P-10898

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES
E. S. D.

Re: Expediente No. **15-270.184**
Solicitud de patente a nombre de **UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO**

Yo, CAROLINA DAZA MONTALVO identificada como aparece al pie de mi firma, domiciliada en la Carrera 11 No. 86-53, piso 3, obrando como apoderada de la sociedad **UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO**, domiciliada en Ciudad de México, México, solicitante de la patente de la referencia, en respuesta al Oficio No. **4882** notificado por comunicación electrónica el 4 de abril de 2017 y relacionado con concepto técnico de fondo, me permito manifestar:

1. Modificaciones al capítulo reivindicatorio

Primeramente, con fundamento en la facultad que confiere el Artículo 34 de la Decisión 486/00 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, me permito presentar junto con este memorial un nuevo capítulo reivindicatorio que consta de 16 reivindicaciones y reemplaza completamente al anteriormente presentado, el cual contempla las siguientes modificaciones:

- La reivindicación 1 fue modificada para corregir la ortografía del compuesto activo y restringir el alcance del emulsificante de la formulación.

- La reivindicación 8 anteriormente presentada fue eliminada con el objetivo de incluir su materia en la reivindicación 1.

- La reivindicación 18 anteriormente presentada fue eliminada por tratarse de materia no patentable.

En cuanto a dichas modificaciones, me permito expresar que las mismas no amplían de ninguna manera la materia objeto de la presente solicitud y se realizaron con el ánimo de superar las objeciones planteadas por el examinador en su concepto técnico.

2. Pago de tasas

En conexión con lo anterior y en relación con el número de reivindicaciones, atentamente me permito comentar que no se adjunta tasa por reivindicaciones adicionales, toda vez que el nuevo capítulo reivindicatorio cuenta con menos cláusulas que el actualmente pagado.

Por otra parte, me permito adjuntar recibo por concepto de pago de tasa para un nuevo examen de patentabilidad sobre el nuevo pliego reivindicatorio, según lo estipulado en la Resolución No. 3719 de 2016.

3. Reivindicaciones relativas a un uso

En este sentido, con relación a lo señalado por el examinador en cuanto a que: «*A pesar de estar encabezada como una composición, la reivindicación 18 se relaciona con el uso de la misma, lo cual no es patentable de acuerdo al artículo citado*», me permito indicar que, tal y como se expuso en el numeral 1 del presente memorial, la reivindicación 18 anteriormente presentada fue eliminada del nuevo capítulo reivindicatorio. Por tanto, me permito solicitar a ese Despacho que la objeción a este respecto sea retirada, toda vez que las reivindicaciones se ajustan a las disposiciones del Artículo 14 de la Decisión 486/00.

4. Claridad y concisión del capítulo reivindicatorio

Ahora bien, con relación a la claridad y concisión de las reivindicaciones, me permito manifestar que mediante el nuevo capítulo reivindicatorio quedan subsanadas la totalidad de las objeciones a este respecto, ya que las nuevas reivindicaciones proporcionan claridad y concisión a la invención reclamada, y cuentan con pleno soporte en la descripción de la solicitud en estudio, siguiendo además las recomendaciones realizadas por el examinador en su concepto técnico.

En particular, frente a lo mencionado por el examinador en cuanto a que: «*El término "emulsificante" dentro de la reivindicación 1 no es conciso, ya que puede abarcar infinidad de compuestos que cumplan dicha función, de los cuales muchos no se encuentran revelados en la descripción. Igualmente, la proporción de dicho componente no se encuentra dentro del alcance de la reivindicación*»; y que: «*Parece que no está clara la ortografía del compuesto activo, ya que en algunas reivindicaciones se escribe "tilmicocina" y en otras "tilmicosina"*», amablemente me permito resaltar que la reivindicación 1 del nuevo capítulo reivindicatorio fue restringida para especificar que el emulsificante que contiene la formulación reclamada es poloxámero, así como su concentración dentro de la misma. Además, tal y como se expuso en el numeral 1 del presente memorial, la reivindicación 1 del nuevo capítulo reivindicatorio fue modificada para corregir la ortografía del nombre del compuesto activo de la formulación: «tilmicosina».

Por lo anterior, me permito manifestar que el capítulo reivindicatorio adjunto al presente memorial, que incluye 16 reivindicaciones, cumple a cabalidad con las disposiciones del Artículo 30 de la Decisión 486/00.

5. <u>Nivel inventivo</u>

Finalmente, el examinador establece en su concepto técnico la falta de nivel inventivo de la presente solicitud, en vista de las enseñanzas descritas en los documentos CN 101422432 (D1) y US 2006094669 (D2). A este respecto, y teniendo en cuenta la restricción realizada a la reivindicación 1 explicada anteriormente, me permito manifestar lo siguiente:

La solicitud No. US 2006/0094669 (D2) busca protección para materia que se refiere a métodos para tratar una infección bacteriana en un caballo o un cerdo. El método implica administrar al caballo o cerdo una composición que comprende (i) una sal hecha de tilmicosina y un ácido lipófilo y (ii) un solvente farmacéuticamente aceptable combinado entre sí para formar una composición inyectable que precipita cuando se inyecta en agua.

Además de que dicha solicitud y su correspondiente concesión US 7,282,487 B2 hacen referencia a un método de tratamiento y **no a una formulación como lo pretende la presente solicitud**, en todo el texto de la memoria de dicha solicitud US

2006/0094669 **no se hace mención del uso del emulsificante poloxámero en la formulación de tilmicosina**.

La patente US 7,282,487 B2 expone que las composiciones usadas en los métodos de la invención pueden contener altas concentraciones de tilmicosina. En una modalidad, la tilmicosina se incluye en la composición en una cantidad que varía de aproximadamente 10% a 60% (p/v). No obstante, un experto en la materia comprenderá que este intervalo puede variar ampliamente, dependiendo, por ejemplo, del ácido lipófilo seleccionado, del disolvente seleccionado, de la inyectabilidad del producto final y de cualquier otra necesidad pertinente.

El porcentaje de tilmicosina presente y la variación del mismo dependen de otros elementos o factores presentes en el documento norteamericano D2. La presente invención no varía su porcentaje de tilmicosina en los mismos elementos o factores, toda vez que se trata de formulaciones completamente diferentes.

En otras modalidades, las composiciones utilizadas en los métodos de la invención incluyen tilmicosina en una cantidad tan baja como aproximadamente 10%, aproximadamente 5%, o incluso aproximadamente 1% y todavía proporcionan un efecto útil. De forma similar, la tilmicosina se puede incluir en las composiciones usadas en los métodos de la invención en cantidades de 70%, o incluso más altas según requieran las necesidades. No se requieren excipientes ni portadores exóticos. Las composiciones se filtran fácilmente, simplificando así el proceso de fabricación. También se cree que la exclusión del agua de las composiciones imparte una mayor estabilidad a las composiciones e inhibe el crecimiento de microorganismos. Los procedimientos para preparar las composiciones, como se describen aquí, son simples, y la administración de acuerdo con la presente invención típicamente da como resultado reacciones más leves en el sitio de inyección debido a la neutralización del compuesto farmacológicamente activo.

Como se podrá observar, las formas de liberación de la sustancia activa son completamente diferentes, principalmente ante la presencia del poloxámero de la presente invención.

Por otro lado y con relación al documento CN101422432 (D1) citado por el examinador, me permito destacar que en él se describe un medicamento

antibacteriano de tilmicosina nano-emulsión cuyo diámetro de grano está entre 1 y 100 nm; los componentes y el porcentaje en peso de los mismos son los siguientes: 0,01 a 6,0 por ciento de Tilmicosina, 20,0 a 40,0 por ciento de agente tensioactivo/cotensioactivo, 2,2 a 10,0 por ciento de aceite; el resto es agua destilada; y la suma del porcentaje en peso de los componentes es del 100 por ciento. El medicamento es un líquido claro y transparente en la apariencia, se caracteriza por baja viscosidad, buena estabilidad, alta dispersabilidad y rápida absorción y similares, puede mejorar la biodisponibilidad del medicamento, prolongar la semivida del medicamento en un cuerpo, potencian el efecto curativo, reducen la toxicidad y tienen los efectos de la liberación sostenida y la focalización; simultáneamente, la técnica de preparación es simple; el consumo de energía es bajo; y la toxicidad es baja, la seguridad es alta y la producción grande se puede llevar a cabo sin dispositivos especiales.

En este sentido, el documento chino CN101422432 describe un medicamento antibacteriano de tilmicosina nano-emulsión que consiste de un líquido claro y transparente de baja viscosidad, buena estabilidad, alta dispersabilidad y rápida absorción y similares; sin embargo, y de la misma manera que D2, **en todo el texto de la memoria del documento D1 no se hace mención del uso del emulsificante poloxámero en la formulación de tilmicosina**.

La concentración de fosfato de tilmicosina presente en la formulación de la presente invención va de 35 a 50% en peso de la composición total, a diferencia del medicamento que busca protección en D1, el cual expone **de 0,01 a 6,0 por ciento de tilmicosina en una emulsión antibacterial**.

