INFORME FINAL

Presentado por

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Epidemiología molecular de la bacteriana mastitis en el ganado vacuno en la provincia de El Oro, Ecuador: Impacto económico y medidas de control

ÁREA DE DESARROLLO:
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Contenido

INFORME FINAL DE ACTIVIDADES

INTRODUCCIÓN

La cría intensiva de ganado se asocia con la prevalencia de varias enfermedades que originan problemas de salud tanto en los animales como en la comunidad, más que la contaminación ambiental. La mastitis es una enfermedad bovina grave que afecta a la ubre y por ende resulta en un mal estado de salud de los animales, el deterioro de la calidad de la leche producida y el potencial problema de salud pública en la mayoría de los casos. Aunque la mastitis representa una pesadilla para la industria ganadera, poco se sabe sobre su incidencia, surgimiento microbiano y la diversidad molecular de los agentes causantes en Ecuador. La mastitis bacteriana es la principal forma común de la enfermedad debido a la abundancia y diversidad de agentes microbianos en el ambiente de las granjas. Su transmisión incluye el contacto de la tetina con el agente infeccioso (s), ya sea desde el ambiente circundante o de un animal a través de prácticas de ordeño o contaminación de la piel del pezón. El nivel de higiene es un factor inductivo significativo, mientras que la transferencia de los animales infectados entre diferentes granjas es un factor importante de diseminación. El manejo de las granjas y las condiciones de higiene en el entorno de los animales son factores cruciales en la tasa de incidencia de la enfermedad. El tratamiento depende principalmente de un amplio uso de agentes antimicrobianos. Sin embargo, el desarrollo de resistencia a los medicamentos por parte de los microbios responsables y la acumulación de residuos de drogas tóxicas en los productos alimenticios crean gran preocupación. La diversidad en la comunidad microbiana que causa la mastitis es la suma de las pautas de tratamiento y de control. La identificación precisa de los agentes causantes y la determinación de la sensibilidad de los microorganismos aislados a los fármacos antimicrobianos usados ayudará eficazmente el tratamiento y control de la enfermedad.

Los análisis moleculares de los aislados recolectados abren nuevos horizontes para entender la diversidad de la comunidad microbiana causante de la mastitis, el desarrollo de resistencia y de genes de resistencia, la determinación de la fuente de infección y un seguimiento de la infección. Por lo tanto , el presente estudio se realizó

para visualizar en detalle el análisis sobre la epidemiología molecular de la mastitis en el ganado vacuno en la provincia de El Oro, Ecuador, a partir del otoño de 2013 por 2 años consecutivos , y también el tratamiento preciso y control de la enfermedad conocida como mastitis : (1) identificación exacta de los microbios causantes de mastitis a través de procedimientos bacteriológicos estándar ; (2) La recomendación de uso de agentes antimicrobianos basados en la sensibilidad / resistencia a un amplio grupo de agentes antimicrobianos ; (3) la determinación de la diversidad molecular de las bacterias con mastitis y la inferencia de sus filogenias; (4) la determinación estadística de los factores de efecto recíproco sobre la presencia de mastitis; (5) la elucidación de la presencia / ausencia de genes de resistencia en cepas microbianas individuales / aisladas; (6) la determinación de la relevancia de los microbios recuperado como un problema de salud pública; (7) construir un análisis de los factores de riesgo para ayudar a los tomadores de decisiones a emitir planes estratégicos de salud veterinaria y pública; (8) diseñar una granja (ganadera) basado en un régimen de gestión para ayudar a controlar la enfermedad y aumentar la sostenibilidad agrícola y la rentabilidad. Sobre la base de las bases de datos generadas, la gestión y control adecuados, los regímenes aplicables de mastitis se diseñarán, no sólo en la provincia de El Oro, pero pueden ser aplicables a nivel nacional.

MARCO TEÓRICO

La mastitis (inflamación de las glándulas mamarias) es una grave enfermedad de la ubre de ganado bovino que resulta en una mala salud del animal, disminución de la fertilidad y la productividad, deterioro de la calidad de la leche producida y causa un potencial problema de salud pública. La mastitis se origina como una reacción del tejido de la ubre al ataque bacteriano, o a una lesión química, térmica, o mecánica; sin embargo, la mastitis inducida por agentes microbianos sigue siendo la forma más común. La enfermedad sigue siendo un desafío importante para la industria lechera en todo el mundo a pesar de la aplicación generalizada de estrategias de control de la mastitis. La mastitis subclínica se asocia a menudo con el recuento de aumento de

células somáticas y un cambio de la calidad física y química de la leche producida [Malek dos Reis et al., 2013], mientras que, la mastitis clínica es una grave dolencia animal ya que se asocia con el sufrimiento y reducción del bienestar de los animales afectados [Leslie et al., 2012].

En tanto, en la mastitis clínica y subclínica hay una pérdida sustancial en la producción de leche [Blum et al., 2014], incluso después del tratamiento debido a los daños irreversibles en el tejido mamario [Zhao et al., 2008]. La baja calidad de los resultados de leche en un daño económico significativo debido a la pérdida de calidad superior de la leche [Seegers et al., 2003], o descarte de la leche con baja calidad obvia es usado para la alimentación de los terneros o descarte completa en los casos graves. Además, no hay costes adicionales de tratamiento de pérdida económica debido , incluyendo los costos de los diagnósticos, medicamentos , consultas y servicios veterinarios , así como los costos de la mano de obra adicional necesaria gestión de la mastitis por [Seegers et al. , 2003]. Los residuos de antibióticos en la leche son un serio desafío para la industria láctea que podría conducir a la exclusión de los animales a altas dosis de antibióticos del ordeño para el consumo humano.

La mastitis es la enfermedad multifactorial, que está ampliamente asociado con la presión de infección debido al sistema de gestión, el nivel de higiene, sistema de ordeño, la edad y la raza del animal y/o factores externos como los cambios de clima [Jansen et al., 2009]. La mastitis se clasifica habitualmente como formas clínicas y subclínicas. Mastitis clínica se caracteriza por anormalidades obvias de la ubre (por ejemplo, hinchazón y enrojecimiento, picor..... etc.), y/o cambios en la apariencia de la leche y la composición. Identificación de mastitis subclínica se basa principalmente en el recuento de células somáticas (SCC) en la leche producida y el cultivo bacteriológico. Individuales cuartos de la ubre con un SCC por encima de un determinado umbral (por ejemplo, 200.000 / ml) se considera que tienen la mastitis subclínica [Schukken et al., 2003]. A menudo, un cuarto de la ubre está infectado, sin embargo, ni la ubre ni la leche son clínicamente anormales. Mastitis subclínica es la principal forma de mastitis en vacas lecheras modernas, con rangos de tasa de infección de 20 a 65% en los rebaños dado en todo el mundo [Gitau et al., 2014;

Ramírez et al., 2014]. Incidencias reportadas difieren ampliamente entre-intra países, basado en el concepto del estudio desde el nivel del rebaño al nivel vaca (es decir, la tasa de infección por trimestre). En África, la alta incidencia de ~ 85%, 63% y 51% en las vacas lecheras se informó de Tanzania y Nigeria [Mdegela et al., 2009; Gebrekrustos et al., 2012] frente a 28% en Argelia [Saidi et al., 2013]. En Asia, se informó alta tasa de infección (~ 88-55%) en Vietnam y Corea del Sur [Ostensson et al, 2013.; Sharma et al., 2013], tasa moderada (48%) en Sri Lanka [Gunawardana et al., 2014], y la tasa relativamente baja (~ 20%) en Bangladesh [Sarker et al., 2013]. Comparativamente, la incidencia en los países de América Latina es más o menos similar en otros continentes, con ~ 49% en Brasil [dos Reis et al., 2011] y ~ 37% en Colombia [Ramírez et al., 2014]. En los países industrializados, en los que se presta más atención a la salud de la ubre, la incidencia de las infecciones intramamarias parece bajo. La prevalencia en los rebaños lecheros holandeses se informó en un 25% frente al 3% en Canadá [Watters et al., 2014]. Curiosamente, una fracción considerable de la infección ocurre durante el IMI período seco, lo que agrava la situación después del parto y durante la lactancia. Incidencia trimestre promedió 12,8% en el no tratado a 8,0% en los trimestres tratados en el período seco de ganado lechero [Robert et al., 2006].

Etiología de la enfermedad

La mastitis microbiana es causada por una amplia variedad de bacterias Gram positivas y Gram negativas; hongos, micoplasmas y con menor frecuencia por las algas. Sin embargo, el trauma mecánico, traumatismo térmico, y la irritación química predisponen la glándula a la infección intramamaria. Una amplia gama de microorganismos puede causar la mastitis en las vacas; Sin embargo, los principales patógenos de mastitis (*Estafilococo aureus, Estreptococo agalactia, Estreptococos uberis, Estreptococos dysgalactiae* y *Escherichia coli*) son responsables de aproximadamente el 80% de los casos de mastitis en comparación con los menores tales como (CNS) estafilococos coagulasa-negativos [Reyher et al., 2012]. La distribución de la deformación de los patógenos de mastitis difiere en cada animal en el rebaño, y entre rebaños, países y especies hospedadoras. A pesar de la naturaleza

del agente causante, la patogenicidad de determinada cepa (s) se rige por factores patogénicos que aumentan su invasividad y bajo los patrones de genes específicos. Estos patrones difieren de raza a raza y están fuertemente asociados con la magnitud de la respuesta inflamatoria resultante, y directamente en el número de recuento de células somáticas [Zecconi et al., 2005].

Como la respuesta del huésped a la infección intramamaria, mastitis depende de la interacción de anfitrión, agente infeccioso, y los factores ambientales [Zhao et al., 2008]. Los agentes etiológicos pueden variar de un lugar a otro dependiendo del clima; especies animales y la cría de animales. Está claro que algunas bacterias que causan mastitis puede haber aumento de la aptitud, es decir, una mayor capacidad de causar mastitis, que los otros.

Mastitis contagiosa

Patógenos contagiosos transmiten de vaca a vaca, a menudo en el momento del ordeño a través del contacto con las herramientas de ordeño-equipo contaminado. Normalmente, los patógenos contagiosos se caracterizan con infecciones persistentes y dominio de una cepa a través de la manada. El patógeno más común es contagiosa Estafilococos aureus, y Estreptococos agalactia.

La mastitis ambiental

Patógenos ambientales que normalmente se encuentran en el entorno de granja tales como estiércol, material de cama y/o el suelo. Mastitis ambiental se caracteriza por infecciones transitorias y multitud de cepas. Los patógenos ambientales más comunes son los coliformes, tales como *Escherichia coli, estreptococos*, tales como *Estreptococo dysgalactiae y Estreptococo uberis,* y estafilococos coagulasa negativos. La etiología bacteriológica de la mastitis en algunos países muestra una tendencia de cambio desde contagiosa a los patógenos ambientales, lo que ha reducido la eficacia de las estrategias de control de la mastitis tradicionales [52].

CONCLUSIÓN Y PERSPECTIVAS FUTURAS

Mastitis sustancialmente los impactos de la industria lechera, que puede apoyar el desarrollo, especialmente en los países en desarrollo. La mastitis es causada por una amplia gama de microorganismos, con la transmisión varía de contagiosa a los modos ambientales. Principales producen mastitis patógenos incluyen Estafilococos aureus, Estreptococos agalactia, Estreptococos uberis, Estreptococos dysgalactiae y Escherichia coli. Sin embargo, los patógenos menores aún jugadores eminentes en las infecciones intramamarias. Varios informes indican fuerte asociación entre ciertas cepas microbianas y manifestación clínica y el pronóstico de la enfermedad. El tratamiento depende principalmente del uso de anticuerpos, sin embargo, los regímenes de gestión, nivel de higiene y prácticas de salud de la ubre son factores cruciales en el control de la enfermedad. Mejora de la vigilancia de la enfermedad y una mejor comprensión de la multiplicidad de agentes patógenos, factores de virulencia bacteriana, y mecanismos de patogénesis son cruciales para el control de la enfermedad. Mejora la resistencia a enfermedades y mejorar el conocimiento de la inmunología de la glándula mamaria son esenciales para la prevención de enfermedades y el desarrollo de vacunas.

PLANTEAMIENTO DEL PROBLEMA

En los países de América del Sur, la mastitis bovina es la enfermedad muy común, multifactorial con alto impacto en la industria ganadera. En estos países la mastitis es causada por diferentes patógenos, sin descripción inequívoca de los agentes microbianos causantes. Sin embargo, las bacterias contagiosas incluyendo Estafilococos aureus y Estreptococias agalactia son los agentes más reportados implicados en la mastitis bovina. Estreptococias Ambiental uberis También se ha informado repetidamente. Dicha diversidad profunda está bien documentada en Estafilococos aureus de diferentes países de América del Sur, con indicación de la posición dominante de algunos clones en la mayoría de las infecciones. Transferencia horizontal de genes de algunos genes de virulencia asociados se reporta en algunas cepas de estafilococos distintos involucrados en la mastitis

bovina, dándoles una ventaja selectiva al colonizar las glándulas mamarias. La mayoría de las cepas de estafilococos aislados son cepas toxigénicas potenciales que presentan el riesgo potencial adicional para la salud pública. Además, gran diversidad de perfiles genéticos y virulencia se documentan entre estreptococo bovino, de las cepas deuberis. Sin embargo, las bacterias coliformes fueron los agentes menos reportados. En conclusión, la presencia de estas infecciones intramamarias parece estar asociado con ciertas condiciones de manejo, que destacó la necesidad de mejorar las medidas de diagnóstico y control de la mastitis bovina.

En Ecuador están disponibles en la incidencia o la etiología de la mastitis bovina no hay datos. Sin embargo, las prácticas de campo en las granjas de animales tanto por los veterinarios y propietarios de fincas indican a la alta incidencia de la mastitis bovina que drena la economía agrícola por el costo de la consulta veterinaria y medicamentos, así como el deterioro de la salud de los animales y la productividad. El uso excesivo de antibióticos para controlar la mastitis bovina plantea grandes dudas sobre la calidad de la leche y la carne producida a partir de estas granjas en la salud pública debido a los residuos de drogas. También, como el uso intensivo de antibióticos es la mayoría de los factores que promueven para la aparición de las nuevas generaciones de las bacterias resistentes a los fármacos que sería un reto importante para la salud pública y animal. Este estudio se llevó a cabo en algunas granjas de todo el área municipal de la ciudad de Machala, provincia de El Oro, donde la agricultura intensiva está creciendo como una industria bien y lucrativo.

