a week/twice a week
Type of floor of cow housing	Concrete/earthen/raised wood
Availability of veterinary service; Data record of past diseases	Yes/no
Milking area hygiene	Clean/slightly dirty/very dirty
Frequency of cleaning milking area	Before every milking/once per day/thrice per week/twice per week /once per week /other
Fly control	Yes/no

[a] Kraal: enclosure for cattle.

(clean, moderately dirty, very dirty). Herd level information included in the questionnaires are presented in Table 1. These factors were included in the questionnaire based on previous studies in Rwanda and in East Africa (Abrahmsén et al., 2014; Iraguha et al., 2015; Mekonnen et al., 2017; Ndahetuye et al., 2019) and their relevance for practices commonly used in the Rwandan dairy industry. Eight experts of various background reviewed the questionnaire for relevance of each question to the mastitis outcomes studied. Trained research team members interviewed farmers in Kinyarwanda language on herd characteristics, management practices, milking routines and hygiene using closed ended questions. The farmers responded freely without aid of the interviewer.

2.4. Screening for mastitis and milk sampling

Udder quarter milk samples were collected during ongoing milking by the research team. First, the initial two or three streams of milk were inspected for milk abnormality (colour, clotting) and discarded, followed by CMT screening. Subclinical mastitis was indirectly evaluated by CMT using the Scandinavian scoring system grade 1–5, where 1 indicates negative result (no gel formation, no indicative colour change), 2 is traceable (possible inflammation), 3 or above indicates a positive result where 5 has the most gel formation and deep blue/violet colour change (Schalm et al., 1971, Saloniemi, 1995). A cow was defined as positive for SCM if she had at least one positive quarter (CMT $\geq$ 3) and with no signs of illness and/or visible inflammatory signs of the udder, and without visible abnormality in milk. Quarters with CMT $\geq$ 3 were sampled for bacteriological analyses for culturing and identification of SCM causative agents according to the National Mastitis Council (NMC, 2017). After cleaning the teat ends with 70 % alcohol, an aseptic milk sample was collected in 10 mL sterile sampling tube. The samples were transported on the same day in an ice-cooled box and stored in −80 °C before being cultured within 48 h at the microbiology laboratory of the University of Rwanda, College of Agriculture, Animal Sciences and Veterinary Medicine, Busogo Campus.

2.5. Bacteriological analyses of milk samples

All milk samples were cultured on blood agar plates (5% bovine blood with 0.5 % esculin) and incubated in aerobic incubators at 37 °C for 24 h. Plates with unclear growth or no visible growth were allowed an extra 24 h before final examination. Microbial growth density and purity (single vs multiple colony morphology on the same plate) were checked. All isolates were initially characterized based on colony morphology, α-, β-, or double hemolysis. To be classified as a positive bacterial growth, the presence of at least one colony-forming unit (CFU) was required for the following major udder pathogens: *S. aureus*, *Streptococcus (Str.) uberis* and *Klebsiella* spp. and at least 5 CFUs for the other genera. Samples were classified as contaminated if two or more bacterial types were isolated from one milk sample and growth of one of the mentioned major udder pathogens was not identified. If growth of a major udder pathogen was found in combination with contaminating species and if the CMT was high, the sample was diagnosed as positive for growth of the major udder pathogen. The method described by Björk et al. (2014) was adapted for transport of all isolates to the National Veterinary Institute (SVA) in Uppsala, Sweden, for final identification at the species level using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). At SVA, each bacterial sample was first re-cultured on horse blood agar, and material from single pure colonies were spotted on a MALDI-plate without pre-treatment. The spots were covered with 1 μL matrix solution consisting of α-cyano-4-hydroxycinnamic acid (HCCA). Subsequently, isolates on MALDI-plate were analysed by the MALDI Biotyper system (Bruker Daltonics, Bremen, Germany) to identify the species. Mass spectra were compared against 4613 spectra in the MALDI Biotyper database using the MALDI Biotyper 3.0 Realtime Classification (RTC) software (Bruker Daltonics, Bremen, Germany).

2.6. Antimicrobial susceptibility testing

All staphylococci isolates were examined for β-lactamase production by the “clover-leaf” method as described by Bryan and Godfrey (1991). Furthermore, 60 *S. aureus* isolates (one isolate per herd) were selected and tested for antimicrobial susceptibility by determination of minimum inhibitory concentration (MIC) using a micro-dilution method according to recommendations from the Clinical and Laboratory Standards Institute using VetMIC™ panels (SVA, Uppsala, Sweden). The panels contained the following antibiotics: Penicillin