Así pues, **es importante mencionar que los documentos D1 y D2 solos o en combinación no logran los resultados inesperados de la formulación de la presente invención, además que ocupan vehículos farmacéuticos comunes ya reportados en el estado de la técnica como vehículos presentes en medicamentos de acción prolongada y no utilizan poloxámero**.

El poloxámero consiste de un polímero de alto peso molecular que le distingue ser termorreversible, es decir, es líquido a temperatura ambiente y gelifica al entrar al organismo, provocando una matriz de donde se libera la tilmicosina por mucho tiempo.

La liberación de tilmicosina con la presencia del poloxámero evita la formación de concentración pico y con ello la carga toxica. Es importante tomar en cuenta del arte previo que la tilmicosina es cardiotóxica.

Lo anterior se encuentra comprobado y expuesto en la memoria descriptiva de la presente solicitud, particularmente en los siguientes ejemplos:

Ejemplo 1 - Protocolo de las pruebas de seguridad para tilmicosina de acción prolongada mejorada (TIL_{APM})
Ejemplo 2 - Estudio farmacocinético de la tilmicosina de acción prolongada mejorada (TIL_{APM}) en bovinos
Ejemplo 3 - Estudio clínico comparativo de la eficacia clínica de la tilmicosina de referencia (TIL_{REF}) y la tilmicosina
de acción prolongada mejorada (TIL_{APM}) en el tratamiento del complejo respiratorio bovino (CRB).
Ejemplo 4 - Uso de la tilmicosina de acción prolongada mejorada (Til_{APM}) en el tratamiento de las vacas secas

No obstante, con el objeto de aportar mayor claridad y viabilidad a la presente invención, me permito anexar dos artículos científicos que demuestran lo que se logra con la presencia del poloxámero (incluso de diferente peso molecular). Dichos artículos incluyen información y datos experimentales comparativos probando el mejor efecto logrado por la invención reclamada, con el fin de demostrar su actividad inventiva frente a las composiciones de los documentos D1 y D2.

Al respecto, es posible notar que las distinciones y características de la presente invención se relacionan directamente con un producto donde se aprovecha eficientemente la tilmicosina como antibiótico reduciendo el riesgo de toxicidad, al estar presente en una formulación donde la presencia del poloxámero otorga las bondades y ventajas previamente mencionadas.

De esta manera, me permito señalar que los documentos D1 y D2 no afectan el nivel inventivo de la presente solicitud y la objeción a este respecto se ve superada, toda vez que no existe motivación alguna para que una persona del oficio normalmente versada en la materia derive de manera obvia la presente invención a partir de dichas anterioridades, demostrándose así que las reivindicaciones 1 a 16 del nuevo capítulo

reivindicatorio cumplen con el requisito de nivel inventivo según los lineamientos del Artículo 18 de la Decisión 486/00.

6. Conclusión

Finalmente, a la luz de las modificaciones al capítulo reivindicatorio y los argumentos presentados, es posible concluir lo siguiente:

6.1. La presente solicitud es clara, concisa y está debidamente soportada por la descripción, cumpliendo así con los requerimientos del Artículo 30 de la Decisión 486/00.
6.2. La presente solicitud es patentable sobre los documentos D1 y D2, de acuerdo con los requerimientos del Artículo 18 de la Decisión 486/00.

Por tanto, atentamente me permito solicitar a ese Despacho otorgar el privilegio de patente solicitado a la solicitud en estudio, toda vez que cumple a cabalidad con los requerimientos de la Decisión 486/00.

7. Anexos

7.1. Nuevo capítulo reivindicatorio que comprende 16 reivindicaciones.
7.2. Comprobante de pago de tasa para nuevo examen de patentabilidad.
7.3. Artículo científico: Gutiérrez L, *et al*, 2016. Pharmacokinetics of a new parenteral formulation of Tilmicosin-La in cows. Pak Vet J, 36(2): 165-168.
7.4. Artículo científico: Mendoza J, *et al*, 2016. Concentrations of tilmicosin in mammary gland secretions of dairy cows following subcutaneous administration of one or two doses of an experimental preparation of tilmicosin and its efficacy against intramammary infections caused by Staphylococcus aureus. AJVR, 77(9): 922-930.

Señor Director,

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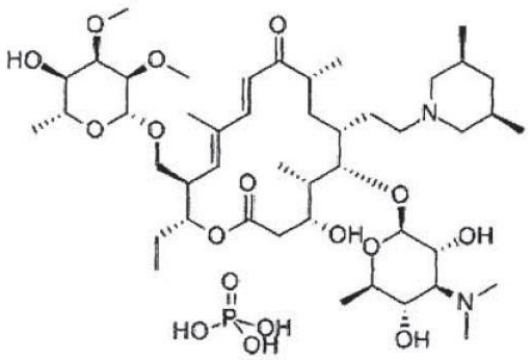
P-10898
Nuevo capítulo reivindicatorio
Julio 4, 2017

REIVINDICACIONES

1. Una formulación de liberación prolongada de tilmicosina que tiene la fórmula:



,

caracterizada porque la formulación comprende:

- fosfato de tilmicosina en una concentración desde 35 a 50% en peso de la composición total;
- un primer co-solvente en una concentración desde 8 a 20% en volumen;
- un segundo co-solvente en una concentración de 5 a 15% en volumen; y
- un emulsificante en una concentración de 1.5 a 15% en peso del peso total de la composición;

en donde el primer co-solvente se selecciona del grupo que comprende sorbitol, glicerina, derivados de esteres glicéridos, alcohol etílico, alcohol isopropílico, propilenglicol, alcohol bencílico, polietilenglicol de bajo peso molecular (100, 200, 300, 400, 600 y 800) y derivados de polietilenglicol, dimetilsulfóxido, glicerilformal, glucofurol, carbonato de etilo, lactato de etilo, dimetilacetamida o 2-metilpirrolidona;

en donde el segundo co-solvente se selecciona del grupo que comprende sorbitol, glicerina, derivados de ésteres glicéridos, alcohol etílico, alcohol isopropílico, propilenglicol, alcohol bencílico, polietilenglicol de bajo peso molecular (100, 200, 300, 400, 600 y 800) y derivados de polietilenglicol, dimetilsulfóxido, glicerilformal, glucofurol, carbonato de etilo, lactato de etilo, dimetilacetamida o 2 metilpirrolidona; y

en donde el emulsificante es poloxámero.

2. La formulación de tilmicosina de conformidad con la reivindicación 1, caracterizada además porque el fosfato de tilmicosina se encuentra en una concentración de 42% en peso de la composición total.

3. La formulación de tilmicosina de conformidad con la reivindicación 1, caracterizada además porque el primer co-solvente se encuentra en una concentración de 10% en volumen.

4. La formulación de tilmicosina de conformidad con la reivindicación 3, caracterizada además porque el primer co-solvente es propilenglicol.

5. La formulación de tilmicosina de conformidad con la reivindicación 1, caracterizada además porque el segundo co-solvente se encuentra en una concentración de 10% en volumen.

6. La formulación de tilmicosina de conformidad con la reivindicación 5, caracterizada además porque el segundo co-solvente es alcohol etílico.

7. La formulación de tilmicosina de conformidad con la reivindicación 1, caracterizada además porque el emulsificante se encuentra en una concentración de 3% en peso del peso total de la composición.

8. Un método para obtener la formulación de tilmicosina de acción prolongada mejorada tal como la que se reclama en las reivindicaciones 1 a 7 precedentes, caracterizado porque comprende las etapas de:

(a) Transferir de 45 a 60ml de agua destilada a un vaso de precipitados;

(b) Añadir al vaso de precipitados que contiene el agua destilada una cantidad de 8 a 20ml de un primer co-solvente que se selecciona del grupo que comprende sorbitol, glicerina, derivados de esteres glicéridos, alcohol etílico, alcohol isopropílico, propilenglicol, alcohol bencílico, polietilenglicol de bajo peso molecular (100, 200, 300, 400, 600 y 800) y derivados de polietilenglicol, dimetilsulfóxido, glicerilformal, glucofurol, carbonato de etilo, lactato de etilo, dimetilacetamida o 2-metil pirrolidona, entre otros; y agitar hasta obtener una solución homogénea;

(c) Adicionar una cantidad de 5 a 15ml de un segundo co-solvente que se selecciona del grupo que comprende sorbitol, glicerina, derivados de esteres

glicéridos, alcohol etílico, alcohol isopropílico, propilenglicol, alcohol bencílico, polietilenglicol de bajo peso molecular (100, 200, 300, 400, 600 y 800) y derivados de polietilenglicol, dimetilsulfóxido, glicerilformal, glucofurol, carbonato de etilo, lactato de etilo, dimetilacetamida o 2-metil pirrolidona, entre otros; y tapar el vaso con papel parafilm mezclando hasta obtener una solución homogénea;

(d) Incorporar una cantidad que va de 35 a 50g de fosfato de tilmicosina, haciéndolo lentamente a fin de evitar la formación de grumos, y agitar hasta obtener una solución homogénea;

(e) Agregar una de 1.5 a 15g de un emulsificante, haciendo la adición de una manera lenta para lograr que se vaya homogenizando la solución;

(f) Refrigerar la solución anterior a una temperatura de 2 a 8ºC por espacio de 1 hora, sacar de refrigeración y agitar por 5 minutos;

(g) Refrigerar nuevamente por 24 horas agitando hasta obtener la completa disolución del poloxámero, obteniéndose el producto final.