Este proyecto se realiza en colaboración con el departamento de veterinaria, facultad de ciencias agrícolas en UTMATC, y en algunos casos con la consulta con los miembros de la rama del ministerio de Agricultura en Machala. Las visitas de campo para recoger las muestras fueron organizadas por el personal UTMATCH (Dr. Lenin Aguilar; Dr. Evan Leudena).

Este proyecto trató de contribuir en la comprensión de los agentes bacterianos responsables de mastitis en el ganado vacuno en la provincia de El Oro. Los resultados y conocimientos generados ayudarán al veterinario para tomar la decisión correcta sobre las políticas de tratamiento de los tratamientos de campo, ayudarán a

los académicos y la comunidad científica para conocer en profundidad la diversidad genética y la heterogeneidad de los microbios causantes de mastitis, ayudarán a los propietarios de fincas para adoptar las condiciones de medición rentables y políticas de tratamiento rentables para aumentar la productividad, la rentabilidad y la sostenibilidad de las explotaciones.

DELIMITACIÓN DE LA INVESTIGACIÓN

Trabajar con las enfermedades infecciosas necesita varias normas comunes de las medidas de bioseguridad para la seguridad del personal, pública y el medio ambiente. Por lo tanto, un buen aislamiento y de laboratorio equipado con gabinetes de seguridad e instalaciones de esterilización para el aislamiento. El acceso de tales laboratorios debe estar restringido al personal bien entrenado. Además, se necesita un flujo continuo y suave de los reactivos y productos químicos necesarios para el cultivo, el aislamiento y la identificación bioquímica. Los análisis moleculares necesitan equipos y reactivos más sofisticado, así como un lugar físico como de laboratorio confinado. Más importante aún, viajes de campo, granjas visitas, así como la recogida de datos y muestras necesitan equipo de conocer y activa. Todos estos problemas y limitaciones fueron dirigidos al SENESCYT, especialmente durante la visita del SENESCYT equipo encabezado por la Excelentísima Sra. Susana Toro on Noviembre 12/2014.

UTMATCH hizo lo posible para resolver estos problemas, pero mucho fuera de las manos. Lo hicieron de suministro con varios medios de comunicación necesarios para el cultivo y aislamiento, además con personal (privada) que fijan otros reactivos y productos químicos. Además, en la Facultad de Agricultura, el nuevo laboratorio acaba de llegar y ahora bajo la instalación de los equipos. Otro laboratorio en el departamento de veterinaria está en marcha, donde definimos las especificaciones de equipos y después de la aprobación seleccionado los equipos y terminamos los últimos pasos con la empresa proveedora. Sin embargo, estos son demasiado tarde para este proyecto. En diciembre de 2014, se perdieron 4 meses debido a la falta de reactivos y productos químicos en lugar broncearse los equipos. Esto corto período aprobado por SYNECYTE es de la partida de Enero, 12 / 2015- hasta Abril 26/2015.

En todos los casos que visitamos con éxito varias fincas, evaluó la tasa de incidencia de mastitis clínica y subclínica. Según la sensibilidad de las bacterias aisladas a amplio panel de antibióticos (antibiograma), especificamos el antibiótico apropiado para el tratamiento de los animales infectados y el curso del tratamiento. Seguimiento se hizo para evaluar la eficiencia de régimen de tratamiento. Además, durante todo el proyecto hemos obtenido un gran número de cepas bacterianas. Debido a la falta de instalaciones moleculares, hemos cooperado con mi mentor el Prof. Hongxuan Él (Director del Laboratorio), en el Instituto de Zoología de la Academia China de Ciencias, China, para analizar molecularmente los aislamientos obtenidos. Hemos enviado a (no infecciosas) muestras representativas fijos para la identificación molecular del gen 16S rDNA. Actualmente estamos recibiendo los datos.

JUSTIFICACIÓN

El control de la mastitis bovina es la meta suprema en términos de productividad animal, economía agrícola, el bienestar animal, así como la salud pública. Para ello, la investigación de campo de los animales es el factor clave para determinar la incidencia y la magnitud de la enfermedad. Cultivo, aislamiento e identificación del agente infeccioso es elemento esencial conocer la etiología de la enfermedad y las medidas apropiadas de manejo y régimen tratamiento. El análisis molecular identificó con precisión los microbios aislados y la diversidad molecular de estos patógenos. Prueba de sensibilidad a los antibióticos es la aplicación real para el tratamiento eficaz y el curso para el tratamiento. Seguimiento de los animales bajo tratamiento es importante evaluar la eficiencia del régimen tratamiento. Debido a ninguna base de datos en a mastitis bovina en Ecuador está disponible, este estudio hace referencia sobre la base de la literatura disponible. Curiosamente, se identificó como agentes Responsable de la mastitis clínica y subclínica amplia gama de la bacteria ambiental. Patógenos contagiosos prevalecían en algunas granjas, mientras que los patógenos ambientales eran comunes en otras granjas, lo que implica el sistema de gestión. Pruebas de sensibilidad Anti-microbianos indicaron que todas las cepas aisladas de resistencia a fármacos mostraron durante al leas un antibiótico, pero la resistencia a múltiples fármacos no se detectó. La falta de

bacterias resistentes a múltiples fármacos es una buena característica del perfil de la enfermedad, a pesar de la alta tasa de incidencia. Sin embargo, el uso incontrolado mal y de los antibióticos son alarmantes para la posibilidad de la aparición de patógenos resistentes a múltiples fármacos que constituiría un serio peligro a la salud pública y veterinaria.

OBJETIVO GENERAL

- Teniendo en cuenta la falta total de información sobre la epidemiología de la mastitis bovina, el presente estudio tuvo como objetivo determinar las tasas de incidencia y las medidas de intervención para controlar la enfermedad.
- Generación de una base de datos microbiana, tanto en los niveles bacteriológicos y moleculares será beneficioso tanto para uso regional y nacional.
- Bases de datos precisos permitirán regímenes de tratamiento rentables, regímenes de gestión adecuados para el control de la mastitis que aumentará la productividad y la sostenibilidad financiera de la fincas / rebaño.
- Sobre la base de los informes periódicos y bases de datos generados a partir de este proyecto, los responsables políticos podrán tomar las decisiones estratégicas racionales y planes relacionados con la salud animal.
- Participar en la enseñanza de las nuevas generaciones de investigadores y graduados, la transferencia de conocimientos, diferentes habilidades de investigación y desarrollo de capacidades.

OBJETIVOS ESPECÍFICOS

- Base de datos epidemiológicos : Creación de la base de datos epidemiológicos de incidencia y los factores asociados como la higiene , los factores de manejo y la edad y raza del animal , el estado nutricional, variaciones estacionales ... .etc.
- Base de datos microbiana: Esta base de datos será construido en la identificación bacteriológica de microorganismos causantes de mastitis. Sensibilidad a varios agentes antimicrobianos, la diversidad molecular y la heterogeneidad, factores de virulencia y la presencia de genes de resistencia asociados.

- Factores de riesgo: Basado en las bases de datos generadas, las condiciones ambientales y los regímenes de gestión, los factores de riesgo se desprenderán después de los análisis estadísticos.
- Creación de capacidad y la transferencia de conocimiento: la formación de los profesores, investigadores y estudiantes ayudan en la adquisición de nuevas competencias y la transferencia de conocimiento.

RESULTADOS OBTENIDOS

Investigación:

- Visitar las granjas lecheras de la provincia de El Oro y la investigación de los animales para determinar las infeted queridos, ya sea clínica o subclínica (Anexo 1 CD).
- Recogida de las muestras de leche de animales infectados y cultivo microbiológico de las muestras en el laboratorio (Anexo 2 CD).
- Identificación de los principales patógenos de mastitis en las granjas visitadas (Anexo 3 CD).
- Implicación de bacterias ambientales en la mastitis bovina en Ecuador: un estudio piloto (Anexo 4 CD).
- La mastitis bovina: una actualización (Anexo 5 CD).
- Se incluyeron otros aspectos que contribuyeron a la generación de información científica de gran interés para la ciencia y, por supuesto, al conocimiento de la biodiversidad microbiana de Ecuador y por el cual fue posible producir el tercer artículo (en preparación) sobre la prevalencia de mastitis bovina en el Ecuador, que es la primera descripción en este campo.

Formación científica:

- El establecimiento del Laboratorio molecular en el departamento de veterinaria, Facultad de Agricultura, UTMATCH. (Anexo 6 CD).

- Capacitación a varios miembros de la institución (estudiantes y profesores) de departamento de veterinaria, Facultad de Agricultura, UTMATCH. (Anexo 7 CD).

Docencia:

- Prestar asesoramiento, formación práctica y aportación de trabajo para estudiantes de veterinaria de alto nivel para llevar a cabo la parte práctica de sus proyectos de graduación.

ESCRITOS INDEXADOS O ARTÍCULOS CIENTÍFICOS PUBLICADOS Y/O SOMETIDOS A REVISTAS CIENTÍFICAS

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Implication of environmental bacteria in bovine mastitis in Ecuador: A pilot study

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Abstracto

Este estudio describe la mastitis clínica y subclínica en una granja de ganado lechero en Santa Rosa, provincia de El Oro, Ecuador. Una granja de tamaño medio (N = 65) experimentaron alta incidencia de clínica (10,8%, 7/65) y subclínica (81.5, 53/65) mastitis, según lo determinado por California Mastitis Test (CMT). La incidencia por trimestre fue de 57,7% (150/260), con la frecuencia en cuartos delanteros fue mayor (96/150; 64%) que en los cuartos traseros. Aislamientos bacterianos fueron genotipo basado en el análisis de PCR de la secuencia de fragmento de gen 16S rDNA. Identificación bacteriana indicó a la aparición de los estafilococos coagulasa negativos (CNS) en 50,8% (33/65), Bacilos spp en el 30,8% (20/65), Escherichia (E.) coli en el 6,2% (4/65), Estafilococo aureus en 4,6% (3/65) y Estreptococo agalactia en 4,6% (3/65); y Micrococos lúteos en 3,1% (2/65). Especies CNS identificadas incluyen estafilococo epidermidis, Estafilococo hemolíticos, Estafilococo sciuri, Estafilococo arlettae, Estafilococo hominis y estafilococos gallinarum; mientras que las especies de

Bacillos incluyen Bacillos cereus, B. licheniformis, B. subtilis, B. altitudinis, B. pumilus y Corynebacterium freneyi. En patrones de sensibilidad a los antibióticos in vitro de cepas aisladas fueron sensibles a amoxicilina / ácido clavulánico, ampicilina, cefotaxima, enrofloxacina, gentamicina, neomicina y sulfametoxazol / trimetoprim. Diversos grados de susceptibilidad se registraron a la penicilina, la estreptomicina y la tetraciclina. Los casos clínicos fueron tratados con la administración sistémica de una dosis única de enrofloxacina (10 mg / kg) seguido de una infusión intramamaria de fórmula cóctel de antibióticos (tetraciclina y neomicina), bacteriocina y prednisolona anti-inflamatoria dos veces por día, durante 3 días consecutivos, mientras que subclínica los casos fueron tratados con el régimen de infusión intramamaria. Hasta donde sabemos, este es el primer informe sobre la mastitis en Ecuador.

Palabras clave: Mastitis, estafilococos coagulasa negativos, los bacilos, Ecuador

The first Paper is submitted to the Journal: Tropical Animal Health and Production: https://www.editorialmanager.com/trop/default.aspx

Implication of environmental bacteria in bovine mastitis in Ecuador: A pilot study

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Abstract

This study described clinical and subclinical mastitis in a dairy cattle farm at Santa Rosa, El Oro Province, Ecuador. A medium size farm (N = 65) experienced high

incidence of clinical (10.8%, 7/65) and subclinical (81.5, 53/65) mastitis, as determined by California Mastitis Test (CMT). The incidence per quarter was 57.7% (150/260), with the frequency in fore quarters was higher (96/150; 64%) than that in hind quarters. Bacterial isolates were genotyped based on PCR-sequence analysis of 16S rDNA gene fragment. Bacterial identification indicated to occurrence of coagulase negative staphylococci (CNS) in 50.8% (33/65), *Bacilli* spp in 30.8% (20/65), *Escherichia* (*E*.) *coli* in 6.2% (4/65), *Staph. aureus* in 4.6% (3/65) and *Streptococcus agalactiae* in 4.6% (3/65); and *Micrococcus luteus* in 3.1% (2/65). Identified CNS species included *Staph. epidermidis*, *Staph*. *haemolyticus*, *Staph*. *sciuri*, *Staph*. *arlettae*, *Staph*. *hominis* and *Staph*. *gallinarum*; whereas *Bacillus* species included *Bacillus cereus, B. licheniformis, B. subtilis, B. altitudinis, B. pumilus* and *Corynebacterium freneyi*. *In vitro* antibiotic sensitivity patterns of isolated strains were susceptible to amoxicillin/clavulanic acid, ampicillin, cefotaxime, enrofloxacin, gentamicin, neomycin, and sulfamethoxazole/trimethoprim. Varying degrees of susceptibility were recorded to penicillin, streptomycin and tetracycline. Clinical cases were treated with systemic administration of single dose of enrofloxacin (10 mg/kg) followed by intramammary infusion of cocktail formula of antibiotics (tetracycline and neomycin), bacteriocin and anti-inflammatory prednisolone twice per day, for 3 consecutive days, whereas subclinical cases were treated with intramammary infusion regimen. To our knowledge, this is the first report on mastitis in Ecuador.

Key words: Mastitis, Coagulase Negative *Staphylococci*, *Bacilli*, Ecuador

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El Segundo artículo (Presentado a la revista: PLOS ONE: http://www.plosone.org/)

Mastitis Bovina: actualización

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Abstracto

Antecedentes

Mastitis, respuesta inflamatoria del huésped a las infecciones intramamarias, es la enfermedad más costosa bovina y sustancialmente los impactos de la industria láctea, que puede apoyar el desarrollo, especialmente en los países en desarrollo. Este artículo de revisión dirigido a actualizar los conocimientos en la mastitis bovina.