(Pc), cephalothin (Ct), cefoxitin (Fox), enrofloxacin (Ef), fusidic acid (Fu), erythromycin (Em), clindamycin (Cl), gentamicin (Gm), nitrofurantoin (Ni), tetracycline (Tc), trimethroprim (T). Twelve isolates were selected from each of the four regions, and additionally 12 isolates were included from a previous SCM study carried out by the same authors in herds located in peri-urban areas of Kigali (Ndahetuye et al., 2019) where samples are comparable and sampling was done according to the same protocol as in the current study. Each isolate was selected randomly from individual herds within each region. Only one isolate was taken from each herd. Initially material from 3 to 5 fresh colonies of each isolates was suspended in 5 mL cation adjusted Mueller-Hinton broth (Becton Dickinson, Cockeysville, MD, USA) and incubated for 3–5 hours at 37 °C to reach at least 10^8 CFU/mL. Subsequently around 10 µl was further transferred into a broth of cation adjusted Mueller-Hinton broth to obtain a final inoculum density of approximately 5×10^5 CFU/mL. Finally, 50 µl of the inoculum from each isolate was dispensed in a distinct well of VetMIC™ panels. The wells were sealed with transparent tape and panels were incubated for 16 − 18 hours at 37 °C. As quality control strains, *S. aureus* ATCC 29213, *S. aureus* ATCC 25923 and *Escherichia (E.) coli* ATCC 25922 were used. The MIC values were determined and defined as the lowest concentration of an antimicrobial that inhibited any visible growth of an isolate. The MIC distributions were studied, and isolates were reported as resistant or susceptible based on species-specific epidemiological cut-off (ECOFF) values issued by European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org).

2.7. Data analysis

The prevalence of SCM on cow level was calculated as the number of mastitis-positive cows (with one or more quarters with SCM) divided by the total number of cows tested. The quarter SCM prevalence was calculated as number of quarters with SCM divided by the total number of quarters investigated. As almost half of the herds had only one lactating cow, we decided to divide the data set into herds with a single cow and multiple cows and to evaluate the data set separately motivated by the fact that many of potential risk factors would not apply to single cow herds. To evaluate cow and herd risk factors associated with SCM on cow level (cow with one or more quarters with SCM), as well as with NAS (cow with one or more quarters with NAS IMI) and *S. aureus* IMI on cow level (cow with one or more quarters with *S. aureus* IMI), unconditional associations between each independent variable and the dependent variable, first with cow SCM status (0 = negative and 1 = positive), and subsequently in a separate analysis with cow NAS IMI status (0 = negative and 1 = positive) or cow *S. aureus* IMI status (0 = negative and 1 = positive), were investigated using univariable logistic or univariable mixed-effect logistic regression analysis. Statistical significance in this step was assessed at P-value < 0.20. Factors that were significant in the univariable analyses were then tested whether each binary factors are associated using e.g. chi-square test and then based on that, the one with the lowest P-value was then offered to the multivariable regression models. Multivariable mixed-effect logistic regression models, with MCC and province as two random factors in single cow herd models and with herds, MCC and province as three random factors in multiple cow herd models. If the random factor was not significant ($P \geq 0.05$), an ordinary logistic regression model was used. Random effects in the model for SCM in single cow herds (Table 5), and in the model for non-aureus staphylococci intramammary infections in dairy cows from multiple-cow herds (Table 7) were not significant. Furthermore, herd was only random factor included in the final multivariable mixed-effect logistic regression model for SCM in multiple-cow herds since province and MCC did not produce CI or SE because the variance components was very small and close to zero therefore excluded in the model as random factors (Table 6). The multivariable models were reduced using a manual, stepwise backward variable selection procedure where the initial model included all independent variables as main effects. Variables with a significant association ($P \leq 0.05$) with the dependent variable were kept in their respective final models. In each model, all variables with $P \leq 0.20$ in the univariable analyses were then re-tested one at a time in their respective final model and kept in the model if they were significantly associated with the dependent variable. In parallel, confounding was checked if removal of a variable in final multivariable models changed the regression coefficients of the remaining variables (> 25 %). All plausible two-way interactions between the significant main effects were tested in all final models. Model fit was assessed by Hosmer-Lemeshow goodness-of-fit test. The statistical analyses were performed using Stata 15 (Stata Corp LLC, College Station, USA).

3. Results

3.1. Descriptive data

Cows were of the following breeds: local breed (3.8 %), Holstein-local crossbreed (77.8 %), Jersey-local crossbreed (5.1 %), Holstein breed (11.1 %) and Jersey breed (2.3 %). Out of 572 animals, 331 cows (57.9 %) were from single lactating-cow herds and 241 (42.1 %) were from multiple lactating-cow herds. There were 79 herds with multiple lactating cows recruited in this study, with an average herd size of 7 cows (range 2–65). The average number of cows sampled in each multiple cow herd range was 3, (range 2–14). Hand milking was practiced in all herds. The use of dry-cow therapy, post-milking teat dipping, regular check of mastitis status of the cows using CMT and culling due to mastitis were absent in all visited herds. All herd owners recognized clinical mastitis, however only 4.3 % recognized SCM. Average daily milk production was 4.3 L/cow in Rugobagoba, 5.7 L/cow in Muyira in southern province, 4.8 L/cow in Rubengera 5.7 L/cow in Mudende in western province, 6.3 L/cow in Rwamagana and 8.9 L/cow in Nyagatare in eastern province and 6.6 L/cow in Nyankenke and 6.7 L/cow in Rubaya in northern province.