9. Un método para obtener la formulación de tilmicosina de acción prolongada mejorada de conformidad con la reivindicación 8, caracterizado además porque la cantidad de agua destilada transferida es de 53ml.

10. Un método para obtener la formulación de tilmicosina de acción prolongada mejorada de conformidad con la reivindicación 8, caracterizado además porque el primer co-solvente se añade en un volumen de 10ml.

11. Un método para obtener la formulación de tilmicosina de acción prolongada mejorada de conformidad con la reivindicación 10, caracterizado además porque el primer co-solvente es propilenglicol.

12. Un método para obtener la formulación de tilmicosina de acción prolongada mejorada de conformidad con la reivindicación 8, caracterizado además porque el segundo co-solvente se añade en un volumen de 10ml.

13. Un método para obtener la formulación de tilmicosina de acción prolongada mejorada de conformidad con la reivindicación 12, caracterizado además porque el segundo co-solvente es alcohol etílico.

14. Un método para obtener la formulación de tilmicosina de acción prolongada mejorada de conformidad con la reivindicación 8, caracterizado además porque el fosfato de tilmicosina se agrega en una cantidad de 42g.

15. Un método para obtener la formulación de tilmicosina de acción prolongada mejorada de conformidad con la reivindicación 8, caracterizado además porque el emulsificante se agrega en una cantidad de 3g.

16. Un método para obtener la formulación de tilmicosina de acción prolongada mejorada de conformidad con la reivindicación 15, caracterizado además porque el emulsificante es poloxámero.

Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Pharmacokinetics of a New Parenteral Formulation of Tilmicosin-La in Cows

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ARTICLE HISTORY (15-302)

Received: June 17, 2015
Revised: November 02, 2015
Accepted: December 10, 2015
Published online: February 06, 2016

Key words:
Cow
Long-acting
New-preparation
Pharmacokinetics
Tilmicosin

A B S T R A C T

The blood serum pharmacokinetics of a Poloxamer-407 based new pharmaceutical preparation of 39% tilmicosin was determined in cows. Two dose levels injected SC, were assessed: 10 mg/kg (Til-LA_{10}) and 26 mg/kg (Til-LA_{26}). Tilmicosin was determined using HPLC, also electrocardiographic (EKG) monitoring in all animals before and at 1, 2 and 4 h after the injection of the drug was performed to measure key EKG parameters, including heart rate. Maximum serum concentration values were 2.6 µg/mL and 1.23 µg/mL, occurring 4.9 and 5.1 h after the injection of Til-LA_{26} and Til-LA_{10}, respectively. Mean residence time was statistically larger for Til-LA_{26} (50.4±5.8 h) than Til-LA_{10} (37.4±4.7 h) ($P<0.05$), with $T½_{el}$ of 39.8 h for Til-LA_{26} and 33.2 h for Til-LA_{10}. There were no differences in relative bioavailability as adjusted for dose (AUMC of 8663 µg/mLžh^2 for Til-LA_{26} and 2858 µg/mLžh^2 for Til-LA_{10}). This new formulation as dosed for Til-LA_{26} possesses a long $T½_{el}$ of tilmicosin and resulted in a lack of changes in the EKG and heart rate. Based on PK/PD ratios tilmicosin is regarded as a time-dependent antibacterial drug. Considering a theoretical minimum therapeutic serum concentration (MTC) of 0.1 µg/mL useful concentrations can be achieved for up to 192 h with Til-LA_{26} and an AUC/MTC ratio of 1514 without cardiac toxicity. Further studies are necessary to correlate PK/PD parameters obtained with clinical efficacy and a more thorough analysis of cardiac toxicity is required to determine the suitability of this preparation in bovine medicine.

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To Cite This Article: Gutiérrez L, Soriano R, Martínez-Cortes I, Miranda-Calderon J and Sumano H, 2016. Pharmacokinetics of a new parenteral formulation of Tilmicosin-La in cows. Pak Vet J, 36(2): 165-168.

INTRODUCTION

Tilmicosin, a semi-synthetic macrolide antibacterial drug shows adequate pharmacokinetic features to treat respiratory bacterial diseases of bovines and has also been used for parenteral dry-cow therapy (Dingwell *et al*., 2002). Tilmicosin has outstanding tissue diffusion to respiratory tissues and mammary gland (Scott 1995; Brumbaugh *et al*., 2002; Lombardi *et al*., 2011; Soliman and Ayad, 2014). Due to its large apparent volume of distribution, useful serum concentrations have been considered to be at or above 0.1 µg/mL (Ramadan, 1997). Reference pharmaceutical preparation (Micotil®, Elanco Animal Health, Mexico) has a half-life of approximately 30 h (Ziv *et al*., 1995; Lombardi *et al*., 2011). These features explain the resolution of a reasonable amount of respiratory infections with a single injection of 10 mg/kg (Scott, 1995; Soliman and Ayad, 2014). Also, tilmicosin has been appointed as treatment for *Staphyloccus* spp. induced mastitis during the dry-cow period (Owens *et al*., 1999). Nevertheless, a certain number of cases need a second dose to achieve bacteriological and clinical cure. To minimize handling of animals, it is here postulated that the pharmacokinetics/pharmacodynamic (PK/PD) ratios of the drug, as a time-dependent antibacterial agent (Ziv *et al*., 1995; Lombardi *et al*., 2011), can be optimized by further extending its mean residence time. Such a manoeuvre will also take advantage of the anti-inflammatory effects of tilmicosin (Buret, 2010; Munic *et al*., 2011), and of its ability to stimulate phagocytosis (Brumbaugh *et al*., 2002; Shinkai, 2008; Buret, 2010). Tilmicosin, as prepared in Micotil®, is based on propylene glycol to achieve useful serum concentrations for 48 to 72 h, setting minimum therapeutic concentrations (MTC) at 0.1 µg/mL (Ziv *et al*., 1995; Ramadan, 1997). The experimental preparation of

tilmicosin here proposed contains the hydrogel Poloxamer 407, a vehicle that allows a long acting effect of some drugs (Brayden, 2000; Sun *et al*., 2004). Poloxamer-407 has thermosensitive characteristics that produce hydrogel-based formulations with flow at low room temperature and will form a gel-like matrix with minimum inflammation at body temperature (Hoffman, 2002). Hence, this paper describes the pharmacokinetics (PK) of a new preparation of tilmicosin injected subcutaneously at two dose levels: 10 mg/kg (Til-LA_{10}), and 26 mg/kg (Til-LA_{26}).

MATERIALS AND METHODS

Study design and animal handling complied with Mexican prescripts (NOM-062-ZOO-2001). This trial was implemented at a dairy farm under intensive production with Holstein/Friesian cows. Twenty, two to five years old, treatment-free cows, with a mean weight of 545±12 kg and entering the dry-cow period were randomly divided in two groups of 10 cows each: group Til-LA_{10}, treated with a single SC injection of tilmicosin (10 mg/kg; 1 mL/39 kg of body weight) utilizing the experimental preparation of 39% tilmicosin phosphate (Patent 212148; *Instituto Mexicano de la Protección Industrial*, Mexico City); and group Til-LA_{26} cows injected with the same preparation but at a dose of 26 mg/kg (1 mL/15 kg of body weight). No more than 10 mL/injection site were administered at either side of the neck region with 16 gauge needles, 3 cm long. Injection was directed downwards, while pulling the skin; to allow the formation of a cavity where the drug preparation was deposited. Intravenous injection of tilmicosin is not recommended due to acute cardiac toxicity.