Resultados

La mastitis es causada por una amplia gama de microorganismos, con la transmisión varía de contagiosa a los modos ambientales. Principales producen mastitis patógenos incluyen Staphylococcus aureus, Streptococcus agalactiae, Streptococcus uberis, Streptococcus dysgalactiae y Escherichia coli. Sin embargo, los patógenos menores aún jugadores eminentes en las infecciones intramamarias.

Conclusiones

La incidencia de la enfermedad y el agente (s) causal, así, son muy diferentes, tanto inter-países intra e y principalmente asociado con la presión infecciosa, de acogida y los factores ambientales. El tratamiento depende principalmente de una anticuerpos, sin embargo, los regímenes de gestión, nivel de higiene y prácticas de salud de la ubre son factores cruciales en el control de la enfermedad.

Palabras clave: la mastitis bovina, Medidas Epidemiología, etiología, tratamiento, control.

The second paper is submitted to Journal: PLOS ONE: http://www.plosone.org

Bovine mastitis: an update

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Abstract

Background

Mastitis, inflammatory response of the host to intramammary infections, is the most costly bovine disease and substantially impacts the dairy industry, which can support development especially in developing countries. This article review aimed to update the knowledge on bovine mastitis.

Results

Mastitis is caused by a vast range of microorganisms, with transmission varies from contagious to environmental modes. Major mastitis causing pathogens include *Staphylococcus aureus*, *Streptococcus agalactiae, Streptococcus uberis*, *Streptococcus dysgalactiae* and *Escherichia coli*. However, minor pathogens still eminent players in the intramammary infections.

Conclusions

Incidence of the disease and the causative agent(s) as well, differ greatly both intra and inter-countries and mainly associated with infectious pressure, host and environmental factors. Treatment depend mainly on an antibodies, however, management regimens, hygiene level and udder health practices are crucial factors in the control of the disease.

Key words: Bovine mastitis, Epidemiology, Etiology, Treatment, Control Measures.

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La Tercera Segundo artículose encuentra bajo la redacción (se presentará a la revista: **PLOS ONE: http://www.plosone.org/**)

La prevalencia de la mastitis bovina en la provincia de El Oro, Ecuador

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Abstracto

Este estudio describe la mastitis clínica y subclínica en 5 granjas de ganado lechero en la provincia de El Oro, Ecuador. Casos clínicos fueron diagnosticados de base en los signos clínicos comunes, mientras que la mastitis subclínica fue diagnosticado por Claifornia Mastitis Test "CMT. Un total de 980 muestras de leche trimestre se obtuvieron de ganados lecheros fueron seleccionados CMT. Los resultados indicaron que la incidencia de la clínica (5-10%) y subclínica (20-81%) mastitis varía de una granja a otra. El análisis molecular está hecho y aún no terminó. En patrones de sensibilidad a los antibióticos in vitro de cepas aisladas fueron sensibles a amoxicilina / ácido clavulánico, ampicilina, cefotaxima, enrofloxacina, gentamicina, neomicina y sulfametoxazol / trimetoprim. Diversos grados de susceptibilidad se registraron a la penicilina, la estreptomicina y la tetraciclina. Los casos clínicos fueron tratados con la administración sistémica de una dosis única de enrofloxacina (10 mg / kg) seguido de una infusión intramamaria de fórmula cóctel de antibióticos (tetraciclina y neomicina), bacteriocina y prednisolona anti-inflamatoria dos veces por día, durante 3 días consecutivos, mientras que subclínica los casos fueron tratados con el régimen de infusión intramamaria. Granja hyagien juega el factor crucial en la incidencia de mastitis.

Prevalence of bovine mastitis in El Oro Province, Ecuador

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Abstract

This study described clinical and subclinical mastitis in 5 dairy cattle farms at El Oro Province, Ecuador. Clinical cases were diagnosed base on common clinical signs, whereas subclinical mastitis was diagnosed by Claifornia Mastitis Test “CMT. A total of 980 quarter milk samples were collected from dairy cattles were screened CMT. Results indicated the incidence of clinical (5-10%) and subclinical (20-81%) mastitis varied from farm to farm. **Molecular analysis is currently done and not yet finished**. *In vitro* antibiotic sensitivity patterns of isolated strains were susceptible to amoxicillin/clavulanic acid, ampicillin, cefotaxime, enrofloxacin, gentamicin, neomycin, and sulfamethoxazole/trimethoprim. Varying degrees of susceptibility were recorded to penicillin, streptomycin and tetracycline. Clinical cases were treated with systemic administration of single dose of enrofloxacin (10 mg/kg) followed by intramammary infusion of cocktail formula of antibiotics (tetracycline and neomycin), bacteriocin and anti-inflammatory prednisolone twice per day, for 3 consecutive days, whereas subclinical cases were treated with intramammary infusion regimen. Farm hyagien plays the crucial factor in the incidence of mastitis.

CONTRIBUCIÓN AL PLAN DEL BUEN VIVIR

Las enfermedades animales socavan la economía del país, el bienestar del animal y la salud pública. Ecuador como un país tropical es propenso a brotes de muchas enfermedades infecciosas, tanto para los animales y los seres humanos. La mastitis afecta a todas las facetas de buenos aspectos de vida, tanto para los animales y los humanos. La biodiversidad de los microbios etiológicos determinar las medidas de control de la enfermedad. Estas medidas contribuyen a un desarrollo sostenido, el bienestar y el buen vivir. El gobierno ecuatoriano es consciente de que la biodiversidad microbiana alta del país es un aspecto importante de la salud y la enfermedad, y toma las medidas para desarrollar un conjunto de conocimientos, habilidades y aplicaciones , tanto tradicionales como potenciales científica , establecida en el Plan Nacional para el Buen Vivir de la necesidad para poner en práctica iniciativas para fortalecer la investigación y la aplicación del bio - conocimiento y , por lo tanto , los resultados se enmarcan en los intereses del plan Nacional para el Buen Vivir .

En este orden de ideas, y también en el marco del Plan Nacional para el Buen Vivir , la posibilidad de hacer un análisis de la situación actual de la gestión de la industria animal en Ecuador , teniendo en cuenta el elevado número de animales y los enormes recursos disponibles para poner en Ecuador la lista de los países exportadores de animales que podrían hacer los cambios económicos dramáticos y contribuir al desarrollo sostenido de gran sector de la nación , junto con las industrias relacionadas.

PRODUCTOS ALCANZADOS

Investigación:

A pesar de la falta de productos químicos, reactivos, insumos y equipos, utilizamos herramientas muy pequeñas y sencillas a la cultura y aislar la bacteria a partir de muestras con mastitis. Por último tenemos número satisfactorio de aislamientos para la identificación y el análisis molecular. Sorprendentemente, la mayoría de los aislados fueron bacilos Gram positivos o estafilococos y todo lo perteneciente a los

patógenos ambientales que reflejan la fuerte relación entre la higiene granja y la incidencia de la enfermedad (véase el anexo Manuscrito).

Este manuscrito se presenta a diario de la salud y la producción animal tropical, y ahora bajo revisión por pares.

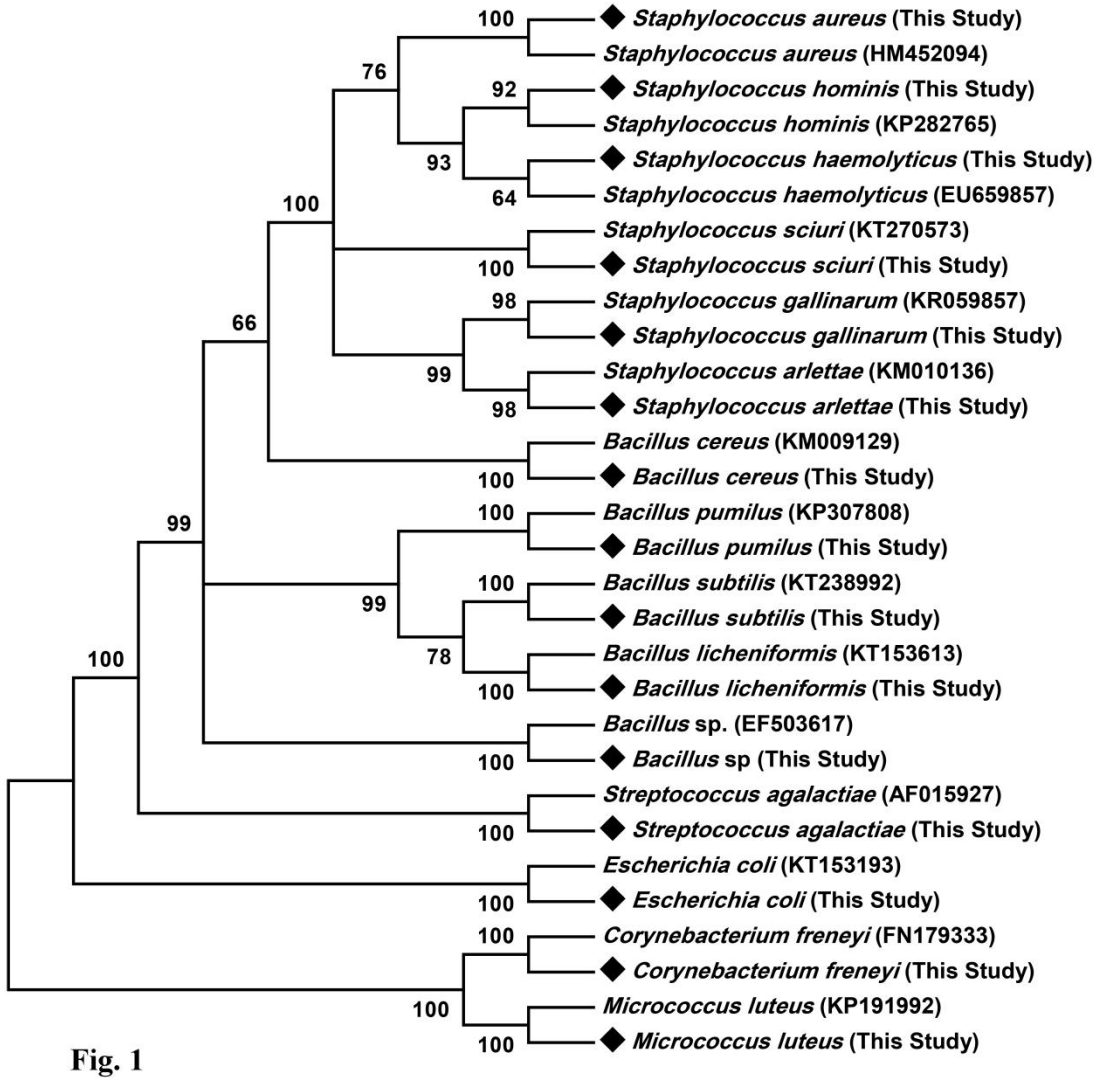

Fig. 1

- **<u>Actualizar los conocimientos</u>** sobre la mastitis bovina y contribuir en la producción ya través UTMATCH , un manuscrito en forma de revisión de se presentó a la Revista de Investigación Veterinaria, pero este diario recomendado presentación de este manuscrito a la Investigación Veterinaria BMC para las cuestiones relacionadas con la alcance general de las revistas . Ahora, me re-formato de este artículo Presentado a la revista: PLOS ONE:

http://www.plosone.org. Artículo adicional está en preparación, para su presentación a la misma revista "PLOS ONE". **Gracias a UTMATCH para COMPROMISO que pagar los gastos de publicación de PLoS ONE.** En tercer lugar, otro manuscrito está en preparación.

Formación científica: Durante mi estancia me capacitó a varias personas en la institución (estudiantes y profesores), microbiología general y microbiología mastitis, así como de microbiología de alimentos. El más importante es que el Dr. Lenin Aguilar, que fue asignado a mí como contrapartida, se convierte así conocer diferentes aspectos de la microbiología mastitis y control de la mastitis, que contribuye al aumento de capacidades y la producción de profesionales y científicos en UTMACH.

Docencia:

1. Taller teórico y práctico sobre la microbiología y la mastitis microbiología general, fue organizado para los estudiantes y el personal del departamento de veterinaria.
2. Proporcionar asesoramiento, formación práctica y aportación de trabajo para 3 estudiantes de veterinaria sénior (Decimo Ciclo) para hacer la parte práctica de sus proyectos de graduación (Michel Fabián Vargas Zambrano, Christian Fernando Cordero y Selma Avila).

También, he contribuido en diferentes actividades solicitadas por SENESCYT para evaluar proyectos científicos y propuestas.

CONCLUSIONES Y RECOMENDACIONES

- Hemos llevado a cabo todas las excursiones a las granjas lecheras necesarias para la recogida de muestras de leche con mastitis de animales infectados. Historia de la magnitud de la infección en la granja fue tomada por el director granja / pesebre (s). Los animales fueron investigados en las granjas de los síntomas de mastitis clínica. Animales no sintomáticos (aparentemente sanos) fueron investigados por la mastitis subclínica. Cuartos infectados fueron desinfectados y se tomaron muestras en tubos estériles para bacteriológica cultivo, aislamiento e identificación del agente (s) causal. La sensibilidad a los antibióticos se llevó a

cabo para seleccionar correctamente el antibiótico para el tratamiento. El seguimiento fue realizado para la misma finca para comprobar los animales previamente infectados después del curso de tratamiento para evaluar la eficacia del régimen de tratamiento. Se obtuvieron más de 250 cepas bacterianas e identificados. Se seleccionó un total de 60 aislamientos y se envía a la identificación molecular basado en el análisis de PCR de secuencia de segmento de gen 16S rDNA. Actualmente estamos recibiendo los resultados de secuencia. Esta ha sido una pesada carga de trabajo para las horas pasadas en el campo y en el laboratorio, consultar la literatura, la edición de imágenes digitales y la organización del material recogido, pero se le ha permitido tener un ciernes objetivos de recogida en el futuro, se convierten en material de referencia para los estudiantes y maestros de la UTMACH y Ecuador.