In total, 2288 quarters from 572 cows from 404 herds were examined, 23 quarters were blind, and thus 2265 were screened for SCM. In total, 664 quarters were milk sampled, 516 had IMI and 148 were culture negative. On average, 2 quarters per cow were sampled and on average 1 quarter per cow was infected.

The overall prevalence of SCM was 37.3 % at the quarter level and 62.0 % at the cow level. Cow level prevalence of SCM was 63.6 % and 59.8 % in single and multiple cow herds respectively. The SCM prevalence in cows delivering to MCCs differed as shown in Fig. 1.

3.2. Udder pathogens

Bacteria were isolated in 73.7 % of the cultured milk samples, whereas 23.3 % were culture-negative and 3.0 % were contaminated. Overall, NAS and *S. aureus* were the most prevalent bacterial species in examined samples, represented in more than half of all SCM cases in dairy cows in all MCCs (Table 2). The remaining findings were minor environmental udder pathogens. Within NAS, *S. chromogenes* and *S. epidermidis* were the most prevalent udder pathogens in SCM in all MCCs, representing 82 % of all identified NAS. The remaining NAS consisted of the following species from the most to the less prevalent: *S. xylosus*, *S. haemolyticus*, *S. saprophyticus*, *S. durans*, *S. sciuri*, *S. pasteuri*, *S. hyicus*, *S. capitis* and *S. warneri*. The distribution of udder pathogens was similar across the MCCs based on their prevalence.

3.3. Antimicrobial susceptibility testing

The most prevalent staphylococci exhibited β-lactamase production at a level of more than 50 % except *S. chromogenes* where prevalence of β-lactamase production was 45 % (Table 3.) In total, 83.3 % of the *S. aureus* strains were resistant against penicillin and 100 % were resistant against clindamycin. Overall, 48 out of 60 isolates were resistant to

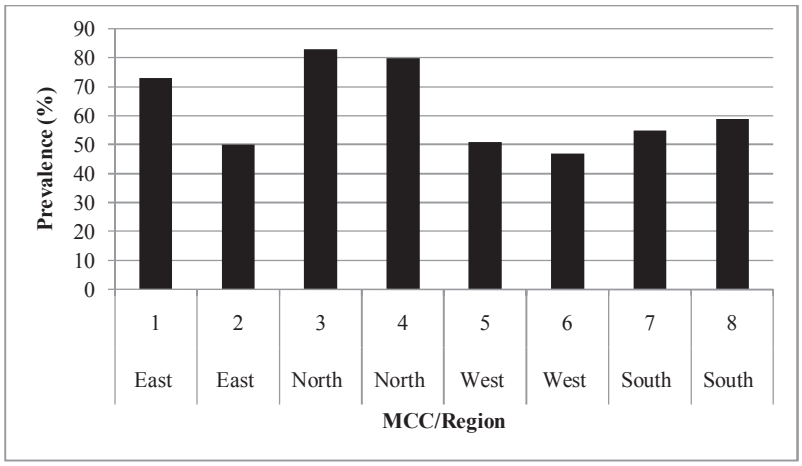

Fig. 1. Cow level prevalence of subclinical mastitis, defined as a California Mastitis Tests score of ≥3 in at least one udder quarter of a cow, in 572 dairy cows linked to 8 milk collection centers (MCC 1, n = 73, MCC 2, n = 72, MCC 3, n = 69, MCC 4, n = 66, MCC, 5 n = 71, MCC 6, n = 72, MCC 7, n = 75, MCC 8, n = 74) in four provinces in Rwanda in 2017.

Table 2
Udder pathogens isolated from subclinical cases of mastitis, defined as a California Mastitis Test score of ≥3 in at least one quarter of a cow, in 572 dairy cows from 404 herds linked to 8 milk collection centers in Rwanda in 2017.

Bacterial species	Total	Percentage
Non-aureus staphylococi	230	36.2
Staphylococcus aureus	195	30.7
Culture negatives	148	23.3
Lactococcus spp.[a]	23	3.6
Contaminated	19	3.0
Enterobacter asburiae	3	0.5
Streptococcus uberis	3	0.5
Klebsiella variicola	3	0.5
Other[b]	11	1.7
Total	635	100.0

[a] *Lactococcus lactis*, *Lactococcus raffinolactis*, *Lactococcus garvieae*.
[b] Other: *Acinetobacter johnsonii*, *Brevundimonas diminuta*, *Pseudomonas fluorescens*, *Enterococcus raffinosus*, *Klebsiella oxytoca*, *Enterococcus cloacae*, *Aerococcus viridans*, *Acinetobacter Iwooffii*, *Enterococcus durans*.