Blood samples were obtained by direct jugular puncture with Vacutainer tubes before inyection and at 0, 0.5, 1, 2, 6, 8, 10, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 180, 192, 204 h post-injection. Serum was obtained by centrifugation (3000 *g* for 15 min), samples labelled and stored at -20°C until analyzed. Tilmicosin was determined through high-performance liquid chromatography (HPLC), as reported by Parker and Patel (1994). Based on Akaike's criterion (Olofsen and Dahan, 2014) and with computerized curve-stripping program (PKAnalyst®; Micromath Scientific Software, SLM, USA) best fitting was obtained with a two compartment model with first–order input and first–order output (Model 13, r≥0.98), whose formula is:

$$\text{Concentration (Time)} = Ae^{-\alpha \cdot \text{Time}} + Be^{-\beta \cdot \text{Time}} + Ce^{-KAB \cdot \text{Time}}$$

Pharmacokinetic variables obtained were: $T½_{el}$=elimination half-life; $T½_{ab}$=absorption half-life; C_{MAX}=maximum serum concentration; T_{MAX}=time to reach C_{MAX}; AUC=area under the concentration-time curve; AUMC=area under the first moment of the concentration-time curve; MRT=mean residence time; Fr=relative bioavailability of Til-LA_{10} *vs* Til-LA_{26}, corrected for dose [Fr=($AUC_{Til\text{-}LA26}$ · Til-LA_{10} dose) ÷ ($AUC_{Til\text{-}LA10}$ · Til-LA_{26} dose) × 100]. Also the following PK/PD ratios were obtained: AUC/MTC (MTC=minimum arbitrary therapeutic concentrations so far accepted i.e., 0.1 µg/mL), and T>MTC. Descriptive statistical data for these parameters was calculated using SPSS 11.0 (SPSS Inc., Chicago, IL). Significant differences (P<0.05) between Til-LA_{10} and Til-LA_{26} parameters was assessed with Wilcoxon rank sum test and Student's *t*-test.

Possible cardio-toxicity of tilmicosin was studied with electrocardiography (ECG), obtained with an Edan iM8B Vet AG30 apparatus (EDAN Instruments, Inc., Nanshan - Shenzhen, China). Cows were restrained and placed on a thick plastic sheet. Needle electrodes were placed in forelegs in the anterolateral skin fold immediately above the olecranon process and in the skin fold at knee level in rear legs to obtain recordings at DI, DII and DIII derivations. Parameters studied were: heartbeat rate and cardiac rhythm as well as amplitude, morphology and duration of P and T waves, QRS complex, duration of P-R, Q-T and R-R intervals, the S-T segment and the electrical axis of the heart, as described by Macfarlane *et al*. (2010). Tracings were obtained before the injection of either tilmicosin preparation and 1, 2 and 4 h after. Significant differences were studied before and after injection of tilmicosin and differences analyzed through variance and Student's *t*-tests.

After 30 days from injection, all animals were slaughtered as stipulated by Mexican regulations (NOM-0033-ZOO-1995), injection sites dissected, macro-scopically inspected for lesions and processed for routine histopathology.

RESULTS

Mean±SD serum concentrations *vs* time profiles of Til-LA_{10} and Til-LA_{26} after a single SC injection of either 10 or 26 mg/kg, respectively, are presented in Fig. 1. Table 1 shows mean±SD values of the pharmacokinetic parameters obtained, as well as their statistical comparison.

Maximum serum concentration (C_{MAX}) was 1.23±0.09 µg/mL for group Til-LA_{10} and 2.6±0.13 µg/mL for group Til-LA_{26}. Terminal phase half-lives ($T½_{el}$) were 33.2±5.1 h and 39.8±4.5 h, respectively. Mean residence time was slightly larger for Til-$LA_{26,}$ as compared to Til-LA_{10} (50.4±5.8 h *vs* 37.4±4.7 h) (P>0.05). Relative bioavailability (Fr) of the Til-LA_{26} group, as compared to Til-LA_{10}, corrected for dose level, was negligible (98% for Til-LA_{26}). Absorption half-life ($T_{½ab}$) was also very similar between groups (0.90 h vs 0.91 h for Til-LA_{10} and Til-LA_{26}, respectively). However C_{MAX} was higher in the Til-LA_{26} as compared to Til-LA_{10} (2.6 µg/mL vs 1.23 µg/mL); yet, T_{MAX} was not statistically different.

All cows from both groups remained clinically healthy throughout the study and no signs of visible pain were detected. However, a clearly distinctive bulging response in the injection sites was observed in both groups. This bulge was more an occupying mass than an inflammation. It disappeared completely after 72 h in both groups. Histopathological analysis of injection sites showed neither macroscopic signs of inflammation nor of scar tissue.

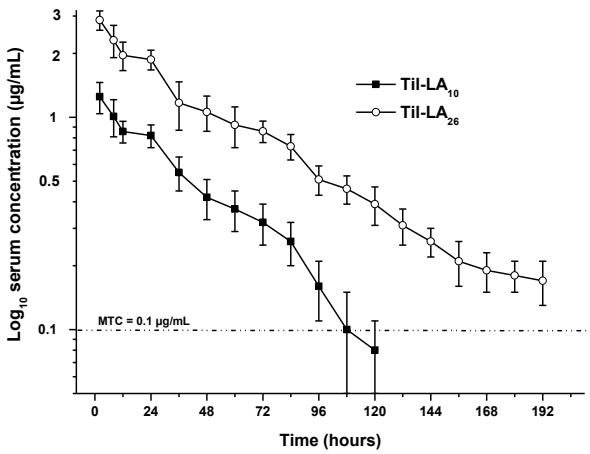
All ECG tracings were noticeably flat as expected for this species and basal values showed lack of stress as these animals were accustomed to daily handling. Morphologies of the P wave, QRS complex, and T wave, revealed no apparent changes induced by either preparation of tilmicosin. P waves were, either positive or negative with some isoelectrical tracings. Also, duration of P and T waves, of the QRS complex, and of the Q-T, P-R, and R-R intervals showed no differences between

groups, neither before nor after the injection of either preparation of tilmicosin. Also, as expected, the S-T segment was particularly long, with T waves showing variable morphology. Yet such morphologies were unrelated to injection of either Til-LA_{10} or Til-LA_{26}. Similarly rS configuration did not show significant differences neither before nor after injection of tilmicosin. Basal cardiac rates in the Til-LA_{10} and the Til-LA_{26} groups were 87.6±6.5 and 86.8±5.5, respectively and remained almost unchanged after the three posterior ECG readings.

DISCUSSION

Microbiological determination of tilmicosin concentrations in biological samples reveals only 20-25% of the actual concentration of the drug (Stobba-Wiley *et al*., 2000). Hence, HPLC analysis was chosen as analytical technique and it can be safely assume that the fraction detected is the relevant analyte to assess biological activity (Lombardi *et al*., 2011) and can be safely utilized to calculate the pharmacokinetic behaviour of the active fraction of the drug.

Cardiotoxicity of tilmicosin precluded the determination of PK of the drug after IV administration. Consequently, neither absolute bioavailability nor apparent volume of distribution values were determined. Also, the tissue irritation capability of this macrolide drug impeded PK studies after IM injection. This study compares the PK of two dose levels of an experimental preparation of tilmicosin. Setting MTC at 0.1 µg/ml, based on data derived from other studies (Ziv *et al*., 1995; Ramadan, 1997) and considering that tilmicosin is distributed outside the central compartment at least 10-20 times (Helton-Groce *et al*., 1993; Modric *et al.,* 1998; Soliman and Ayad, 2014), the time period with serum concentrations of tilmicosin at or above a therapeutic level, was statistically different between Til-LA_{26} (192 h) and Til-LA_{10} (120 h) ($P<0.001$). Also, AUC/MTC values were higher in the Til-LA_{26} group, as compared to the Til-LA_{10} group (1514 and 592) ($P<0.01$). Figure 1 shows this time-extension of useful serum concentrations of tilmicosin. In contrast, Lombardi *et al*. (2011) demonstrated that when the dose of a standard preparation of tilmicosin was increased from 10 to 20 mg/kg in light or heavy beef calves, proportional increase in both AUC and C_{MAX} were obtained. However, the MTC (0.1 µg/mL) did not surpassed 72 h with either dose. It is likely to assume that Poloxamer-407 in the experimental preparation increases time above MTC, allowing an initial priming absorption lacking cardiac toxicity. Correspondingly MRT for Til-LA_{26} was 50.4±5.8 h and 37.4±4.7 h for Til-LA_{10}; and $T½_{el}$ values were roundly 39.8 h *vs* 33.2 h for Til-LA_{26} and Til-LA_{10}, respectively. These values are both higher than the ones obtained in former studies using the standard preparation of tilmicosin ($T½_{el}$=30 h) (Modric *et al.,* 1998). Poloxamer-407 improves solubility of some drugs, reduces their hydrolytic degradation and provokes their controlled release, often resulting in improved bioavailability (Vargas *et al*., 2008). Poloxamer-407 as thermo-reversible vehicle becomes gel at body temperature (37-40ºC), entrapping tilmicosin which is then release in a sustained manner while allowing an initial priming absorption.



MTC = minimum accepted serum therapeutic concentration.
Fig. 1: Mean±SD plasma concentrations *vs* time profiles of tilmicosin in Holstein/Friesian cows after the SC injection of an experimental, poloxamer 407-based preparation of tilmicosin (390 mg/mL), intended for extended long action, at two dose levels 10 mg/kg (Til-LA_{10}) and 26 mg/kg (Til-LA_{26}).