- Investigación de campo, toma de muestras y análisis de laboratorio de microbiología y archivo de los aislados obtenidos sería permitir así a la contraparte institucional (Dr. Lenin Aguilar) para adquirir estas habilidades y tiene la ventaja como profesor - investigador para desarrollar y tales estudios directos.
- Debido a que el personal de la granja de animales son los estrechos contactos con los animales y saber cualquier condición o enfermedad signos anormales de los animales, se recomienda establecer una asociación u organización, tanto en los niveles nacional y provincial para la formación continua y la mejora de los conocimientos y competencias del personal de la granja en las prácticas agrícolas modernas y actualizarlos, para familiarizarse con los diferentes enfermedades y cómo contrarrestar cualquier incidente. Además, se les pide reportar cualquier condición grave o enfermedad de declaración obligatoria y buscar la ayuda logística y médica. Estas asociaciones pueden proporcionar los propietarios de fincas y personal con folletos periódicos sobre los diferentes aspectos de la industria de la agricultura bajo la supervisión de expertos del Ministerio de Agricultura, y la contribución de investigadores como los Prometeos. Estas medidas son muy importantes para la salud animal, la economía agrícola, la epidemiología y control de enfermedades agrícolas.

- También, una recomendación importante es que el SENESCYT resolver el problema de permiso autorizado para acceder a las granjas lecheras.
- Como un archivo nacional, se recomienda establecer una instalación de almacenamiento a nivel nacional para recibir y conservar la novela microbiana aísla para crear un centro nacional para el archivado de cepas microbianas patógenas que circulan en el país, que son necesarios para cualquier programa de vigilancia y seguimiento
- Más importante aún, las autoridades deben imponer regulaciones estrictas sobre la calidad y el transporte de la leche suministrada a fábricas de productos lácteos en términos de pH, conductividad, recuento de células somáticas, recuento de bacterias, etc. y los precios deben ser clasificados en la primera calidad de la leche.
- Además, tres días taller teórico-práctico sobre la microbiología mastítica fue organizado para los miembros del personal y los estudiantes de pregrado de departamento de veterinaria en la Facultad de Agricultura, UTMATCH permitió reconocer la transferencia a la nueva generación de profesores / investigadores de UTMATCH.
- También, como contribución enseñanza y transferencia de conocimiento, yo contribuyó y dirigió el trabajo de laboratorio de tres estudiantes de veterinaria de alto nivel para el desarrollo de sus proyectos de graduación sobre diferentes temas de la microbiología. Creo, es muy recomendable para asignar las actividades de supervisión de algunos estudiantes de pregrado y postgrado a cada Prometeo.
- Este tipo de investigación debe ser generalizado a nivel nacional para dejar claro mapa epidemiológico cubre todo el país, en un intento de control de enfermedades y prevención.
- Tal vez lo más importante es la preparación de tres artículos científicos, uno de los cuales fue sometido a la Investigación Veterinaria Diario, sin embargo esta revista recomienda someterse a BMC veterinaria para tema del ámbito de aplicación. Ahora, me re-formato de este artículo Presentado a la revista: PLOS ONE: **http://www.plosone.org**. Artículo adicional está en preparación, para su

presentación a la misma revista "PLOS ONE". **Gracias a UTMATCH para COMPROMISO que pagar los gastos de publicación de PLoS ONE.**

- En referencia de la regulación SENESCYT (sin queja u objeciones), creo que el informe mensual es de consumo muy angustiante y hora que hacen que el investigador y la contraparte para recopilar evidencias de los trabajos para preparar el informe mensual. Creo que sería más conveniente preparar un informe provisional y final preparado por la contraparte para evaluar con precisión y reportar las actividades y el desempeño de la Prometeo (esto se aplica comúnmente en todo el mundo). Si prefiere SENESCYT informes a corto plazo, se sugiere que cada 3 o cada 4 meses y preparado por la contraparte, no el Prometeo.
- Además, te sugiero un presupuesto respetuosa investigación para cada Prometeo se asigne a la universidad de acogida para ayudar a la ejecución del proyecto de investigación e impulsar la universidad de destino para la alta competencia y facilitar los procedimientos de investigación.
- Otro aspecto que considero importante destacar es que se refiere a los cambios de la SENESCYT de plazos Vinculación Informe Final. Es de destacar que, tras haber recibido la aprobación para desarrollar una matriz de actividades fue revisado y auditado tanto por la Universidad y por la SENESCYT repetidamente me alertando que los plazos para la realización del proyecto son inamovibles me someto a una presión en mi opinión que pagar lapsos excesivos informes técnicos fuera del inicialmente estipulado. Si bien se reconoce que los cambios en el calendario obedecen a una intención de liquidar los pagos de Prometeo, esta situación crea condiciones de estrés y daño innecesario y por lo tanto sugiere que en el futuro tal vez anticipar la ejecución presupuestaria y los procedimientos administrativos con suficiente antelación para evitar este tipo de cambios posibles en los horarios o, cuando Prometeo completar sus actividades en diciembre, pruebe estos casos el investigador ofreciendo condiciones especiales que permiten el uso del tiempo de manera más eficiente en la generación de productos finales, es decir, los artículos científicos que requieren la paz para hacer girar las ideas y traducirlas en un manuscrito para su difusión en revistas científicas.

LIMITACIONES

Una de las principales limitaciones que confrontan el presente proyecto es el permiso oficial para el acceso sin problemas en las granjas lecheras. A falta de unidad de almacenamiento como unidad de nitrógeno líquido mantener sub aislados como cepas de campo de referencia para el futuro seguimiento y vigilancia. Además, el envío oficial de las muestras garantizadas y seguras para el análisis fuera del país, que limitan alcanzar los objetivos del proyecto debido a la falta de instalaciones en la universidad y los problemas de envío simple. Lago de consorcio o asociación agrícola para comunicarse con ellos y explicar la magnitud de los problemas agrícolas y los métodos convenientes de medidas de control y prevención. A falta de equipo asociado para ayudar en el desarrollo del campo y de laboratorio obras entre técnicos, paramédicos y trabajadores de limpieza, ya que es difícil pasar del 25% del tiempo en la limpieza de vidrios lleva, personal de laboratorio y herramientas para el trabajo. Todos estos inconvenientes están más allá de mi capacidad y responsabilidad como Prometeo. Espero que estas observaciones no se consideren como queja, pero aspira a tomarse como una crítica constructiva para mejorar el funcionamiento y operación del Proyecto Prometeo, que es una iniciativa digna de admiración y apoyo incondicional, pero para mantener esa sensación de nuevo, considero que es necesario emitir mi opinión como investigador.

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ANEXOS

ANEXOS 1: Phylogenetic relathioshship of the obtained strains of bacteria from mastitic cows and the reference databse in the genebank based on 16S rDNA sequences

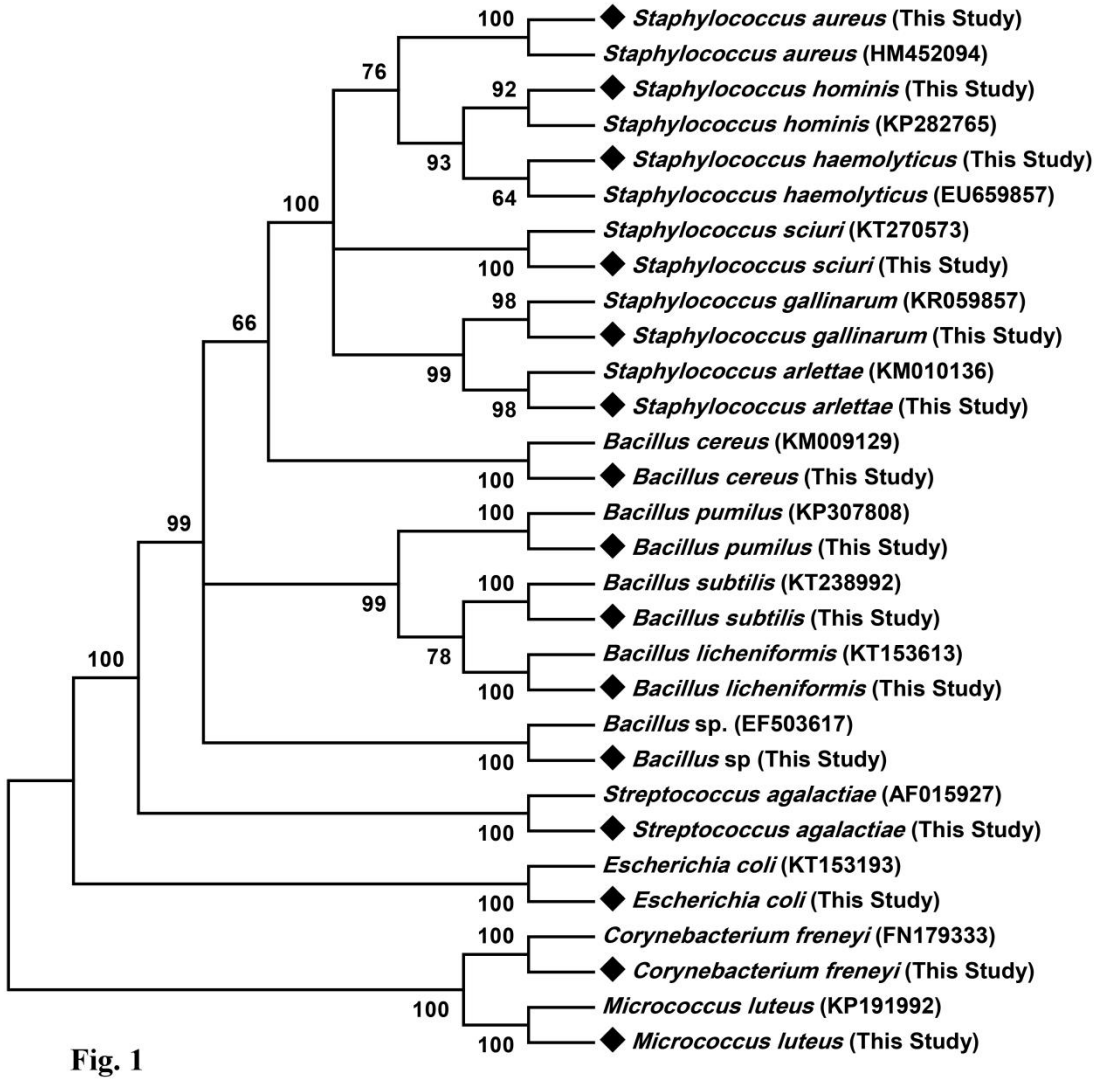

Fig. 1

ANEXOS 2
An Article submitted for publication in the Journal of "Tropical Medicin and Animal Production" https://www.editorialmanager.com/trop/default.aspx

Implication of environmental bacteria in bovine mastitis in Ecuador: A pilot study

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Abstract

This study described clinical and subclinical mastitis in a dairy cattle farm at Santa Rosa, El Oro Province, Ecuador. A medium size farm (N = 65) experienced high incidence of clinical (10.8%, 7/65) and subclinical (81.5, 53/65) mastitis, as determined by California Mastitis Test (CMT). The incidence per quarter was 57.7% (150/260), with the frequency in fore quarters was higher (96/150; 64%) than that in hind quarters. Bacterial isolates were genotyped based on PCR-sequence analysis of 16S rDNA gene fragment. Bacterial identification indicated to occurrence of coagulase negative staphylococci (CNS) in 50.8% (33/65), *Bacilli* spp in 30.8% (20/65), *Escherichia* (*E*.) *coli* in 6.2% (4/65), *Staph. aureus* in 4.6% (3/65) and *Streptococcus agalactiae* in 4.6% (3/65); and *Micrococcus luteus* in 3.1% (2/65). Identified CNS species included *Staph. epidermidis*, *Staph*. *haemolyticus*, *Staph*. *sciuri*, *Staph*. *arlettae*, *Staph*. *hominis* and *Staph*. *gallinarum*; whereas *Bacillus* species included

Bacillus cereus, B. licheniformis, B. subtilis, B. altitudinis, B. pumilus and *Corynebacterium freneyi*. *In vitro* antibiotic sensitivity patterns of isolated strains were susceptible to amoxicillin/clavulanic acid, ampicillin, cefotaxime, enrofloxacin, gentamicin, neomycin, and sulfamethoxazole/trimethoprim. Varying degrees of susceptibility were recorded to penicillin, streptomycin and tetracycline. Clinical cases were treated with systemic administration of single dose of enrofloxacin (10 mg/kg) followed by intramammary infusion of cocktail formula of antibiotics (tetracycline and neomycin), bacteriocin and anti-inflammatory prednisolone twice per day, for 3 consecutive days, whereas subclinical cases were treated with intramammary infusion regimen. To our knowledge, this is the first report on mastitis in Ecuador.

Key words: Mastitis, Coagulase Negative *Staphylococci*, *Bacilli*, Ecuador

Introduction

Mastitis is a serious udder disease of different livestock resulting in poor animal condition, decreased fertility and productivity, deterioration of the quality of the produced milk and potential public health challenge (Seegers et al. 2003), rather than treatment-related expenditures. Mastitis originates as a reaction of the udder tissues to bacterial, chemical, thermal, or mechanical injury. However, microbial induced mastitis remains the most common form. Clinical mastitis is a serious animal welfare issue as it is associated with suffer and reduced well-being of the affected animals (Leslie and Petersson-Wolfe 2012), whereas, subclinical mastitis is often associated with increase somatic cell count and change in the physical and chemical quality of the produced milk (Malek et al. 2013). Subclinical mastitis is the main form of mastitis in dairy herds, with frequency ranges from 20 to 65% in a given herd around the world (Ostensson et al. 2013; Saidi et al. 2013; Ramirez et al. 2014; Leon-Galvan et al. 2015).

A vast range of microorganisms may cause mastitis in cattle; with the major mastitis pathogens (*Staphylococcus aureus*, *Streptococcus agalactiae, Streptococcus uberis*, *Streptococcus dysgalactiae* and *Escherichia coli*) are historically responsible for the majority of cases (Reyher et al. 2012). However, attention in the ‘minor pathogens’ including coagulase negative *Staphylococci* (CNS) and *Bacilli* is

increasingly growing (Pitkala et al. 2004) due to frequent isolation from clinical and subclinical cases of mastitis (Waage et al. 1999; Taponen et al. 2007; Supre et al. 2011), and persistence of the infection(s). It is well established that CNS and great variety of *Bacillus* spp are present in different environmental compartments (Piessens et al. 2011), proposing the name of environmental mastitis versus the contagious mastitis caused mainly by major mastitis pathogens. The bacteriological etiology of mastitis in many countries show a trend of change from contagious to environmental pathogens, which has reduced the efficacy of the traditional mastitis control strategies (Pyorala 2002). The strain distribution of mastitis pathogens differs within individual animals in the herd, and between herds, countries and host species.