Table 3
β-lactamase production evaluated by clover leaf method in staphylococci species isolated from subclinical mastitis cases, defined as a California Mastitis Test score of ≥3 in at least one quarter of a cow, in 572 dairy cows from 404 herds linked to eight milk collection centers in Rwanda in 2017.

Bacterial species	Number of isolates tested	Number of β-lactamase positive isolates	Prevalence of β-lactamase positive isolates (%)
S. aureus	175	138	78.8
S. epidermidis	49	32	65.3
S. sciuri	3	3	100.0
S. chromogenes	126	58	45.3
S. xylosus	12	9	75.0
S. haemolyticus	6	3	50.0
Other	12	9	75.0
Total	383	252	65.8

Other: *S. pasteuri*, *S. warneri*, *S. hyicus*, *S. equorum*, *S. equorum*, *S. simulans, S. saprophyticus, S. sciuri*.

both drugs. Only a few of the *S. aureus* strains analysed were resistant to other drugs, except resistance towards tetracycline (20 %) (Table 4.). Among *S. aureus* isolates that were negative on β-lactamase production, 2 out of 8 isolates had also MIC ≤ ECOFF of 0.125 mg/l for penicillin. The results for *S. aureus* isolates distribution according to MIC are presented in Table 4.

3.4. Cow and herd level risk factors

Factors associated with the SCM ($P < 0.2$) in single cow herds and consequently offered to the full models were: Parity ($P = 0.119$), stage of lactation($P = 0.000$), calf suckling ($P = 0.157$), udder and leg hygiene ($P = 0.007$), grazing type ($P = 0.051$), type of person who milk the cows ($P = 0.064$), farm hygiene ($P = 0.011$), bedding materials ($P = 0.132$), frequency of bedding replacement ($P = 0.168$), data record of past diseases ($P = 0.131$) and milking area hygiene Age ($P = 0.050$). Factors were associated with the NAS infection ($P < 0.2$) in single cow herds and consequently offered to the full models were: stage of lactation ($P = 0.000$), udder and leg hygiene ($P = 0.115$), type of cattle kraal ($P = 0.163$), grazing type ($P = 0.006$), type of person who milk the cows ($P = 0.144$), fore-milking stripping ($P = 0.121$), farm hygiene ($P = 0.117$), frequency of bedding replacement ($P = 0.007$), type of floor of cow housing ($P = 0.133$), data record of past disease ($P = 0.021$) and milking area hygiene ($P = 0.024$). Factors were associated with the *S. aureus* ($P < 0.2$) in single cow herds and consequently offered to the full models were: Age ($P = 0.154$), milk production ($P = 0.053$), udder and leg hygiene ($P = 0.006$), separate calving area ($P = 0.100$),type of person milking the cows ($P = 0.114$),knowledge of clinical mastitis ($P = 0.098$), farm hygiene ($P = 0.021$), availability of veterinary service ($P = 0.077$) and milking area hygiene ($P = 0.002$). Factors were associated with the SCM ($P < 0.2$) in multiple cow herds and consequently offered to the full models were: Parity ($P = 0.078$), stage of lactation ($P = 0.167$), calf suckling ($P = 0.000$), udder and leg hygiene ($P = 0.001$), separate milking area ($P = 0.103$), feed cow after milking ($P = 0.076$), farm hygiene ($P = 0.090$), bedding materials ($P = 0.144$), type of floor of cow housing ($P = 0.011$), milking area hygiene ($P = 0.062$) and frequency of cleaning milking area ($P = 0.003$). Factors were associated with the NAS ($P < 0.2$) in multiple cow herds and consequently offered to the full models were: Stage of lactation ($P = 0.030$),calf suckling ($P = 0.014$), udder and leg hygiene ($P = 0.064$), type of cattle kraals ($P = 0.007$), grazing type ($P = 0.035$), type of person milking the cows ($P = 0.137$), hand washing between cows ($P = 0.126$), fore-milking stripping ($P = 0.166$), type of floor of cow housing ($P = 0.092$) and frequency of cleaning milking area ($P = 0.016$). Factors were associated with the *S. aureus* ($P < 0.2$) in multiple cow herds and consequently offered to the full models were: Parity ($P = 0.021$), udder and leg hygiene ($P = 0.164$), type of cattle kraal ($P = 0.137$), the type of person milking the cows ($P = 0.061$), hand pre-washing ($P = 0.108$), fore-milking stripping ($P = 0.072$), knowledge of subclinical mastitis ($P = 0.187$), farm hygiene ($P = 0.143$) and flies control ($P = 0.047$)

Stage of lactation and udder and hind-leg hygiene were significantly associated with SCM in dairy cows in single cow herds in the multivariable model (Table 5). The odds for SCM increased significantly for

Table 4
Resistance and distribution of minimum inhibitory concentration of *Staphylococcus aureus* (n = 60) from subclinical mastitis cases in dairy cows Rwanda.