Table 1: Pharmacokinetic values and PK/PD ratios of tilmicosin in Holstein/Friesian cows from a new long-acting preparation of tilmicosin administered, subcutaneously, at two doses: 10 mg/kg (Til-LA_{10}) or 26 mg/kg (Til-LA_{26})

Pharmacokinetic variable	Til-LA_{10}	Til-LA_{26}
$T½_{el}$ (h)	33.2±5.1a	39.8±4.5b
$T½_{ab}$ (h)	0.90±0.05a	0.91±0.04a
C_{MAX} (µg/mL)	1.23±0.09a	2.6±0.13b
T_{MAX} (h)	5.1±0.4a	4.9±0.4a
AUC (µg/mL·h)	59.2±3.8a	151.4±8.4b
AUMC (µg/mL·h^2)	2858±17.4a	8663±21.1b
MRT (h)	37.4±4.7a	50.4±5.8b
Fr %	-	98
AUC/MTC	592a	1514b
T>/MTC (h)	120a	192b

Values (Mean±SD) with different letter in a row differ significant ($P<0.05$). n=10 cows/group; $T½_{el}$=elimination half-life; $T½_{ab}$=absorption half-life; C_{MAX}=maximum serum concentration; T_{MAX}=time to reach C_{MAX}; AUC=area under the concentration-time curve; AUMC=area under the first moment of the concentration-time curve; MRT=mean residence time; Fr=relative bioavailability of Til-LA_r *vs* Til-LA_{exp}, corrected for dose [Fr=($AUC_{Til\text{-}LA26}$ · Til-LA_{10} dose) ÷ ($AUC_{Til\text{-}LA10}$ · Til-LA_{26} dose) × 100]. AUC/MTC (MTC=minimum accepted therapeutic serum concentration, taken in this study as 0.1 µg/ml) and T>MTC=PK/PD ratios.

Presently, the time mark of 192 h to reach MTC merits further work by examining the concentrations of the drug in lungs and/or in mammary tissue and milk. Notwithstanding the above, advantages of injecting Til-LA_{10} or Til-LA_{26} will include a reduction in work-load if long treatment schemes are required. Also potentially improved clinical outcome can be expected based on AUC/MTC ratios obtained (592 and 1514 for Til-LA_{10} and Til-LA_{26}, respectively), a key feature required for macrolide antibacterial drugs (Burch, 2010). This requires confirmation under various clinical settings *i.e*., parenteral dry-cow therapy.

Tilmicosin is extensively distributed outside the central compartment into respiratory tissues (Soliman *et al*., 2014), mammary gland (Helton-Groce., 1993) and even at the intracellular level (Brumbaugh *et al*., 2002), and for longer time, as compared to serum concentrations (Ibrahim, 2011). Consequently, a low MTC has been accepted (0.1 µg/mL) (Ziv *et al*., 1995; Ramadan, 1997; Ibrihim, 2011). Tissue concentrations of tilmicosin were

not determined in this trial. Yet, it is tempting to speculate that clinical efficacy for respiratory and mammary infections could be improved with Til-LA_{26} due to its long residence time and assuming a similar behaviour of tilmicosin once it is released from Poloxamer-407. Nevertheless, tissue concentrations of tilmicosin as well as efficacy studies are warranted.

All cows from both groups remained clinically healthy throughout the study without signs of visible pain, even in Til-LA_{26} cows. Electrocardiographic findings and cardiac rates were very much in agreement with set standards (Macfarlane *et al*., 2010) and were not different when comparing either, both groups or data before and after administration of either dose of tilmicosin. Tracings obtained tended to be flatter compared to classical ECG ones in other species. This phenomenon has been attributed to the diffuse organization of Purkinje fibers in the bovine myocardium. Yet, the ECG in cows can be useful to differentiate arrhythmias; being sinus arrhythmias, the most common dysrhythmias observed when tilmicosin is overdosed (Macfarlane, 2010). However in the 20 cows studied, no sinus arrhythmias was detected. Mean frontal plane electrical axis of the QRS complex varied from 79° to 275°, and did not change substantially after the injection of either preparation of tilmicosin. Although these results are encouraging for low cardiac toxicity of the experimental formulation tested, further studies also measuring blood creatine phosphokinase are necessary. Also, it is mandatory to determine safety of this pharmaceutical design in all types of cattle i.e., debilitated animals as well as, drug residues of the drug and metabolites before this preparation can be regarded as potentially useful in bovine medicine.

Acknowledgements: We are grateful for the financial support received from DGAPA-UNAM Program of Post-Doctoral Scholarships in the UNAM and from PROINNOVA/CONACYT in Mexico.

Author's contribution: LG and HS conceived and designed the study. IM-C and RS executed the experiment and JM-C analyzed the sera samples.

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Concentrations of tilmicosin in mammary gland secretions of dairy cows following subcutaneous administration of one or two doses of an experimental preparation of tilmicosin and its efficacy against intramammary infections caused by *Staphylococcus aureus*

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Received July 17, 2015.
Accepted December 9, 2015.

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OBJECTIVE
To determine the concentration of tilmicosin in mammary gland secretions of dairy cows following administration of an experimental preparation once or twice during the dry period (45-day period immediately prior to calving during which cows are not milked) and to evaluate its efficacy for the treatment of cows with intramammary infections (IMIs) caused by *Staphylococcus aureus* at dry off (cessation of milking; first day of dry period), compared with that of an intramammary infusion of ceftiofur.

ANIMALS
172 cows.

PROCEDURES
Milk samples were collected for microbiological culture 5 days before dry off and at calving and 15 and 30 days after calving. Cows with *Staphylococcus* IMIs were randomly assigned to receive an experimental preparation of tilmicosin (20 mg/kg, SC) once at dry off (n = 58) or at dry off and again 20 days later (56) or receive a long-acting intramammary preparation of ceftiofur (500 mg/mammary gland; 56) at dry off. Mammary gland secretions were collected from 5 cows in the tilmicosin-treated groups every 5 days after dry off until calving for determination of tilmicosin concentration.

RESULTS
Mean maximum concentration of tilmicosin in mammary gland secretions ranged from 14.4 to 20.9 µg/mL after the first dose and was 17.1 µg/mL after the second dose. The bacteriologic cure rate was 100% for all 3 treatments. Tilmicosin was detectable for 0 and 18 days after calving in the milk of cows treated with 1 and 2 doses of tilmicosin, respectively.

CONCLUSIONS AND CLINICAL RELEVANCE
Administration of an experimental preparation of tilmicosin (20 mg/kg, SC) once to dairy cows at dry off might be useful for the treatment of *S aureus* IMIs. (*Am J Vet Res* 2016;77:922–930)

In dairy cattle, the purpose of dry cow treatment is to treat and prevent IMIs during the 45- to 60–day period immediately prior to calving in which cows are not milked (dry period).[1–3] Dry cow treatment minimizes the number of clinical cases of mastitis during the subsequent lactation.[4–6] Thus, the dry period is considered a key intervention point for the prevention of future cases of mastitis and an opportunity to lessen the economic impact of mastitis on dairy farms.[7,8] Although it is not without controversy, the most accepted method for the prevention of IMIs in dairy cows during the dry period is the administration of antimicrobials, generally by the intramammary route,[3,9] and most commercially available dry-cow treatments contain an antimicrobial. Anecdotal reports suggest that up to 71% to 85% of IMIs present at the time of dry cow treatment (first day of dry period; dry off) will be resolved; however, results of a meta-analysis[1] indicate that 37% to 56% of IMIs present at dry off will resolve spontaneously without antimi-

ABBREVIATIONS

AUC	Area under the mammary gland secretion concentration–time curve
C_{max}	Maximum concentration
CMT	California mastitis test
HPLC	High-performance liquid chromatography
IMI	Intramammary infection
MRT	Mean residence time
SCC	Somatic cell count
$t_{1/2\beta}$	Elimination half-life
t_{max}	Time to maximum concentration

crobial administration. The cure rate for IMIs caused by *Staphylococcus aureus* following antimicrobial administration is much lower than that for IMIs caused by other bacterial pathogens and ranges from 27% to 80%.[10] The breadth of that range is understandable given the various antimicrobials administered and treatment protocols used and the varying health status of treated cows. Factors that affect the cure rate for IMIs include, but are not limited to, the age of the cow being treated, whether the fore or rear mammary glands (quarters) are infected, overall size of the mammary gland, duration of the current infection, and the IMI history and immune status of the cow being treated.[11,12] Intramammary infections caused by *S aureus* are particularly difficult to treat because the pathogen often becomes sequestered within neutrophils, macrophages, and epithelial cells where it can replicate and remain viable even in the face of antimicrobial administration.[11,13]

Tilmicosin, a macrolide, has been used as an intramammary infusion (1,500 mg/quarter) to treat cows with IMIs caused by *S aureus* at dry off, and reported cure rates range from 62% to 74.2%.[10,14] Conversely, parenteral administration of tilmicosin (5 mg/kg, SC) to cows with IMIs caused by *S aureus* at dry off and again 4 days later resulted in a cure rate of only 9.1%.[10]

A new experimental polymer-based 40% tilmicosin preparation has been described.[15] This preparation releases tilmicosin from the thermoreversible poloxamer 407 with zero-order kinetics, which results in a mean $t_{1/2\beta}$ of 39.8 hours.[15] In cows, clinically relevant serum concentrations of tilmicosin are achieved for 8 to 10 days following administration of 1 dose (20 mg/kg, SC). The constant release of tilmicosin from the poloxamer 407 gel formed in situ prevents the buildup of toxic concentrations of the drug in the serum and prevents adverse changes in the heart rate and other signs of cardiac toxicosis. The pharmacokinetics of this experimental preparation of tilmicosin, in addition to the antimicrobial spectrum of tilmicosin (particularly against *Staphylococcus* spp),[16] its immunomodulating capabilities,[17] and its ability to readily diffuse into the bovine mammary gland,[18] suggests that parenteral administration of this experimental preparation to dairy cows at dry off might be a beneficial dry cow treatment. The purpose of the study reported here was to determine the concentration of tilmicosin in mammary gland secretions of dairy cows following administration of this experimental preparation of tilmicosin (20 mg/kg, SC) once or twice during the dry period and to evaluate its efficacy for the treatment of cows with IMIs caused by *S aureus* at dry off, compared with that of an intramammary infusion of ceftiofur.