Ecuador is a developing country that promotes animal farming, especially small stockholders. Indeed, small and medium dairy herds and enterprises can profoundly impact the development process in developing countries (FAO 2010). However, very little is known on incidence and etiology of bovine mastitis in Ecuador. The present study investigated an outbreak of mastitis in a medium size dairy farm (N = 65). Identity of etiological agents was determined based on conventional bacteriological and molecular analyses. Treatments were done based on systemic and intramammary infusion of antibiotics

Materials and Methods

Study Farm

The farm under study was a medium size dairy farm (N = 65), located at Santa Rosa city, El Oro Province, Ecuador. Visit of the farm was done upon consultation request of the farm owner to veterinary department, UTMATCH, on background of mastitis complains. Cows in the farm differed greatly in the number of days in milk, number of calving, age, and level of milk yield.

Collection of sample

Clinical cases (CLM) presented moderate to severe symptoms of mastitis in the form hotness, redness, hardness of the udder and strong pain reaction, with one animal

had a gangrenous form that died within one week of detection. The milk derived from these animals was serous fluid or bloody. Subclinical mastitis (SCM) was determined using California Mastitis Test (CMT; Milktest, Arthur Schopf Hygiene GmbH & Co. KG, Germany). The results were interpreted in score range of 0-4; with 0 for no reaction, 1 for trace, 2for weak positive, 3 for distinct positive, and 4 for strong positive. Aseptic milk samples were collected from clinical and subclinical (grad 3 and 4 CMT) cases, after disinfection of udders. Individual mastitic quarter milk samples (10-15 ml) were collected after discarding the first 5-7 streams of milk into sterile numbered screwed tubes, then cooled in ice box and transferred to the laboratory at UTMATCH.

Microbiological Culture and Isolation

Ninety milk samples from mastitic udder quarters were subjected to microbiological analysis. A loop-full milk samples were inoculated on blood agar base enriched with 5% sterile sheep blood and MacConkey agar (Difco). Plates were aerobically incubated at 37°C for 24 to 48 h. Culture plates showed growth of more than 2 colony types were considered as contaminated and excluded. Subcultures were done to obtain pure isolates for morphological, biochemical and molecular identification (Sears et al. 1993). Gram positive *cocci* were identified by means of hemolytic pattern and secondary tests [growth pattern on Manitol Salt Agar (MSA; Difco), catalase and coagulase reaction with rabbit plasma (positive or negative)] (Bautista-Trujillo et al. 2013). *Bacillus* spp. were confirmed by Gram staining (Persson et al. 2011). For molecular identification, subcultures of the obtained isolates were grown in LB liquid at 37°C for 24 h, centrifuged at 3000 rpm for 5 min, bacterial pellet washed thrice with cold sterile PBS buffer (pH 7.2), and finally fixed in 95% ethanol.

Molecular identification based on 16S rDNA

Molecular identity of the isolated strains was done based on PCR-sequence analysis of 16S rDNA. PCR reactions were done as described previously (Weisburg et al. 1991; Lange et al. 2015), using the primer set 27F: 5´-AGAGTTTGATCMTGGCTCAG-3´ and 1492R: 5´-TACGGYTACCTTGTTACGACTT-3´. PCR conditions consisted of initial denaturation step at 94℃ for 5min, followed by

30 cycles of denaturation at 94℃ for 30s, annealing at 55℃ for 30s, and extension at 72℃ for 1.5 min. A final extension step at 72℃ for 7min was included. An aliquot of 5 μL of the PCR products was subjected to electrophoresis in 1% agarose gels and stained with ethidium bromide to visualize the amplified products.

DNA sequence analyses

PCR products were sequenced directly using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI 3130 Genetic Analyzer (Applied Biosystems). Sequences were assembled using the ChromasPro (version 1.5) software (http://www.technelysium.com.au/ChromasPro.html). The sequences obtained were aligned with each other and reference sequences in the GenBank "NCBI nonredundant nucleotide database; nt." using ClustalX (ftp://ftp-igbmc.u-trasbg.fr/pub/ClustalX/). Phylogenetic tree was constructed using Maximum Likelihood method as implemented in the MEGA6.06 (http://www.megasoftware.net). The robustness of the tree was assessed with 3000 bootstrap replicates.

In vitro antibiotic sensitivity test

Representative strains (10 CNS, 10 *Bacilli*, 3 coagulase positive *Staphylococci*, 3 *Streptococci* and 3 coliform) of isolated organisms were spread on Mueller Hinton agar plates (Difico) and tested against the following antibiotics (Oxoid): amoxicillin (AMX; 10 μg/disc), amoxicillin/clavulanic acid 2:1 (AMC; 30 μg/disc), ampicillin (AMP; 10 μg/disc), cefotaxime (CTX; 30 μg/disc), enrofloxacin (ENR; 5 μg/disc), gentamicin (GEN; 30 μg/disc), neomycin (NEO; 30 μg/disc), penicillin G (PEN; 10 units/disc), streptomycin (STR; 10 μg/disc), sulfamethoxazole/trimethoprim (SXT; 25 μg/disc) and tetracycline (TET; 30 μg/disc). Zones of inhibition (in mm) were recorder after ~18 h of incubation at 37°C. Size of zones of inhibition was interpreted following CLSI (2008) tables. Results were expressed as susceptible (S) or resistant (R), followed by the number of the isolates responded out of the number tested.

Treatment course

Treatment of clinical cases was done using systemic administration of a single dose of Baytril® 100 **(**enrofloxacin; i.m of 10 mg/kg), followed by intramammary infusion of Mastijet Fort [tetracycline (200 mg), neomycin (250 mg), bacitracin (2000 IU) and prednisolone (10 mg); 2 times/day; for 3 consecutive days]. Udders were stripped out of milk after i.m injection of 20 IU Oxytocin before intramammary administration of antibiotic (Hillerton and Semmens 1999). All cases, but one, responded well and improved within one week, and cured within 3 weeks of treatment as determined by CMT, and bacterial culture. The gangrenous mastitic animal did not respond to treatment and died with one week of detection. Subclinical cases were treated with intramammary infusions of infected quarters with Mastijet Fort after milking, for 3 days. Milk from clinical cases was completely discarded till normalized in consistency. Milk from all animals under treatment was used for feeding of young calves up to 3 days after treatment cessation.

Results and discussion

Small and medium dairy herds and enterprises are the dominant feature in animal industry in the developing world (FAO 2010), representing an important figure in the national economies. In the present study, medium sized (N = 65) dairy farm experienced an outbreak of clinical (10.8%, 7/65) and subclinical mastitis (81.5%, 53/65), accompanied with the decrease of animals' milk production. The incidence per quarter was 57.7% (150/260), with the frequency in fore quarters was higher (96/150; 64%) than that in hind quarters. Clinical and subclinical mastitis is very common in dairy cattle (Bludau et al. 2014; Boujenane et al. 2015), in buffalo (Elhaig and Selim 2015), sheep (Mork et al. 2007; Narenji et al. 2015) and in goats (McDougall et al. 2014; Zhao et al. 2015), worldwide. The frequency of the infection differs greatly from farm to farm and from country to country (Bludau et al. 2014; Thompson-Crispi et al. 2014; Boujenane et al. 2015; Leon-Galvan et al. 2015).

Out of the cultured 90 milk samples, 15 were excluded due to contamination and 10 showed no growth. Bacterial identification indicated to occurrence of CNS in 50.8% (33/65), *Bacilli* spp in 30.8% (20/65), coagulase positive *Staphylococci* in 4.6% (3/65), coliform in 6.2% (4/65), and *Streptococci* in 4.6% (3/65), and *Micrococcus* in

3.1% (2/65). Genotyping based on analysis of 16S rDNA is a reliable way for identification of mastitic bacteria up to species level (Lange et al. 2015). Molecularly identified CNS species included *Staph. epidermidis*, *Staph*. *haemolyticus*, *Staph*. *sciuri*, *Staph*. *arlettae*, *Staph*. *hominis* and *Staph*. *gallinarum*; whereas *Bacillus* species included *Bacillus cereus, B. licheniformis, B. subtilis, B. altitudinis, B. pumilus* and *Corynebacterium freneyi*. Coagulase positive *Staphylococci* were identified as *Staph. aureus*, coliform as *E. coli*, *Streptococci* as *Strept. agalactiae* and *Micrococcus* as *Micrococcus luteus* (Fig.1). More or less similar results were reported in different countries, in terms of incidence and identity of isolated strains. CNS were implicated for 49.6% in total mastitis cases in Finland (Pitkala et al. 2004) and 27% of subclinical mastitis in dairy cattle in Sweden (Persson et al. 2011), 31.7% in Uganda (Bjork et al. 2014), 42% in Mexico (Leon-Galvan et al. 2015), and 30% in China (Yang et al. 2015). CNS were frequently isolated from different dairy environmental compartments, including teat skin, milker's skin and gloves as well as farm floors, which represent reservoirs for intramammary infections (Piessens et al. 2011; De Visscher et al. 2014). However, persistence, and virulence of the different CNS species associated with intramammary infections are a matter of much debate (Vanderhaeghen et al. 2014).

Bacillus spp were the dominant pathogens isolated from mastitic samples from dairy cattle in Finland, with significant occurrence of to toxinogenic *Bacillus pumilus* and *Bacillus licheniformis* (Nieminen et al. 2007). *Bacillus* sp (*Bacillus cereus*) was implicated for gangrenous mastitis in goats (Mavangira et al. 2013) as well. Although Pitkala et al. (2004) reported high incidence of *Corynebacterium* spp (34.4%) as a mastitis pathogen in Finland, Persson et al. (2011) reported that *Corynebacterium* spp is uncommon and seldom recognized as a mastitis pathogen in Sweden. Nonetheless, results reported herein are in contradiction to several studies showed that mastitis caused by *Bacillus* spp. is rare in dairy cows (Mekonnen et al. 2005; Sori et al. 2005; Abera et al. 2012), which might reflect the differences in management regimens, husbandry factors and farm hygiene. Laying dawn of animals, under study, after milking may help colonization of the opened teat canals with environmental pathogens.

In vitro antibiotic sensitivity patterns of isolated strains indicated that they were susceptible for AMC, AMP, CTX, ENR, SXT, GEN and NEO. However, penicillin was insensitive to all *Bacilli* and coliform isolates, sensitive in 3/10 CNS and 1/3 in coagulase +ve *Staph*. Streptomycin were sensitive in 3/10 *Bacilli*, 7/10 CNS, 2/3 coagulase +ve *Staph*., 1/3 coliform and 3/3 *Streptococci*. Tetracycline was sensitive in 7/10 bacilli, 9/10 CNS, 2/3 coagulase +ve *Staph* and 3/3 *Streptococci* and coliform. Obtained results are comparable to those reported previously for mastitis-isolated bacteria from different animal species in different geographical places (Dhakal et al. 2007; Ranjan et al. 2010; Bhatt et al. 2011; Ruegg et al. 2015). Generally, it is very difficult to generalize a robust conclusion on the sensitivity patterns, in the light of the low number of tested isolates, especially for coliform, coagulase +ve *Staph.* and *Streptococcus*.

In the present study, systemic administration of single dose of enrofloxacin followed by intramammary infusion of cocktail formula of antibiotics (tetracycline and neomycin), bacteriocin and anti-inflammatory prednisolone twice per day, for 3 consecutive days, proved effective in treatment of the clinical cases as judged by CMT and bacteriological culture after 3 weeks of treatment. Similar regimen of intramammary infusion proved officious in treatment of subclinical cases. The animals greatly improved and the incidence declined up to 17% within 3 weeks after treatment as determined by CMT and bacteriological culture. In concordance, therapy with enrofloxacin and nimesulide was found more efficacious (92.30%) in treating mastitic cows in India (Joshi and Gokhale 2006). In addition, treatment with enrofloxacin and flunixin enhanced elimination of bacteria in experimentally induced *E. coli* mastitis (Rantala et al. 2002). On the other hand, results reported by Suojala et al. (2010) and Persson et al. (2015) did not support the use of enrofloxacin to treat acute clinical *E. coli* mastitis. Further, bacteriocins proved to inhibit the growth of multi-antibiotic resistance bacteria derived from mastitis animals (Pieterse and Todorov 2010; Gutierrez-Chavez et al. 2015; Leon-Galvan et al. 2015), which might be beneficial in the applied regimen. Notably, the combination of systemic administration and intramammary infusion with antibiotics may induce higher bacteriologic cures than did intramammary infusion alone (Owens et al. 1988), especially with clinical cases.

Enrofloxacin is not yet approved by FDA for use in lactating cattle; therefore additional studies are needed to determine more optimal routes, doses, and treatment durations for antimicrobial therapy with enrofloxacin alone or in combinations in clinical and subclinical mastitis. For safety issues, milk from animals under treatment was abstained from human consumption for 3 days after the last administration.

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Conflict of interest

The authors declare that they have no conflict of interest.

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ANEXOS 3: An Article is submitted to to journal PLOS ONE (http://www.plosone.org/)

Bovine mastitis: an update

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Abstract

Background

Mastitis, inflammatory response of the host to intramammary infections, is the most costly bovine disease and substantially impacts the dairy industry, which can support development especially in developing countries. This article review aimed to update the knowledge on bovine mastitis.

Results

Mastitis is caused by a vast range of microorganisms, with transmission varies from contagious to environmental modes. Major mastitis causing pathogens include *Staphylococcus aureus*, *Streptococcus agalactiae, Streptococcus uberis*, *Streptococcus dysgalactiae* and *Escherichia coli*. However, minor pathogens still eminent players in the intramammary infections.

Conclusions

Incidence of the disease and the causative agent(s) as well, differ greatly both intra and inter-countries and mainly associated with infectious pressure, host and environmental factors. Treatment depend mainly on an antibodies, however, management regimens, hygiene level and udder health practices are crucial factors in the control of the disease.