Antimicrobial agent	No of Isolates tested	% Resistance	ECOFF	Number of isolates with indicated MIC												
				≤ 0.002	0.0040	0.008	0.0150	0.0300	0.0600	0.1200	0.2500	0.5000	1.0000	2.0000	4.0000	8.0000	16.0000	32.0000	64.0000
Penicillin (Pc)	60	83.33	0.125	0	0	0	0	7	3	0	2	3	45	0	0	0	0	0	0
Cephalothin (Ct)	60	0.017	1	0	0	0	0	0	0	0	0	0	59	0	1	0	0	0	0
Cefoxitin (Fox)	60	0.017	4	0	0	0	0	0	0	0	9	0	0	7	43	1	0	0	0
Enrofloxacin (Ef)	60	.		0	0	0	0	0	0	0	59	1	0	0	0	0	0	0	0
Fusidic acid (Fu)	60	0	0.5	0	0	0	0	0	0	0	0	60	0	0	0	0	0	0	0
Erythromycin (Em)	60	0	1	0	0	0	0	0	0	0	0	60	0	0	0	0	0	0	0
Clindamycin (Cl)	60	100	0.25	0	0	0	0	0	0	0	0	60	0	0	0	0	0	0	0
Gentamicin (Gm)	60	0	2	0	0	0	0	0	0	0	0	0	60	0	0	0	0	0	0
Nitrofurantoin (Ni)	60			0	0	0	0	0	0	0	0	0	0	0	0	0	60	0	0
Tetracycline (Tc)	60	20	1.5	0	0	0	0	0	0	0	47	1	0	1	11	0	0	0	0
Trimethroprim (T)	60	3.33	0.5	0	0	0	0	0	0	0	55	3	1	1	0	0	0	0	0

Table 5
Final multivariable logistic regression models describing the associations between risk factors and subclinical mastitis defined as a score of ≥ 3 in the California Mastitis Tests score in single-cow herds in dairy cows in eight milk collection centers in Rwanda.

	Coeff.	S.E.[a]	OR[b]	95 % CI (OR)	*P*-value
Stage of lactation (cow)					0.003
≤3months			Ref.[c]		
4 – 7 months	0.49	0.27	1.84	0.97; 2.78	0.07
≥8 months	2.09	0.64	3.24	2.29; 28.6	0.001
Udder and leg hygiene					0.02
Clean			Ref.		
Moderately dirty	0.80	0.29	2.27	1.25; 3.99	0.007
Very dirty	0.79	0.43	1.84	0.95; 5.07	0.07

[a] Standard error.
[b] Odds ratio.
[c] Reference category.

cows ≥8 months in lactation compared to cows ≤3 months in lactation (OR = 8.08, CI = 2.29–28.61, P = 0.001). No other significant differences were seen between other categories of month in lactation. Moreover, the odds for SCM increased significantly for cow with moderately dirty udder and hind-legs (OR = 2.22, CI = 1.25–3.99, P = 0.007) compared with cows having a clean udder and hind-legs. No other significant differences were seen between other categories of udder and hind-leg hygiene.

Poor udder and hind-leg hygiene, lack of calf suckling the dam and not feeding cows after milking were significantly associated with SCM in multiple cow herds in the multivariable model (Table 6). The odds of SCM increased significantly for cows in multiple cow herds that did not have a calf suckling them (OR = 3.30, CI = 1.88–17.75, P = 0.002) compared with cows that had a calf suckling them. Moreover, the odds for SCM increased significantly for cow in multiple cow herds with moderately dirty udder and hind-legs (OR = 3.83, CI = 1.65–8.88, P = 0.002) or a very dirty udder and hind-legs (OR = 7.51, CI = 1.81–31.07, P = 0.005) compared with cows in multiple cow herds having clean udder and hind-legs. Furthermore, the odds for SCM significantly decreased for cows not fed after milking (OR = 0.39, CI = 0.17 – 0.94, P = 0.036) compared to cows fed after milking.

Only stage of lactation remained in the multivariable analysis of risk factors associated with NAS IMI in single cow herds. There was an increased odds of NAS IMI for cows 4 – 7 months in lactation (OR = 2.71, CI = 1.47–4.98, P = 0.001) or ≥8 months in lactation (OR = 6.11,

Table 6
: Final mixed-effect multivariable logistic regression models adjusted for repeated measurements within herd describing the associations between risk factors and subclinical mastitis defined as a score of ≥ 3 in the California Mastitis Tests score in multiple-cow herds in dairy cows in eight milk collection centers in Rwanda.