Materials and Methods

Animals

All study procedures and animal care activities were conducted in accordance with Mexican Official Regulations[19] under the oversight of the Institutional Committee of Research, Care and Use of Experimental Animals of the National Autonomous University of Mexico. The study was conducted on a commercial dairy farm located in the State of Mexico from January through November 2015. The herd consisted of 1,500 Holstein-Friesian cows with a mean milk production of 23 kg/cow/d. To be eligible for the study, cows had to be clinically normal as determined on the basis of results of a physical examination, have 4 functioning quarters with teats free of substantial lesions (eg, cuts and deformities), have an IMI caused by *Staphylococcus* spp on the basis of results of microbiological culture of milk samples obtained 5 days prior to dry off, and not have been treated with an antimicrobial during the 30 days prior to dry off. During the 11-month observation period, 360 cows were screened for the study, of which 170 had an IMI caused by *Staphylococcus* spp and were enrolled in the study at dry off, which was approximately 45 days prior to the expected calving date.

Study design

Cows were divided into 3 groups on the basis of the pathogen causing the IMI (*S aureus*, *Staphylococcus intermedius*, or coagulase-negative staphylococci). Within each group, cows were randomly assigned by means of a random number generator to 1 of 3 treatment groups. Cows assigned to the tilmicosin-1 group (n = 58) received the experimental preparation of tilmicosin[a] (20 mg/kg, SC) once at dry off. Cows assigned to the tilmicosin-2 group (n = 56) received the experimental preparation of tilmicosin[a] (20 mg/kg, SC) at dry off and again 20 days later. Cows assigned to the ceftiofur group (n = 56; control) received a commercially available, long-acting, intramammary preparation of ceftiofur[b] (500 mg/quarter) at dry off. Three 20-mL milk (or colostrum) samples (ie, samples that contained an approximately equal amount of milk from each of the 4 quarters) were obtained for microbiological culture from each cow 5 days prior to dry off, within 24 hours after calving, and 15 and 30 days after calving. Additionally, a sample (5 to 10 mL) of mammary gland secretions was obtained for determination of tilmicosin concentration from each quarter of each of 5 randomly selected cows from each treatment group once every 5 days after dry off until calving.

Microbiological culture and determination of SCC

Milk or colostrum samples for microbiological culture and determination of SCC were collected aseptically as described by the NMC.[20] Briefly, each teat was disinfected with chlorhexidine solution. Then, 20 to 30 mL of milk was stripped and discarded from each teat before approximately 8 mL of milk from each teat was stripped into each well of a CMT paddle for performance of the CMT. Each teat was disinfected with chlorhexidine again, and three 20-mL

milk samples were collected into sterile 50-mL sample collection tubes. Milk samples were immediately submitted for microbiological culture. Each sample was stirred with a vortex for 5 minutes to achieve a homogenous sample. Then, 15 to 20 µL of the sample was streaked onto a blood agar culture plate and MacConkey agar culture plate with the quadrant streak method. The plates were incubated in aerobic conditions at 37°C for 48 hours.[21] Intramammary pathogens were distinguished from contamination or microbiota by the presence of at least 3 grossly similar colonies in each of at least 3 of the 4 quadrants of the culture plate as described by Carter.[22] Bacterial colonies were initially categorized on the basis of their morphological features, gram stain result, catalase reaction result, and hemolytic ability. Gram-negative bacilli underwent an oxidase test, and then further species identification was performed as described[22] by the conduction of the triple sugar iron test and the citrate-urea-sulfide-indole motility test. Gram-positive cocci suspected of being *S aureus* were subjected to coagulase, CAMP, and esculin tests. Samples with > 3 bacterial species identified were considered contaminated and were excluded from further analyses. An electronic cell counter[c] was used to determine the SCC in each milk sample. A CMT test was not performed on cows within 24 hours after calving because colostrum causes false-positive results. Likewise, the SCC was not determined for colostrum samples collected within 24 hours after calving.

An IMI at dry off was defined as the isolation of ≥ 3 CFUs of a *Staphylococcus* spp/15 to 20 µL of inoculum (milk) in at least 2 of the 3 samples cultured. An IMI was considered cured when a previously identified pathogen was not cultured in any of the samples collected on the subsequent 3 sampling days (within 24 hours after calving and 15 and 30 days after calving). A new IMI was defined as the isolation of a *Staphylococcus* spp from a gland that had previously cultured negative for that organism.

Dry cow treatment administration

At dry off, the cows assigned to the tilmicosin-1 and tilmicosin-2 groups received a polymer-based preparation of 40% tilmicosin phosphate designed for extended action[a] (experimental preparation; 20 mg/kg, SC). The drug was administered in the lateral aspect of the neck just cranial to the shoulder. The volume of the required dose for each cow ranged between 20 and 30 mL; therefore, each dose was divided into 3 approximately equal volumes and was injected by use of a 10-mL syringe with a 16-gauge 3-cm needle. No more than 10 mL of the drug was administered per injection site. The cows assigned to the tilmicosin-2 group received a second dose of the experimental preparation 20 days later. The cows assigned to the ceftiofur group received an intramammary infusion of ceftiofur hydrochloride sterile suspension[b] (500 mg/quarter) at dry off. Each quarter was infused in an aseptic manner after the cow was milked. Briefly, the entire udder was washed with warm water that contained a chlorhexidine-based antiseptic,[d] with particular attention paid to cleansing each teat. Each teat was dried with a paper towel. Then a separate alcohol pad was used to wipe the end of each teat immediately before the ceftiofur suspension was infused into the streak canal. Aside from dry cow treatment administration and collection of mammary gland secretion samples for determination of tilmicosin concentration, all study cows were managed in accordance with the herd's standard husbandry practices for cows during the dry period, which included isolation of cows immediately after dry off for daily observation to detect mastitis or other diseases and a diet change.

Determination of tilmicosin concentration

Mammary gland secretions (5 to 10 mL) were aseptically collected from each quarter from each of 5 randomly selected cows from each treatment group every 5 days after dry off until calving. No cow was sampled more than once during the dry period. Mammary gland secretions (5 to 10 mL) were collected from each quarter of all study cows 1 day after calving. Each teat was sealed with a barrier teat sealer[e] after each sample collection.

Tilmicosin concentration in mammary gland secretions was determined by use of HPLC as described.[23] All solvents used were HPLC grade; tilmicosin phosphate[f] and tylosin tartarate[f] were used as internal standards. An HPLC system[g] equipped with a quaternary pump,[g] 3.5µM-C18 column (4.6 X 100 mm),[h] C18 precolumn (4.6 X 20 mm),[h] and UV and diode-array detectors[g] was used, and analysis was performed with proprietary software.[i] Tilmicosin was extracted from samples on the basis of its chemical characteristics. During the initial liquid-liquid extraction, tilmicosin remained in a weakly acidic aqueous solution, and matrix impurities were removed in a nonpolar organic phase. A tissue blank and a recovery tube were included for the analysis of each test sample. Three milliliters of each mammary gland secretion sample was placed in a 50-mL polypropylene centrifuge tube and homogenized. Then, 10 to 30 mL of methanol was added to the tube, and the contents were stirred for 5 minutes. Samples were centrifuged for 20 minutes at 3,000 X *g*, and the supernatant was decanted into a 250-mL beaker. An additional 30 mL of methanol was added to the centrifuge tube, and the contents were stirred with a stirring rod for 5 minutes to resuspend the pellet in the methanol. The tube was centrifuged again for 10 minutes at 3,000 X *g*, and the resulting supernatant was combined with that in the beaker. Thirty milliliters of a 10% sodium chloride solution was added to the beaker. The pH of the beaker contents was adjusted as necessary by the use of 1N hydrochloric acid in a drop-wise manner until a pH of 4 was achieved. Fifty milliliters of

Table 1—Descriptive data for 170 Holstein-Friesian cows that were treated with 1 (tilmicosin-1 group; n = 58) or 2 (tilmicosin-2 group; 56) doses (20 mg/kg, SC) of an experimental preparation of tilmicosin or were treated with a long-acting intramammary preparation of ceftiofur (500 mg/mammary gland; ceftiofur group; 56).