Key words: Bovine mastitis, Epidemiology, Etiology, Treatment, Control Measures.

Introduction

Mastitis (inflammation of the mammary glands) is a serious udder disease of different livestock resulting in poor animal health, decreased fertility and productivity, deterioration of the quality of the produced milk and potential public health challenge. Mastitis originates as a reaction of the udder tissue to bacterial, chemical, thermal, or mechanical injury; however, microbial induced mastitis remains the most common form. The disease remains a major challenge to the worldwide dairy industry despite the widespread implementation of mastitis control strategies. Subclinical mastitis is often associated with increase somatic cell count and change the physical and chemical quality of the produced milk [1], whereas, clinical mastitis is a serious animal welfare issue as it is associated with suffer and reduced well-being of the affected animals [2].

In both clinical and subclinical mastitis there is a substantial loss in milk production [3], even after treatment due to the irreversible damage to the mammary tissue [4]. Low quality of the milk results in significant economic damage due to loss of premium quality of the milk [5], or discarding of the milk with obvious low quality either used for feeding calves or complete discard in severe cases. Also, there is additional economic loss due treatment costs including the costs of the diagnostics, drugs, veterinary consultations and services as well as the costs of the additional labor needed due mastitis management [5]. Antibiotic residues in the milk are a serious challenge to the dairy industry which might lead to exclusion of animals under high doses of antibiotic from the milking for human consumption.

Continuous efforts are paid to understand epidemiology, immunology, diagnostics and pathogenesis of mastitis. This review was delineated to update and extend the current knowledge on molecular epidemiology and control measures of bovine mastitis. Conclusion and analyses of this study were made using data retrieved from published literature and sequences available in Multi-Locus Sequence Typing (MLST) database (http://www.mlst.net/).

Epidemiology

Incidence

Mastitis is multifactorial disease, which is broadly associated with the infection pressure due to management system, hygiene level, milking system, age and breed of the animal and/or external factors such as weather changes [6]. Reported incidences differ widely, inter- and intra- countries, based on the concept of the study from the herd level to the cow level (i.e the infection rate per quarter). In Africa, high incidence of ~85%, 63% 52%, and 51% in dairy cows were reported from Tanzania, Rwand and Nigeria [7, 8, 9] versus %28 in Algeria [10]. In Asia, high infection rate (~88-55%) was reported in Vietnam and South Korea [11, 12], moderate rate (48 %) in Siri Lanka [13], and relatively lower rate (22.6%) in China [14]. Comparably, the incidence in Latin American countries is more or less similar in other continents, with ~80 in Ecuador [15], ~49% in Brazil [16] and ~37% in Columbia [17]. In industrial countries, where more attention is paid to the udder health, the incidence of intramammary infections seems low. The prevalence in Dutch dairy herds was reported as 25% versus 3% in Canada [18]. Interestingly, a considerable fraction of IMI infection occurs during dry period, aggravating the situation after parturition and during lactation. Quarter incidence averaged 12.8% in untreated to 8.0% in treated quarters in dry period of dairy cattle [19].

Mastitis is routinely classified as clinical and subclinical forms. Clinical mastitis is characterized by obvious abnormalities of the udder (e.g. swelling and redness, hotness...etc), and/or changes in milk appearance and composition. Difference in annual incidence rate of clinical mastitis in dairy cattle under different management regimens ranges from 11.6% in Switzerland [20] to as low as 0.9% in Kenya [21]. Identification of subclinical mastitis is mainly based on the somatic cell count (SCC) in the produced milk and bacteriological culturing. Individual udder quarters with a SCC above a certain threshold (e.g. 200,000/mL) are considered to have subclinical mastitis [22]. Often, a quarter of the

udder is infected, however, neither the udder nor the milk are clinically abnormal. Subclinical mastitis is the main form of mastitis in modern dairy herds, with infection rate ranges from 20 to 65% in given herds around the world [10, 11, 17, 21].

Etiology of the disease

Microbial mastitis is caused by a wide variety of gram positive and gram negative bacteria; fungi, mycoplasma and less frequently by algae. Nonetheless, mechanical trauma, thermal trauma, and chemical insult predispose the gland to intramammary infection. A vast range of microorganisms may cause mastitis in cows; however, the major mastitis pathogens (*Staphylococcus aureus*, *Streptococcus agalactiae, Streptococcus uberis*, *Streptococcus dysgalactiae* and *Escherichia coli*) are responsible for approximately 80% of mastitis cases compared to the minor ones such as coagulase-negative (CNS) staphylococci [23]. The strain distribution of mastitis pathogens differs within individual animals in the herd, and between herds, countries and host species. In spite of the nature of the causative agent, the pathogenicity of certain strain(s) is governed by pathogenic factors that increase their invasiveness and under specific gene patterns. These patterns differ from breed to breed and are strongly associated with the magnitude of the resulting inflammatory response, and directly on the number of somatic cell count [24].

As the response of the host to intramammary infection, mastitis depends on the interaction of host, infectious agent, and environmental factors [4]. The etiological agents may vary from place to place depending on climate; animal species and animal husbandry. It is clear that some mastitis causing bacteria may have increased fitness, i.e. enhanced ability to cause mastitis, than the others. In a study covered 25,288 milk samples that were collected from dairy cows throughout New Zealand over a period of more than 3 years, the most commonly isolated mastitis causing organisms were *Streptococcus* (*Strep.*) *uberis* (23.6%), *Staphylococcus* (*Staph.*) *aureus* (23.5%), coagulase-negative

staphylococci (7.2%), *Strep. dysgalactiae* (6.2%), *Bacillus* spp. (4.0%), and coliforms (3.7%) [25]. The profile seems somewhat different in Colombia, with the most common *Strep. agalactiae* (34.4%), *Corynebacterium* spp. (13.2%), and *Staph. aureus* (8.0%) [17]. A wide range of bacteria are implicated in cattle mastitis in Algeria including *Staphyl. aureus* (40%), *Streptococcus* spp. (12.5%), Enterobacteriaceae (2.5%), *Pseudomonas* spp. (2.5%). Mixed bacterial infections on quarter, cow and herd level is frequently reported [10]. Based on the major mode of transmission, mastitis pathogens can be classified as either contagious or environmental.

Contagious mastitis

Contagious pathogens transmit from cow to cow, often at the time of milking trough contact with contaminated milking tools/equipment. Typically, contagious pathogens are characterized with persistent infections and dominance of one strain across the herd. The most common contagious pathogen is *Staphylococcus aureus*, and *Streptococcus agalactiae*.

***Staphylococcus aureus* mastitis**

Staphylococcus aureus is identified as a major contagious mastitis-causing pathogen. Extramammary sources of *Staph. aureus* include skin (especially hock skin), barn environment, insects, people, non-bovine animals, feedstuffs and air [26]. Despite the debate which is the cause and which is the result i.e. the extramammary sources initiate the intramammary infection or the intramammary infection contaminates the extramammary sites [27], transmission across the herd is thought to occur mainly by milking machine, udder towels or milkers' hands [28]. Occurrence of *Staph. aureus* was reported as high as ~ 77.0% of mastitis-causative agents in Kenya [21], 56.0% in cow herds in Switzerland [29] with seasonal fluctuation patterns and lower as ~20.0% in Iran [30]. Data on *Staph. aureus* from

different herds with mastitis indicate the occurrence of infection by a single strain or the dominance of one strain in a given herd [27,29, 31].

MLST database (http://saureus.beta.mlst.net/) and phylogenetic analysis revealed that mastitis causing strains of *Staph. aureus* are distributed in 28 different clonal complexes, 12 of which have intercontinental distribution (Table 1), with CC97, CC133, CC705, CC8, CC5 and CC1 are the most common in terms of frequency, diversity, distribution and multidrug resistance. Mastitis related *Staph. aureus* sequences are distributed into 2 clades (Fig.1). The first clade arbitrary includes mostly or exclusively apparent live-stock associated *Staphylococcus* clonal complexes and/or sequence types, such as CC130, CC133, CC479, CC504, CC522 and CC705, which may indicate some sort of host adaptation [32-34]. It is reported that some bovine associated strains such as CC705 appeared to have a higher transmission probability compared to a human adapted CC8, suggesting that the greatest indirect effects of *S. aureus* control would be realized through elimination of bovine-adapted strains from herds [35]. The second clade includes the clonal complexes and/or sequences that have been reported to be more or less human associated lineages including CC1, CC5, CC8, CC25 and CC97 [36**,** 37], suggesting that they have features allowing enhanced capacity of spread among human beings [38] and the livestock may play an important role of reservoir for such strains. However, this is not conclusive, since some *Staph. aureus* isolates from cases of bovine mastitis sometimes resemble human variants of *Staph. aureus* more than other bovine variants [39]. Strong association has been established between particular strains (as determined by its ST) of *Staph. aureus* and transmissibility, clinical manifestations, severity of the disease, treatment response and prognosis [34, 40]. Virulence gene profiles associated with enterotoxins production such as gelatinase, hemolysins, lipase and the ability to produce biofilm are very heterogeneous and differ between regions and countries [41, 42].

Emergence of new variants of *Staph. aureus* with potential resistance to currently used antibodies, is a serious challenge for veterinary and public health. Out of total *Staphylococcus aureus* isolates, high percentage (47.6%) of methicillin-resistant (MRSA) strains was detected in mastitic milk

in China [43] and 11.6% in Iran [30] versus very low occurrence (1.5%) in Finland [44]. Prevalence of 2.2% and 4% of MRSA was detected in bulk tank milk samples in Great Britain and USA, respectively [34, 45], imposing a significant concern for both animal health and public health. Emergence of methicillin resistance seems to be more associated with particular lineages (Table 1). Additionally, some animal-adapted clonal lineages of *S. aureus* such as CC130, CC599, CC59, CC1943 and CC425 are showing increase in methicillin resistance profile [33, 34, 46]. Livestock-associated MRSA may be responsible for increased rates of infection among people working on farms or dairy industry [47], addressing the potential impact on the public health.

***Streptococcus agalactiae* mastitis**

In cattle, mastitis is the only disease associated with *Strep. agalactiae* infection [28]. Ostensson et al. [11] reported that the pronounced subclinical mastitis problems in dairy cows in Vietnam were mainly due to *Strep. agalactiae* infection. A Danish study of 10 years surveillance concluded that there was an increasing trend in both the incidence and prevalence of *Strep. agalactiae* [48]. Transmission is contagious through contaminated tools and manipulating personnel, resulting in predominance of single strain in a given herd [49]. Significant variables associated with increase *Strep. agalactiae* infection including higher parity, increased months in milk, and compromised milking hygiene [17].

MLST database of *Strep. agalactiae* (http://pubmlst.org/sagalactiae/) showed that 757 sequence types (STs) have been identified and made available on the MLST website as of August 2015, although information about bovine strains is very limited. In individual molecular studies indicated that ST1, ST2, ST6, ST17, ST19, ST23, ST61, ST67, ST86, ST103, ST153, ST297, ST301, ST313, ST352, ST483, ST568 and ST570 are the dominant strains in clinical and subclinical cases [49-52], however, they belong to few CCs. Molecular evidence indicated existence of exclusively host adapted strains of *Strep. agalactiae* [49, 53], worldwide. Multidrug resistance is a significant challenge to control bovine

Strep. agalactiae-derived mastitis. Shift of prevalence from the macrolide resistance phenotype to the macrolide-lincosamide-streptogramin B resistance phenotype has been reported in Brazil [54].

Environmental mastitis

Environmental pathogens are normally found in the farm milieu such as manure, bedding material and/or soil. Environmental mastitis is characterized with transient infections and multitude of strains. The most common environmental pathogens are coliforms such as *Escherichia coli*, streptococci such as *Strep. dysgalactiae* and *Strep. uberis*, and coagulase negative staphylococci. The bacteriological etiology of mastitis in some countries show a trend of change from contagious to environmental pathogens, which has reduced the efficacy of the traditional mastitis control strategies [55].

***Escherichia coli* mastitis**

Mastitis caused by gram-negative infections is of increasing importance in modern and well-managed dairy farms; with *E. coli* tends to be the most important causative agent around parturition and during early lactation [56], and is typically associated with acute, clinical mastitis. The severity of *E. coli* mastitis is mainly determined by cow factors associated with the elements of innate immunity orchestrated in the induction and progress of inflammatory response rather than by *E. coli* pathogenicity [3, 56]. Inflammation due to infection with *E. coli* has long-term detrimental effects on milk quality regardless of clinical or bacteriological cure of affected glands [3].

Broadly, *E. coli* has no specific virulence factors that differentiate strains according to their ability to cause mastitis [56]. However, phylogenetic studies reveal that *E. coli* strains cluster based on their degree of pathogenicity and antimicrobial resistance [57]. Endotoxins or lipopolysaccharides are a non-specific but potent factors in the pathogenesis of *E. coli*, which in ruminants are dose-dependent

and elicit a variety of metabolic and clinical signs [58]. Due to its versatile diversity in the environment, episodes of *E. coli* infections assumed to be caused with different strains; however infections with the same strain have been reported [28]. A small fraction of the incidence of mastitis is due to recurrent *E. coli* infection with the same strain of initial cause, more likely in the same mammary quarter indicating the persistence of infection in some animals and may exhibit some kind of host-adaptation of the pathogen [59].

E. coli MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) showed very limited number of sequence types associated with bovine mastitis compared to the entire *E. coli* database, with ST10, ST58, ST685 and ST1125 are the most frequent sequence types. Few of the reported STs are assigned to clonal complexes (Table 2), but utmost of them are singletons. Noticeably, *E. coli* MSLT database showed very limited geographic distribution mostly in European countries including Ireland and Germany, but this may reflect the interest in multi-locus sequence of *E. coli* isolates from mastitis cases rather than geographical isolation.