	Coeff.	S.E.[a]	OR[b]	95 % CI[d] (OR)	*P*-value
Calf Suckling					
Yes			Ref[c]		
No	1.76	0.57	5.78	1.88;17.75	0.002
Udder and leg hygiene					0.000
Clean			Ref		
Moderately dirty	1.34	0.43	3.83	1.65;8.88	0.002
Very dirty	2.01	0.72	7.51	1.81;31.0	0.005
Feed cow after milking					
Yes			Ref		
No	−0.92	0.44	0.39	0.16;0.94	0.04
Random effects	Coeff.	S.E.[a]		95 % CI (Coeff.)	
Herd, variance	0.48	0.42		0.09; 2.63	

[a] Standard error.
[b] Odds ratio.
[c] Reference category.
[d] Confidence interval.

Table 7
Final multivariable logistic regression models describing the associations between risk factors and non-aureus staphylococci intramammary infections in dairy cows from multiple-cow herds in eight milk collection centers in Rwanda.

	Coeff.	S.E.[a]	OR[b]	95 % CI (OR)	*P*-value
Type of cattle krall					0.03
Individual			Ref[c]		
Grouped	−1.10	0.54	0.33	−2.16; -0.05	0.04
No kraals	−1.77	0.84	0.16	−3.42; -0.13	0.04
Hands washing between cows at milking					
Yes			Ref		
No	−1.43	0.48	0.23	−2.38; -0.49	0.003
Type of the floor in cow housing					0.03
Concrete			Ref		
Earthen	2.20	0.84	9.09	0.57; 3.84	0.008
Raised bed using wood	1.73	1.17	5.66	−0.56; 4.03	0.14

[a] Standard error.
[b] Odds ratio.
[c] Reference category.

CI = 2.61 − 14.30, $P < 0.001$) relative to cows ≤3 month in lactation.

Type of cattle kraal, hand washing between cows during milking, type of the floor of cow housing were all significantly associated with NAS IMI in dairy cows in multiple cow herds in the multivariable model (Table 7). There were decreasing odds of NAS IMI in cows kept in grouped cattle kraal (OR = 0.33, CI = 0.11 − 0.95, $P = 0.041$) and cows not kept in a cattle kraal (OR = 0.17, CI = 0.03 − 0.88, $P = 0.035$) compared with cows kept in individual cattle kraal. In addition, there was a decreased odds of NAS IMI in cows from herds where washing the hands between cows during milking was not practiced (OR = 0.23, CI = 0.09 − 0.61, $P = 0.003$) compared to cows from herds where such practice was present. Furthermore, there were increased odds of NAS IMI in cows from herds where there was earthen floor in cow housing (OR = 9.09, CI = 1.77 − 46.72, $P = 0.008$) compared to cows from herds kept in housing with concrete floor.

The only variable significant in the multivariable analysis of variables associated with S. aureus IMI in single cow herds was hygiene of milking area. There were increased odds ratio for *S. aureus* IMI in cows from single cow herds with slightly dirty milking area (OR = 2.52, CI = 0.99–6.36, $P = 0.05$) and very dirty milking area (OR = 4.37, CI = 1.66 − 11.55, $P = 0.003$) compared with cows from herds where milking area was cleaner.

Lack of foremilk stripping was the only variable significantly associated with *S. aureus* IMI in the multivariable analysis of risk factors for dairy cows in multiple cow herds. The odds of *S. aureus* IMI increased significantly if no foremilk stripping was performed (OR = 3.16, CI = 1.07–9.35, $P = 0.04$).

4. Discussion

In this study, we found a high prevalence of SCM in dairy cows in both single and multiple cow herds linked to the recruited MCCs. The average cow level SCM prevalence of 63 % was higher than the SCM prevalence of 52 % previously reported in eastern (Iraguha et al., 2015) or the 50.4 % in north-western (Mpatswenumugabo et al., 2017) regions of Rwanda, but lower than the 76.2 % (Ndahetuye et al., 2019) in peri-urban areas of Kigali in Rwanda. Prevalence of SCM varied markedly in the studied MCCs with those in the northern region showing the highest proportion' of SCM positive cows. In that particular region, many new farmers joined the trade more recently and probably did not receive trainings in animal husbandry unlike farmers in the eastern province Nyagatare who are known traditionally for being cattle keepers and have been receiving trainings (TechnoServe, 2008). The prevalence of SCM exceeding 50 % reported in this study is probably linked to a low awareness of SCM among farmers, lack of monitoring (as demonstrated by lack of regular udder health screening using CMT) and lack of implementation of elements of the 10-point mastitis control plan such as post milking teat dip or dry cow management. Poor udder and leg hygiene can increase the risk of SCM as the bacterial load at or close to the udder increases; keeping the animals and the surrounding environment clean would reduce the risk of SCM.