				Lactation			
Treatment group	**Age (y)**	**No. of days since last calving**	**Milk production for current lactation (kg)**	**Mean**	**No.**	**No. (%) of cows**	**No. of previous IMIs***
Tilmicosin-1	4.86 ± 1.43	285 ± 20	7,284 ± 1,333	2.64 ± 1.17	1	10 (17)	2.2 ± 0.45
					2	18 (31)	2.8 ± 0.67
					3	16 (28)	3.1 ± 0.64
					4	10 (17)	3.6 ± 0.89
					5	4 (7)	3.7 ± 0.95
Tilmicosin-2	5.07 ± 1.44	290 ± 19	6,916 ± 1,006	2.75 ± 1.26	1	8 (14)	2.5 ± 0.58
					2	20 (36)	2.7 ± 0.67
					3	14 (25)	3 ± 0.82
					4	6 (11)	3.3 ± 0.58
					5	8 (14)	3.8 ± 0.96
Ceftiofur	4.40 ± 1.17	293 ± 16	6,791 ± 1,205	2.25 ± 1.04	1	14 (25)	2.3 ± 0.49
					2	22 (39)	2.8 ± 0.75
					3	14 (25)	3 ± 0.82
					4	4 (7)	3.5 ± 0.71
					5	2 (4)	3 ± 0.5

Values represent the mean ± SD unless otherwise indicated. Cows in both the tilmicosin-1 and tilmicosin-2 groups received 1 dose of tilmicosin at dry off (ie, day that the cows were administered dry cow treatment, and the first day of the dry period [45-day period immediately before calving during which cows are not milked]), and cows in the tilmicosin-2 group received a second dose of tilmicosin 20 days later. Cows in the ceftiofur group were treated once at dry off.

*Intramammary infections caused by *Staphylococcus* spp during the current lactation.

hexane was added to the aqueous extract, which was then stirred for 30 seconds with regular venting; the partitioned hexane layer was discarded. An additional 25 mL of hexane was added to the extract, the partitioning process was repeated, and the partitioned hexane layer was again discarded. The pH of the aqueous layer was adjusted to 9.0 with 0.5M sodium carbonate solution. Then, 20 mL of a 2:1 chloroform-hexane solution was added to the beaker; the sample was stirred and phases were allowed to separate. The chloroform-hexane layer was decanted, and the procedure was repeated. The organic phase was then evaporated to dryness, and the residue was resuspended in 5 mL of methanol and 5 mL of 0.05M dibutylammonium phosphate. The sample was sonicated for 1 minute to ensure complete dissolution. The sample was passed through solid-phase extraction, then through a 0.22-µm filter[j] for analysis.

For the HPLC procedure, 50 µL of the extracted and filtered sample was injected into the system at a flow rate of 1.5 mL/min. Separation was achieved by use of a triple gradient analysis with 50% acetonitrile in water (pH, 2.5), water (pH, 2.5), and 0.08M dibutylammonium phosphate. The run time was 30 minutes with a post time of 10 minutes. Tilmicosin was detected at a wavelength of 280 nm. The limit of detection was 0.015 µg/mL, and the limit of quantification was 0.045 µg/mL. Robustness and tolerance tests indicated that the assay had an absolute difference of 1.54 (< 2.0) and a coefficient of variance of 1.2% (< 2.0%).

Data for tilmicosin concentration in mammary gland secretions were based on individual concentration-versus-time curves and were processed by means of specialized software,[k] which generated results for the following parameters: C_{max}, t_{max}, $t_{1/2\beta}$, AUC, and MRT. Model 13 (r = 0.925) was used with this general formula: concentration (time) = $Ae^{-a(time)} + Be^{-b(time)} + Ce^{-KAB(time)}$.

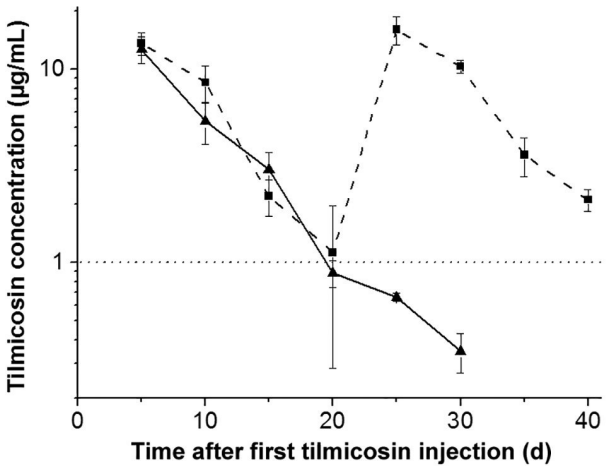

Figure 1—Mean ± SD concentrations of tilmicosin over time in mammary gland secretions obtained from clinically normal Holstein-Friesian cows with subclinical IMIs caused by *Staphylococcus* spp that were treated with an experimental preparation of tilmicosin (20 mg/kg, SC) once at dry off (tilmicosin-1 group; solid line; n = 58) or at dry off and again 20 days later (tilmicosin-2 group; dashed line; 56). The horizontal dotted line represents the minimum concentration of tilmicosin necessary to inhibit 90% of *Staphylococcus aureus* isolates (1.0 µg/mL) determined in another study.[26] Mammary gland secretion samples were collected from 5 randomly selected cows from each treatment group every 5 days after dry off until calving, and no cow was sampled more than once during the dry period. Therefore, the respective mean values represent the mean for 5 different cows at each sampling day.

Additionally, for all study cows, milk samples were analyzed for tilmicosin residues with a commercially

available qualitative test for antimicrobials[l] on a daily basis after calving until a negative result was obtained.

Statistical analysis

At each mammary gland secretion acquisition time, pharmacokinetic parameters for tilmicosin were compared among the 3 treatment groups by use of a 1-way ANOVA. Although all bacterial isolates identified following culture of milk or colostrum samples were recorded, the efficacy of tilmicosin for the treatment of IMIs caused only by *S aureus* was assessed. The efficacy was determined for each individual gland (quarter), and the cure rate for IMIs caused by *S aureus* was compared among the 3 treatment groups by use of a Kruskal-Wallis test. A mixed model was used as described[24,25] to compare the SCC among the 3 treatment groups. Fixed effects included in the model were treatment group, sample acquisition time (time), and the interaction between treatment group and time. The model also included a random effect for cow nested within treatment group to account for repeated measures within cows. The model had the following form:

$$Y_{ijkl} = \mu + \text{treatment}_i + \text{time}_j + \text{treatment} \times \text{time}_{ij} + E_{ijkl}$$

where Y_{ijkl} is the SCC for an individual cow in a particular treatment group at a particular time, μ is overall mean response for the study population, treatment_i is the fixed effect for treatment group (i = tilmicosin-1, tilmicosin-2, or ceftiofur), time_j is the fixed effect for time (j = prior to dry off and 15 and 30 days after calving), treatment X time_{ij} is the fixed effect for the interaction between treatment group and time, and E_{ijkl} is the random error term for cow nested within treatment group. All analyses were performed with a commercially available software program,[m] and values of $P < 0.05$ were considered significant.

Results

Cows

Descriptive statistics for cows within each treatment group were summarized **(Table 1)**. Review of herd records revealed that most of the cows enrolled in the study had at least 2 IMIs caused by *Staphylococcus* spp during the lactation immediately prior to dry off.

Tilmicosin concentration in mammary gland secretions

For cows in the tilmicosin-1 and tilmicosin-2 groups, the mean concentration of tilmicosin in mammary gland secretions over time **(Figure 1)** and pharmacokinetic parameters **(Table 2)** were summarized. The mean ± SD C_{max} for cows in the tilmicosin-2 group after injection of the first dose (20.9. ± 4.9 µg/mL) was greater than that after injection of the second dose (17.1. ± 3.1 µg/mL). Similarly, the mean ± SD t_{max} for cows in the tilmicosin-2 group after injection of the first dose (4.1 ± 0.7 days) was greater than that after injection of the second dose (3.9 ± 0.4 days). However, the mean ± SD $t_{1/2\beta}$ for cows in the tilmicosin-1 group (4.7 ± 2.3 days) and tilmicosin-2 group after injection of the first dose (4.9 ± 1.4 days) was less than that for cows in the tilmicosin-2 group after injection of the second dose (7.7 ± 1.6 days). For cows in the tilmicosin-2 group, the mean ± SD AUC after the second dose of tilmicosin (358.1 ± 51.4 µg/mL•d) was significantly ($P < 0.01$) greater than that after the first dose of tilmicosin (242.1 ± 70.6 µg/mL•d), and those values were in agreement with the AUC that was calculated by the trapezoidal rule. Also, the MRT after the second dose of tilmicosin (8.3 ± 0.4 days) was significantly ($P < 0.01$) greater than that after the first dose of tilmicosin (5.8 ± 0.9 days).