Streptococcus uberis **mastitis**

Streptococcus uberis is strictly an animal pathogen [28, 60], and frequently isolated from dairy environment [61]. Distribution of *Strep. uberis* in a herd seems closely associated with the dominant strains in the environment [28, 62]. Although *Strep. uberis* infection predominantly results in subclinical mastitis (~ 95.0%), it is responsible for up to 16.0% and 33.0% of clinical cases per year in the United States and the United Kingdom [reviewed by 63]. The pattern of mastitis-causing organisms in New Zealand over the last 40 years, showed a substantial increase in the percentage incidence of *Strep. uberis* and decrease in *Strep. agalactiae* [25]. Although several strains can be isolated from a herd, a single strain often could be isolated from a quarter sample [64], indicating a high heterogeneity in the herd(s) with obvious homogeneity on the individual level.

Six hundred and ninety nine sequence types segregated within few clonal complexes is the total MLST database of *Strep. uberis* (http://pubmlst.org/suberis/). Unavailability of data on bovine mastitis associated sequences makes analysis of the database difficult. However, molecular typing studies [61, 62] indicate that some frequent sequence types such as ST5, ST8, ST143, ST153-ST159 are more associated with bovine mastitis. Tomita et al. [65] demonstrated the occurrence of identical sequence types (ST60 and ST184) between different continents (Australia and Europe) and different countries (Australia and New Zealand), contradicting the concept of geographic isolation proposed by Pullinger et al. [66]. Host adaptation was reflected in distinctive molecular differences between mastitis isolates of *Strep. uberis* from ovine and bovine hosts shared the same geographical and temporal origins [67]. Arbitrary, some strains such as those belong to ST143 may be more associated with clinical and subclinical mastitis [50] and others are related to latent infection, i.e. presence of *Strep. uberis* without a discernable inflammatory response [65]. Clinical and/or subclinical associated strains may possess virulence factors promoting invasion of host tissue, survival in the host environment or evasion of the host immune response, and internalization in the mammary gland cells [68].

Klebsiella **Mastitis**

Klebsiella is known environmental pathogen that repeatedly isolated from dairy cattle farms [69]. *Klebsiella*, especially *K. pneumoniae* has been associated with many mastitis outbreaks [69], and clinical cases [70] in dairy farms in USA. Organic-based bedding is often implicated in *Klebsiella* mastitis [71]**.** Molecular typing showed that ~80.0% of the genetic diversity was due to variation of genotypes within herds [72], indicating the environmental nature. Excretion of milk by an infected cow, resulting in seeding of bedding with a large number of colonies from a single strain, leading to what is could tentatively called “cow-to-cow transmission via the environment” [69].

Klebsiella spp. is causing the most severe cases among the gram-negative bacterial causes of mastitis, closely followed by *E. coli*. Mastitis caused by *K. pneumoniae* responds poorly to antibiotic treatment, subsequently, infection(s) tends to be severe and long lasting [72]. Drug resistant, CTX-M-2 extended-spectrum-β-lactamase-producing, *Klebsiella pneumoniae* seems to be obviously occur in bovine mastitis cases in dairy farms in Japan and United Kingdom [73, 74], raising the concerns about the role of such farms in the dissemination of extended-spectrum β-lactamase-producing bacteria in the community.

Streptococcus dysgalactiae **mastitis**

Strep. dysgalactiae is reported as common causative agent of subclinical mastitis in dairy cattle; implicated for important fraction (9.0%) of subclinical mastitis in dairy cows in Sweden [75] and in Austria as well [76]. The major transmission mode of *Strep. dysgalactiae* is not unequivocal. Based on the epidemiological and molecular studies, in the terms of duration of infection and the identity of infecting strains, it might be contagious/environmental [28, 76]. Yet, identification of *Strep. dysgalactiae* at the subspecies level is not yet satisfactory [77]. Bovine mastitis attributed to *Strep. dysgalactiae* may be also associated with teat lesions caused by flying insects [78].

Coagulase-Negative Staphylococci (CNS) causing mastitis

CNS is a greatly versatile group of staphylococci that might impact the udder health. This group includes some common species such as *Staph. chromogenes*, *haemolyticus*, *simulans* and *equorum* with *Staph. epidermidis* is the most common [79, 80]. These bacteria species are commonly associated with the animal at extra-mammary niches [81], and/or environment, with wide heterogeneity in the strains isolated within a herd. Transmission occurs through contamination of teats with the pathogen through

contact with the environment, tools and milkers' hands. Some CNS species seem to be associated with persistent IMI suggesting that they (e.g. *Staph. chromogenes* and *Staph*. *simulans*) are better host-adapted, whereas others may have an environmental reservoir [81].

CNS were reported as the predominant pathogens in the intramammary infections in Dutch [82] and Canadian [18] dairy herds, and with a fraction of ~50.0%, ~ 44.0% and ~ 30.0% of all isolated microorganisms from clinical and subclinical mastitis in Ecuador, Poland, China and Uganda, respectively [15, 79, 14, 83]. Further, CNS are frequently isolated from milk irrespective of the density of somatic cells or the type of farm settings. Emergence of multi-drug resistance in CNS (MR-CNS) is frequently reported. Methicillin-resistant in in coagulase-negative staphylococci ranged from 1.8 to 5.2% of the total CNS from bovine mastitis in Finland [44] versus 20 % of total CNS in mastitis cases in Brazil [84]. These findings are of special significance because MR-CNS can be implicated in mastitis of cows and they constitute a reservoir of resistance genes that can be transferred to other pathogenic bacteria [84].

Miscellaneous bacteria causing mastitis

Mycoplasma mastitis is recognized as an emerging mastitis pathogen worldwide, mostly unresponsive to treatment with antibiotic and/or anti-inflammatory agents [85]. The current prevalence rates in dairy herds [86] and bulk milk [87] are obviously low; however, it is implicated in many outbreaks [88]. Initial transmission might occur via nose-to-nose contact, which may result in out-breaking of *Mycoplasma* mastitis, or it might occur during the milking time [89].

Environmental mastitis due to *Corynebacterium* spp. (mainly *C. bovis*) [17, 23], *Pseudomonas aeruginosa* [10] and *Serratia marcescens* [90] intramammary infections in dairy cows are frequently reported with varying incidences or even sporadic outbreaks. Small fraction of mastitis due to Enterococci (mostly *Enterococcus faecalis*) was reported from different geographical parts.

Environmental farm elements including bedding are natural reservoirs for these pathogens. They are reported as a minor and treatable agent of bovine mastitis, often with mild to moderate clinical symptoms [91]. *Listeria* spp. was also reported in a considerable number of studies associated with bovine mastitis both in clinical and subclinical cases [92]. In rare cases *Bacillus anthracis* was implicated in clinical mastitis [93].

Treatment and Control measures

Treatment

Antimicrobial drug therapy is the most important available tool for producers and veterinarians to prevent the diseases, for growth promotion and to increase production efficiency, as well as to ensure that the animal health and welfare are maintained. Effective treatment of bovine mastitis depends on the causative agent, the clinical manifestation and the antimicrobial sensitivity of that agent; or what is called pathogen, cow and treatment factors [94, 95]. Cure rates decrease with increasing age of the cow, increasing somatic cell count, increasing duration of infection, increasing bacterial colony counts in milk before treatment, increasing number of quarters infected and with high parity [94]. Increased duration of treatment is associated with increased chance of cure, reduced probability of recurrent infection and improved somatic cell count [96]. Notably, the use of antibiotics (intramammary infusions; bacteriocins) and herbs (*Terminalia* spp.) are important for prophylaxis and therapeutics [97]. Recently, immunotherapy using a bead carrier of antibodies directed against the causative

agent(s), facilitating microbial clearance via phagocytosis is newly addressed and growing approach [98].

Emergence of drug resistant strains is a serious challenge for the control of mastitis. Because the resistance profile is often herd specific [99], the choice of treatment regimen should be based on knowledge of the antimicrobial sensitivity of the infecting strain. Although the treatment of choice can be based on the herd-level knowledge of sensitivity pattern of predominant strains, treatment of subclinical infections can be postponed until results of cow-level sensitivity testing are available [94]. Combination of more than one of synergistic antimicrobial drugs may have a privilege than single drug approach with high margin of curability [100]. Potential development of resistance against disinfectants and specific biocides will lead to transmission of infection through teat disinfectants [55].

Control and preventive measures

Although antibiotics are widely used to combat mastitis [55]; the concept of food safety due to excessive usage of antibiotic to control mastitis is receiving growing support. Also, treatment of mastitis is commonly unsuccessful to revert the pathological changes that occur in the udder parenchyma as a result of the inflammatory reaction in response to mastitogenic bacteria [101]. Antimicrobial treatment of gram-negative bacteria has often considered being of limited value and treatment should be more targeted toward cow survival and reduction of clinical symptoms. Therefore, mastitis control strategies in the dairy industry should rely on the concept of preventive measures rather than treatment of clinical disease. Improved disease surveillance and better understanding of the multiplicity of pathogens, bacterial virulence factors, and mechanisms of pathogenesis are crucial factors. Specific prevention program

should depend on the major transmission mode and behavior of the dominant microbial species causing the disease in the herd. Successful implementation of a mastitis control program includes quick identification of mastitic animals, subjection of infected animals to segregation, treatment measures or culling, as soon as possible [55, 94]. Use long acting antibiotics on all quarters of all cows at the end of the lactation to eliminate persisting infections and prevent new infections in the dry period. The concept of using combination therapy with teat sealant and antibiotics seems to be effective in herds that adopt whole herd antibiotic treatment at drying off [100].

Milking procedures occupy the central part in initiation of mastitis infections and control programs as well. Animals are more likely to catch contagious pathogens directly from milking equipment. Therefore, improvement of milking hygiene; regular maintenance of the milking equipment, milking interval, implementation of milking order, adequate premilking udder hygiene, postmilking teat dipping and adequate cleaning of the udder [17, 102], would be the key factors. New milking techniques including robotic milking may provide better possibility for proper milking and improved udder health [103]. Accumulated evidence suggests that the risk of subclinical mastitis, particularly those infections caused by environmental pathogens, is strongly related to postmilking standing and lying patterns. Practices that promote postmilking standing duration of 30 to 120 min, such as the provision of fresh feed or freshly pushed-up feed around the time of milking, and providing ample feed bunk space per cow [18] are important factors to reduce the risk of intramammary infection.

Compromised farm hygiene is a basic factor in mastitis predisposition. Bedding is a primary source of mastitis microbes, especially coliforms. Daily replacement of organic bedding appeared to be an effective approach to reduce exposure to coliforms [71]. Inorganic bedding materials such as sand and crushed limestone in dairy barns harbored less density of environmental bacteria compared to

organic derived ones such as wood shavings, straw, chopped newspaper, recycled manure, and corn fodder [104].

Herd management factors including the variables associated with subclinical mastitis risk such as high parity, high age, increase months in milk, crossbreed versus purebred strain [17, 94] are important to establish an effective control measures. It is reported that the severity of clinical signs, which may range from mild to fatal, is largely attributed to host-characteristics [56]. Therefore, disease resistant breeding using novel biomarkers *viz*. toll like receptors (TLR) 2 and 4, interleukin (IL) 8; breast cancer type 1 susceptibility protein (BRCA1) and calcium channel voltage-dependent alpha 2/delta sub unit 1 (CACNA2D1) are favorable [97]. Generic factors to prevent gram-negative bacteria depend mainly on improving general herd hygiene and reducing exposure of teat ends to environmental contamination [55]. Improving nutrition, housing, bedding materials and environment of dairy cattle are still crucial in the prevention of mastitis, especially during the most susceptible period after parturition.

The risk of introducing new mastitis-causing microbes into a herd may increase by frequent animal movements into the herd [29]. Biosecurity measures should be strictly applied to dairy herds, to prevent not only mastitis but also other potential diseases. New animals must be screened for the main mastitis microbes, especially the contagious ones before introduction into the herd [103]. Animals from different herds of origin should be kept for a limited period of time as a clearance period before they be commingled and share their milking equipment [29].

Although antibiotic remains the main strategy of choice in control of mastitis, excessive usage of antibiotics to treat mastitis may lead to development of resistant strains of bacteria that can be transferred to human. Different methods of immunomodulation for the prevention of mastitis have proved promising results; however it is yet far from commercial applications. The concept of using

vaccination approach to control of mastitis in dairy cattle seems sound. Vaccine formulations including DNA-based and DNA-protein based, recombinant (*Staphylococcal* enterotoxin) or chimeric (pauA); live (*Strep. uberis*) and bacterial surface extracts have been proposed for control of bovine mastitis [97]. Nevertheless, in vitro and in vivo studies suggest that the use of antioxidants and other protective compounds in mastitis control programs is worth investigating, because they may aid in alleviating damage to secretory cells and thus reduce subsequent milk loss [4].

Conclusion and future perspectives

Mastitis substantially impacts the dairy industry, which can support development especially in developing countries. Mastitis is caused by a vast range of microorganisms, with transmission varies from contagious to environmental modes. Major mastitis causing pathogens include *Staphylococcus aureus*, *Streptococcus agalactiae, Streptococcus uberis*, *Streptococcus dysgalactiae* and *Escherichia coli*. However, minor pathogens still eminent players in the intramammary infections. Several reports indicate strong association between certain microbial strains and clinical manifestation and the prognosis of the disease. Treatment depend mainly on an antibodies, however, management regimens, hygiene level and udder health practices are crucial factors in the control of the disease. Improved disease surveillance and better understanding of the multiplicity of pathogens, bacterial virulence factors, and mechanisms of pathogenesis are crucial for disease control. Enhanced disease resistance and improve knowledge of mammary gland immunology are essential for disease prevention and vaccine development.