Our results indicate that NAS and *S. aureus* are the predominant pathogens, which is in agreement with studies in other East African countries (Abrahmsén et al., 2014; Mekonnen et al., 2017; Mpatswenumugabo et al., 2017) but not with Iraguha et al. (2015) who found mainly coliforms. It can be hypothesized that comparable management can contribute to finding similar profile of udder pathogens across MCCs, but also animal trade has been stipulated to result into mastitis pathogen strain being the same in the region where such trade occurs (Capurro et al., 2010). Thus, in the process of farmers buying or selling animals continually without screening for mastitis, the same mastitis pathogen species may prevail and spread between cows or herds. As *S. aureus*, *S. chromogenes* and *S. epidermidis*is can be regarded as contagious or udder adapted pathogens (Capurro et al., 2010), the low awareness of SCM among farmers may have allowed these species to continuously spread among quarters, cows and herds, which can further explain also their predominance. The predominance of *S. chromogenes* in the studied cows is of concern, as Supré et al. (2010) indicated that *S. chromogenes* could cause persistent IMI and result in as elevated SCC comparable to the ones of *S. aureus* IMI. It is also possible that staphylococci-caused mastitis may originate from reservoirs such as extra-mammary sites for *S. aureus* (Capurro et al., 2010) or milkers hands for *S. epidermidis* (Thorberg et al., 2006). Also, lack of hygiene at milking and milkers that work in more than one herd could also result in further spread of these pathogens. In order to gain information on possible transmission routes, genotyping of strains are needed.

Among the risk factors studied, increased stage of lactation was associated with both SCM and NAS IMI in single cow herds. The odds of contracting SCM was eight times higher in the late than early lactation stage. Our data agree with Hortet et al. (1999), suggesting the increased risk can be due to the accumulated exposure to mastitis pathogens cows undergo throughout lactation. Additionally, Abrahmsén et al., (2014) reported higher SCM prevalence in cows in late lactation than cows in early lactation stages in Uganda and Tolosa et al. (2013) found a direct association between late stage of lactation and prevalence of SCM in Ethiopia.

Keeping cows under sub-optimal hygienic conditions increases the bacterial load and potential for transmission of both contagious and environmental mastitis pathogens. Our data corroborated with those of Schreiner and Ruegg (2003) and Abrahmsén et al. (2014) where cows with poor udder or poor hind-leg hygiene were at increased risk of SCM. Sub-optimal hygiene conditions on udder and legs will facilitate udder pathogens gaining entry in the teat canal for example during milking and eventually causing SCM. Schreiner and Ruegg (2003) argues that

dirty udder will make ineffective teat dipping and sanitization. Similarly, poor milking area hygiene was significantly associated with *S. aureus* IMI, in single cow herds. As mentioned above, poor hygiene will first facilitate growth, multiplication of udder pathogens in the cow environment which is a risk for SCM and IMI, furthermore poor hygiene will make ineffective control methods such as teat dipping, and sanitization, this is the main reason of increased odds of cows to contract SCM and *S. aureus* IMI.

Calf suckling is generally practiced in Rwanda to stimulate milk ejection by the cow. Our study found that calf suckling can have a secondary benefit, as cows without this practice had an increased odds for SCM in multiple-cow herds. Other authors reported similar findings (Krohn, 2001; González-Sedano et al., 2010). The beneficial effect of calves suckling their dam might be due to the cow having a more complete milk ejection, a more complete udder emptying or removal of residual milk by the calf. Eventually, complete emptying the udder will remove all residual milk which could otherwise function as a substrate for IMI growth in the udder. In addition, calf saliva might have possible antimicrobial elements that could inhibit growth of mastitis pathogens (González-Sedano et al., 2010). We found that lack of foremilk stripping increased the likelihood of *S. aureus* IMI. It has been stipulated that once mastitis pathogens have gained entry to the teat sinus, lack of foremilk stripping allows pathogens greater access to the mammary gland cistern where it causes infection (Phillips et al., 1969).

Our data indicated that odds for NAS IMI increased significantly in cattle sheds with earthen floor compared to cowsheds with concrete floor. Similarly, there were decreasing odds to contract NAS IMI in grouped cattle kraals or without cattle kraal compared with individual cattle kraal. Individual cattle kraal, an enclosure where individual cow is kept always, favors increased infection pressure than spacious grouped or no cattle kraal. It is possible that concrete floor and lack of cattle kraal maintain better cow hygiene because of better draining of manure on concrete floor and lower infection pressure on cow kept on open ground without cattle kraal. Abrahmsén et al. (2014) reported comparable situation in Uganda where he reported that cows in open grazing on pasture had lower odds for SCM than cows in zero grazing because of better cow hygiene. It is not clear why absence of feeding cows after milking had lower odds for SCM than cows where feeding after milking is practiced since the latter prevent cows from laying down, thus preventing an open teat canal after milking to be infected with udder pathogens. It is possible that the SCM prevalence is to higher degree driven by other factors other than feeding cow after milking. This may include the fact that poor hygiene or uncomfortable lying area in cow shed in cattle kraal motivate less cows for laying down whether or not the cow is fed after milking. Similarly, it is not clear why lack of hand washing between cows during milking has less odds for NAS IMI in cows than actually hand washing between cows. Since some of NAS species such as *S. epidermidis* has been isolated from human skin (Thorberg et al., 2006), it can be postulated that hand washing between cows would minimize prevalence of NAS IMI. It is possible that during hand washing between cows using for example unsanitized water or without drying their hands, water serves as medium for proliferation and spread of NAS species, this might explain why cows in herds where hand washing between cows is practiced were more likely to contract NAS infection than in cows from herds where there is lack of hand washing between cows.

Overall, β-lactamase production was common in staphylococci isolated in the present study (65.8 %). However, this level is lower than the 77 % found in staphylococci in SCM cases in herds located in peri-urban areas of Kigali (Ndahetuye et al., 2019). It is possible that the difference is linked to antibiotic use since owners in farms in Kigali have better financial means and access to antibiotics which may lead to their overuse/misuse. This in turn can eventually have led to a higher selection of resistant clones compared to farms in the studied MCCs in the present study which are located in more rural areas. However, the levels in the present study are higher than the levels below 50 % reported in developed countries (Persson Waller et al., 2011; Persson et al., 2011). β-lactamase production was more prevalent in *S. aureus* than in NAS, highlighting its ability to resist against β-lactam antibiotics. The prevalence of β-lactamase production was higher in *S. aureus* (78.8 %) than in NAS (54.8 %). This is in agreement with Malinowski et al., (2002) who reported higher prevalence of antimicrobial resistance in *S. aureus* than in NAS. It can be hypothesized that this is due to blaZ gene which encodes β-lactamase production being more common in *S. aureus* than in NAS, however more research is needed. High prevalence of resistance in *S. aureus* is of concern because resistant clones of *S. aureus* survive in deep inner tissue of the mammary where antibiotics are hard to reach (Gruet et al., 2001). This has an implication on possible failure of bacterial cure against widely used penicillin (Gruet et al., 2001), which might lead to chronic mastitis and failure to restore infected cells to full milk production capacity even after treatment. Poor hygiene, mentioned earlier, trade without prior mastitis screening and absence of culling of cows infected with resistant clones in Rwanda may be drivers in the spread and dominance of β-lactamase producing staphylococci isolates. Reasons for difference in β-lactamase production in different staphylococci species is not known. Persson Waller et al. (2011) hypothesized that resistant isolates may belong to the same clonal group within each bacterial species, however, further research at molecular level is worthwhile.

The majority of *S. aureus* isolates had high MIC to penicillin and clindamycin, and fewer *S. aureus* isolated (20 %) had high MIC to tetracycline indicating possible reduced clinical susceptibility to these drugs. Resistance to other antimicrobials tested was uncommon. Resistance to clindamycin may be due to genetic potential of *S. aureus* isolates that carry erm genes or isolates acquiring that gene through horizontal gene transfer. The erm genes encodes modification of the clindamycin -binding site on the ribosome thus yielding its resistance in isolates (Lewis and Jorgensen, 2005). Although results of antimicrobial susceptibility from different methods are to be compared with caution, our results are in line with those of Suleiman et al. (2018) who found high levels of *S. aureus* isolates from SCM resistant against penicillin (88 %) and low levels of resistance against tetracycline (16.6 %) in Tanzania. Similarly, Kasozi et al. (2014) reported 100 % resistance to penicillin in *S. aureus* isolates from SCM cases in Uganda, and different trend in tetracycline resistance (71.4 %) than in current study. Ssajjakambwe et al. (2017) reported that tetracycline and penicillin are most common used in treating different infections including mastitis in the Uganda, a country comparable with Rwanda. Since antibiotics for animals can be procured without any veterinary prescription in Rwanda (Manishimwe et al., 2017), it can be hypothesized that the overuse or /misuse of tetracycline and penicillin could equally have contributed to the selection pressure of resistant clones henceforth-high resistance levels reported in this study.

5. Conclusion

This is the first large scale study on SCM in dairy cows across the main dairy producing regions in Rwanda, thus providing information on geographic distribution of the prevalence and etiology of SCM. Our data supports the anecdotal evidence that lack of implementation of the 10-point-mastitis control plan results in high prevalence of SCM and contagious udder pathogens. This plan has helped minimize contagious udder pathogens in developed countries for the last decades (Ruegg, 2017). The prevalence of SCM reported here differed among MCCs and averaged to 63 % at the cow level. We found comparable udder pathogens in SCM positive cows in all MCCs, predominantly *S. aureus* and NAS, and about 65.8 % of these pathogens produced β-lactamase implying resistance to penicillin. The majority of identified risk factors were cow related suggesting that herd management factors were similar in studied herds. A multipurpose approach that includes emphasis on hygiene during milking and practice of biosecurity measures are required in order to limit the spread of both contagious pathogens and

antimicrobial resistant isolates. Education and training to increase awareness of SCM and monitoring at farm and at MCC level, could motivate farmers to control the disease and help increase production and related incomes

Conflict of interest statement

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prevetmed.2020.105007.

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