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Figure ligands

Fig. 1. Clustering pattern of mastitis-derived *Staphylococcus aureus* based on concatenated multi-locus sequences. Relationships of *Staphylococcus aureus* isolates derived from bovine mastitis as illustrated by Neighbour-joining (NJ) tree constructed by MEGA5 software based on concatenated 7-loci of housekeeping gene sequences (Carbamate kinase (**arc**), Shikimate dehydrogenase (**aro**), Glycerol kinase (**glp**), Guanylate kinase (**gmk**), Phosphate acetyltransferase (**pta**), Triosephosphate isomerase (**tpi**) and Acetyle coenzyme A acetyltransferase (**yqi**) retrieved from MLST website. The dendogram shows the clonal complex (CC) followed by sequence type (ST). Clonal complexes that contain many sequences types on the same branch are illustrated as a group and detailed herein as following: **CC1** includes ST1, ST81, ST147, ST743, ST1121, ST2154, ST2493, and ST2518; **CC5** includes ST5, ST744,

ST965, ST2411, ST2510, ST2517, ST2633, ST2659 and ST2825; **CC8** includes ST8, ST630, ST1381, ST1382, ST1652, and 2412; **CC20** includes ST20, ST389, ST1368, and 1370; **CC25** includes ST25, ST26, ST1372, and ST2410; **CC97** includes ST97, ST71, ST124,ST502, ST742, ST746, ST747, ST1072, ST1077, ST1129, ST1367, ST1527, ST1623, ST1624, ST1859, ST2165, ST2187, ST2379, ST2457, ST2519, ST2521, ST2824, and ST2826; **CC130** includes ST700, ST1526, ST1627, and ST2490; **CC133** includes ST133, ST419, ST478, ST745, ST1007, ST1008, ST1075, ST1247, ST1858, ST2488, ST2821, and ST2822; **CC151** includes ST151, ST1078, ST1123, ST1248, ST2185, and ST2186; **CC352** includes ST352, ST1366, ST2010, ST2190, and ST2191; **CC479** includes ST479, ST520, ST1118, ST1380, ST1651, ST2166, and ST2683; **CC504** includes ST504, ST1124, ST1274, ST1712, and ST2823; **CC705** includes ST705, ST1074, ST1076, ST1363, ST1364, ST1520, and ST1365; **CC2459** includes ST2219, ST2368, ST2458, and ST2459.

Table 1: Distribution of mastitis related clonal complexes and associated sequence types. Analysis of the entire *S.aureus* database by eBURST may reflect the limited number of *S. aureus* strains from mastitis/milk source that are registered in the MLST database

Clonal Complex	ST	Country	Source	Methicillin
CC1	1	Japan, Spain, The Netherlands	Milk, Bovine milk	S/ND
	81	Japan, The Netherlands	Bulk milk	S/ND
	147	Norway	Bulk milk	ND
	743	Brazil	Milk	S
	1121	The Netherlands	Milk	S
	2154	China	Milk	R
	2493	Brazil	Milk	S
	2518	Brazil	Bulk tank milk	S
CC5	5	Japan	Bovine milk	S
	744	Brazil	Milk	S
	965	China	Cow's milk	R
	2411	Norway	Bovine milk	ND
	2510	Brazil	bulk tank milk	S
	2517	Brazil	Bulk tank milk	S

	2633	China	Milk	ND
	2659	Sweden	Bulk milk	R
	2825	Germany	Milk	ND
CC6	6	China, Japan	Cow's milk	R/S
	1362	Japan	bulk milk	S
CC7	789	Japan	bulk milk	S
CC8	8	Japan, Switzerland, The Netherlands, USA	Milk, Bovine milk	S/ND
	630	Japan	Bulk milk	S
	1381	The Netherlands	Milk	ND
	1382	The Netherlands	Milk	ND
	1652	Switzerland	Milk	S
	2412	Norway	Bovine milk	ND
CC9	9	China, The Netherlands	Milk, Cow's milk	R
	2454	UK	Raw milk	S
CC12	12	Japan	Bovine milk	S
	1369	Japan	Bovine milk	S
CC20	20	China Japan, USA	Milk bulk, milk	R/S
	389	Switzerland	Milk	S
	1368	Japan	Bovine milk	S
	1370	Japan	bulk milk	S
CC25	25	China Japan USA	Milk, Bovine milk	R/S
	26	Japan Spain	Bovine milk	S
	1372	Japan	Bovine milk	S
	2410	Norway	Bovine milk	ND
CC30	30	Spain	Cow's milk	S
	243	Japan		ND
	895	England	Milk	S
CC45	45	Switzerland	Milk	S
	508	Japan	Bovine milk, Bulk milk	S
CC59	59	Japan	Bovine milk	S
	87	USA	Milk	R
CC72	72	Japan	bulk milk	S

CC88	88	Japan	Bulk milk, Bovine milk	S
	1360	Japan	Bovine milk	S
CC89	89	Japan	Bovine milk	R
CC97	71	Switzerland, The Netherlands	Milk	S/ND
	97	China, Japan, Spain, Switzerland The Netherlands	Cow's milk, Milk	S/R
	124	Japan, The Netherlands	Bovine milk, Milk	S/ND
	502	Portugal	Cow's milk	S
	742	Brazil	Milk	S
	746	Brazil	milk	ND
	747	Brazil	milk	S
	1072	England	Bulk milk	ND
	1077	England	Bulk milk	ND
	1129	The Netherlands	Milk	S
	1367	Japan	bovine milk	S
	1527	UK	Bovine milk	ND
	1623	Brazil	Bovine milk	ND
	1624	Brazil	Bovine milk	ND
	1859	UK	Bovine Milk	ND
	2165	China	Milk	S
	2187	USA	Milk	S
	2379	China	Fresh milk	S
	2457	UK	Un-pasteurized milk	S
	2519	Brazil	Bulk tank milk	S
	2521	Brazil	Bulk tank milk	S
	2824	Germany	Milk	ND
	2826	Germany	Milk	ND
CC121	120	China	Milk	R
	121	China	Milk	R
CC126	741	Brazil	Milk	S
	2270	Cuba	Cow's milk	S
CC130	700	Italy	Cattle milk	S
	1526	UK	Bovine milk	ND
	1627	Italy	Cattle milk	S
	2490	France	Milk	ND
CC133	133	England	Cow's milk	ND
	419	Portugal	Cow's milk	S
	478	Norway	Bulk milk - bovine	ND
	745	Brazil	Milk	S

	1007	Norway	Quarter milk sample	S
	1008	Norway	Quarter milk sample	S
	1075	England	Bulk milk	ND
	1247	England	Bulk Milk	ND
	1858	UK	Bovine Milk	ND
	2488	England	Milk	ND
	2821	Germany	Milk	ND
	2822	Germany	Milk	ND
CC151	151	England, Spain, The Netherlands, USA	Cow's milk, Milk	S/ND
	1078	England	Bulk milk	ND
	1123		Milk	S
	1248	England	Quarter milk sample	ND
	2185	USA	Milk	S
	2186	USA	Milk	S
CC188	188	Japan, Switzerland	Milk, Bovine milk	S
	1519	Japan	Bulk milk	ND
CC352	352	China, Japan, Spain, The Netherlands, USA	Milk, bulk milk , bovine milk	S
	1366	Japan	bovine milk	S
	2010	Cuba	Cow's milk	ND
	2190	USA	Milk	S
	2191	USA	Milk	S
CC398	398	China, Switzerland The Netherlands	Milk	R/S
	2634	China	Milk	ND
CC425	425	England	Cow's milk	ND
CC479	479	Norway, The Netherlands, USA	Milk, Bulk milk - bovine	S/ND
	520		Bulk milk - bovine	ND
	1118	The Netherlands	Milk	S
	1380	The Netherlands	Milk	ND
	1651	Switzerland	Milk	S
	2166	China	Milk	S
	2683	China	Bovine milk	S
CC504	504	Switzerland , The Netherlands	Milk	S/ND
	1124	The Netherlands	Milk	S
	1274	Germany	Bovine	S
	1712	Switzerland	Milk	S
	2823	Germany	Milk	ND

CC522	2489	France	Milk	ND
CC705	705	Japan, The Netherlands	Bovine milk, Bulk milk, Milk	S
	1074	England	Bulk milk	ND
	1076	England	Bulk milk	ND
	1363	Japan	bulk milk	S
	1364	Japan	bovine milk	S
	1520	Japan	Bovine milk	ND
	1365	Japan	bulk milk	S
CC2459	2219	India	Cow's Milk	S
	2368	India	Bovine milk	ND
	2458	India	Cow's milk	S
	2459	India	Cow's milk	S
CC2738	2738	China	Bovine milk	ND

NB.1. S: Susceptible, R: Resistant; ND: Not Determined; S/ND: some strains were reported as susceptible and some strains not determined; R/ND: some strains were reported as resistant and others not determined; S/R: some strains were reported as susceptible and others were reported as resistant.

NB.2. The authors adhered to terms reported on the source of strains at MLST website or individual literature.

Table 2. Distribution of bovine mastitis related sequences of *E. coli* as derived from MSLT

ST Complex	ST	Country	ST Complex	ST	Country	ST Complex	ST	Country
ST10 Cplx	10	Ireland	**None**	1139	Germany	**None**	1493	Germany
ST155 Cplx	58	Ireland	**None**	1141	Germany	**None**	1494	Germany
ST69 Cplx	69	Ireland	**None**	1145	Germany	**None**	1495	Germany
None	141	UK	**None**	1146	Germany	**None**	1496	Germany
None	154	Ireland	**None**	1148	Germany	**None**	1499	Germany
ST165 Cplx	301	Ireland	**None**	1294	Germany	**None**	1508	Ireland
ST278 Cplx	316	Ireland	**None**	1299	Germany	**None**	1508	Ireland
ST29 Cplx	389	UK	**None**	1300	Germany	**None**	1508	Germany
ST398 Cplx	398	Ireland	**None**	1301	Germany	**None**	1510	Germany
ST23 Cplx	410	Ireland	**None**	1302	Germany	**None**	1511	Germany
ST448 Cplx	448	Ireland	**None**	1303	Germany	**None**	1590	Germany
None	540	Ireland	**None**	1304	Germany	**None**	1611	Ireland
ST10 Cplx	548	Ireland	**None**	1307	Germany	**None**	1612	Israel
None	685	Ireland	**None**	1308	Germany	**None**	1617	Israel
None	925	Ireland	**None**	1309	Germany	**None**	2327	Israel
None	1079	Ireland	**None**	1310	Germany	**None**	2328	Israel
None	1088	Ireland	**None**	1395	Germany	**None**	2970	Israel
None	1121	Ireland, Germany	**None**	1396	Germany	**None**	3405	Germany
None	1122	Germany	**None**	1398	Germany	**None**	3406	Germany
None	1123	Germany	**None**	1399	Germany	**None**	3499	Japan
None	1124	Germany	**None**	1400	Germany	**None**	3744	Ireland
None	1125	Germany	**None**	1403	Germany	**None**	4173	Ireland
None	1125	Ireland	**None**	1406	Germany	**None**	4174	Ireland

None	1126	Germany	**None**	1408	Germany	**None**	4175	Ireland
None	1137	Germany	**None**	1414	Germany			
None	1138	Germany	**None**	1415	Germany			

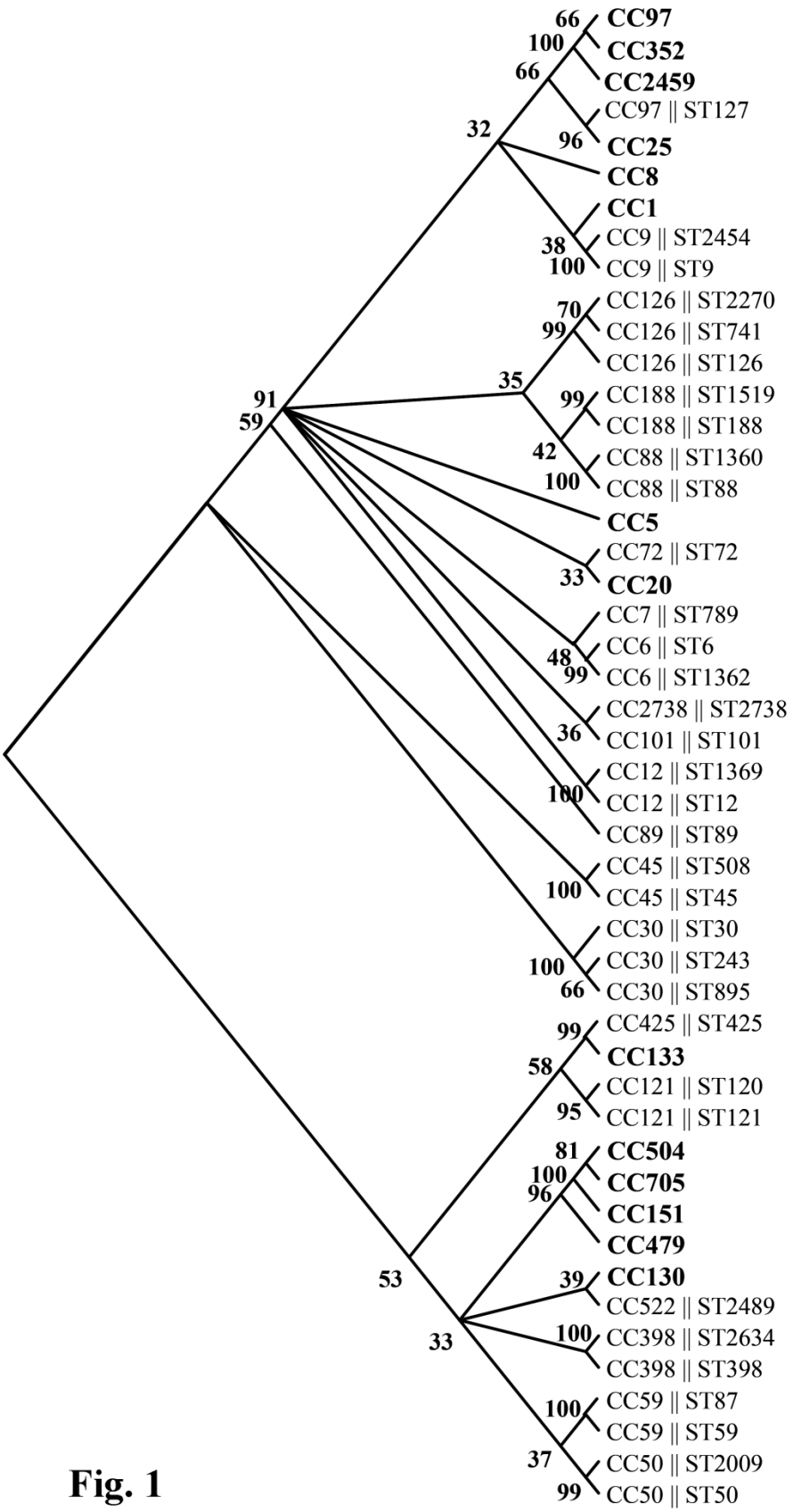
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Fig. 1