Results of daily qualitative testing of milk samples indicated that tilmicosin was detectable in the milk of cows in the tilmicosin-2 group for up to 18 days after calving, whereas tilmicosin was not detectable in the milk of cows in the tilmicosin-1 or ceftiofur groups after calving.

Efficacy of tilmicosin for treatment of IMIs

The numbers of cows with IMIs caused by *Staphylococcus* spp before and after administration of the

Table 2—Mean ± SD pharmacokinetic parameters for an experimental preparation of tilmicosin in mammary gland secretions of dairy cows following administration of 1 (tilmicosin-1 group; n = 58) or 2 (tilmicosin-2 group; 56) doses (20 mg/kg, SC) of the preparation.

		Timicosin-2 group	
Parameter	**Tilmicosin-1**	**First dose**	**Second dose**
C_{max} (µg/mL)	14.4 ± 2.1[a]	20.9 ± 4.9[b]	17.1 ± 3.1[a,b]
t_{max} (d)	3.0 ± 0.5[a]	4.1 ± 0.7[b]	3.9 ± 0.4[a,b]
$t_{1/2\beta}$ (d)	4.7 ± 2.3[a]	4.9 ± 1.4[a,b]	7.7 ± 1.6[b]
AUC (µg/mL•d)	177.3 ± 2.3[a]	242.1 ± 70.6[a]	358.1 ± 51.4[b]
AUC_t (µg/mL•d)	109.8 ± 16.1[a]	146.2 ± 36.1[a]	177.0 ± 28.0[b]
AUMC (µg/mL•d^2)	1,194.4 ± 274.6[a]	1,365.2 ± 194.9[a]	1,477.6 ± 194.7[a]
MRT (d)	6.8 ± 1.7[a]	5.8 ± 0.9[a]	8.3 ± 0.4[b]

AUC_t = AUC calculated by the trapezoidal rule. AUMC = Area under the first moment of the concentration-time curve.

[a,b]Within a row, values with different superscript letters differ significantly ($P < 0.05$).

See Table 1 for remainder of key.

Table 3—Number of dairy cows with an IMI caused by *Staphylococcus aureus*, *Staphylococcus epidermidis*, or coagulase-negative *Staphylococcus* spp (CNS) before and after parenteral administration of 1 (tilmicosin-1 group; n = 58) or 2 (tilmicosin-2 group; 56) doses (20 mg/kg, SC) of an experimental preparation of tilmicosin or administration of a long-acting intramammary preparation of ceftiofur (500 mg/mammary gland; 56).

	Treatment group								
	Tilmicosin-1			Tilmicosin-2			Ceftiofur		
Pathogen	Before treatment	After treatment	Cure rate (%)	Before treatment	After treatment	Cure rate (%)	Before treatment	After treatment	Cure rate (%)
S aureus	24	0	100	24	0	100	22	0	100
S epidermidis	8	0	100	7	0	100	8	0	100
CNS	26	0	100	25	0	100	26	0	100

Three 20-mL milk or colostrum samples for microbiological culture were aseptically collected from each cow 5 days before dry off, within 24 hours after calving, and 15 and 30 days after calving. An IMI before treatment was defined as the isolation of ≥ 3 CFUs of a *Staphylococcus* spp/15 to 20 µL of culture inoculum (milk) in at least 2 of the 3 samples cultured. An IMI was considered cured when a previously identified pathogen was not cultured in any of the samples collected on the subsequent 3 sampling days (within 24 hours after calving and 15 and 30 days after calving).

See Table 1 for remainder of key.

Table 4—Number of cows from Table 3 that developed a new IMI at calving and 15 and 30 days after calving.

		Pathogen		
Treatment group	Sample collection day	*S aureus*	*S epidermidis*	CNS
Tilmicosin-1	Calving	2	1	2
	15 d after calving	2	0	0
	30 d after calving	0	0	0
Tilmicosin-2	Calving	1	0	0
	15 d after calving	3	1	0
	30 d after calving	1	1	2
Ceftiofur	Calving	2	0	3
	15 d after calving	2	2	0
	30 d after calving	1	0	0

A new IMI was defined as the isolation of a *Staphylococcus* spp from a gland that had previously cultured negative for that organism.

See Table 3 for remainder of key.

Table 5—Quadratic mean ± SD SCC (cells/mL of milk) for the cows of Table 1 at dry off and 15 and 30 days after calving.

	Sample collection day		
Treatment group	Dry off	15 days after calving	30 days after calving
Tilmicosin-1	308,679 ± 33,684	209,471 ± 34,073	175,183 ± 34,292*
Tilmicosin-2	253,259 ± 39,638	170,874 ± 39,638	197,759 ± 39,638
Ceftiofur	335,906 ± 35,944	301,484 ± 35,361	210,520 ± 34,913

*Within a column, value differs significantly ($P < 0.05$) from the corresponding values for the other 2 treatment groups.

See Table 1 for remainder of key.

assigned treatment and the associated cure rates for each treatment group were summarized **(Table 3)**. The cure rate for all pathogens was 100% for all 3 treatments. The numbers of cows that developed new IMIs caused by *Staphylococcus* spp in each treatment group were likewise summarized **(Table 4)**.

The qualitative CMT test results (data not shown) were consistent with the quantitative SCCs. The quadratic mean ± SD SCC for cows in each treatment group at dry off and 15 and 30 days after calving were summarized **(Table 5)**. The mean SCC did not differ significantly among the 3 treatment groups at dry off or at 15 days after calving. At 30 days after calving, the mean SCC for the cows in the tilmicosin-1 group was significantly lower than that for cows in both the tilmicosin-2 and ceftiofur groups.

Discussion

Results of the present study indicated that SC administration of a long-acting experimental formulation of tilmicosin to cows with an IMI caused by *Staphylococcus* spp at dry off resulted in a 100% cure rate. The efficacy of a parenterally administered antimicrobial to achieve a bacteriologic cure for an IMI is dependent on its ability to penetrate the udder, diffuse into the infected mammary tissue, and remain

biologically active.[27] This is particularly important in instances when the mammary gland has undergone structural changes such as the development of fibrotic tissue or the formation of abscesses in the infected tissue, both of which are common for IMI caused by *Staphylococcus* spp[10,28]

Traditionally, the recommended course of action for most cows with IMIs caused by L-form *Staphylococcus* spp is culling, or removal from the herd. L-form *Staphylococcus* spp, particularly *S aureus*, are able to reside at the intracellular level, which makes them virtually nonresponsive to in vivo treatment.[10] Furthermore, the structural changes to the mammary gland commonly associated with IMIs caused by *Staphylococcus* spp impair the penetration and diffusion of antimicrobials into the infected areas of the gland.[10,28] This is especially true for antimicrobials that are administered by the intramammary route. Antimicrobials administered by the intramammary route tend to be only marginally effective at achieving a bacteriologic cure for IMIs caused by *Staphylococcus* spp.[27] Additionally, intramammary administration of an antimicrobial requires the insertion of a cannula into the teat, which has been identified as an important cause of iatrogenically induced IMIs.[16,29,30]

Treatment of IMIs by parenteral administration of macrolides has been proposed as a method to ensure homogeneous diffusion of clinically relevant concentrations of an antimicrobial into the bovine mammary gland without the difficulties associated with intramammary infusion.[31,32] Following parenteral administration to cows, tilmicosin readily diffuses into the mammary tissue and milk[18,33] and can also diffuse into the cytoplasm of many cells.[18] In 1 study,[16] cows administered 10 mg of tilmicosin/kg, SC, once, had milk concentrations of the drug that were considered clinically relevant for the treatment of infections caused by *Staphylococcus* spp that persisted for 8 to 9 days. Subsequently, on the basis of the results of that study,[16] many researchers[14,29,34,35] postulated that parenteral administration of tilmicosin to cows will result in concentrations of the drug in milk and mammary gland cells sufficient for the treatment of IMIs caused by *Staphylococcus* spp. Results of another study[26] indicate that the minimum concentration of tilmicosin necessary to inhibit 90% of *S aureus* isolates is 1.0 µg/mL. In the present study, tilmicosin concentrations > 1.0 µg/mL were achieved in mammary gland secretions for 19 and > 40 days following SC administration of 1 and 2 doses (20 mg/kg, every 20 days) of an experimental preparation of the drug, respectively (Figure 1), and the cure rate for naturally occurring IMIs caused by *Staphylococcus* spp was 100% for both dosage regimens. In another study,[10] the cure rate was only 9.1% for cows that were treated with the commercially available formulation of tilmicosin (5 mg/kg, SC) at dry off and again 4 days later, despite the fact that tilmicosin was detectable in mammary gland secretions for 25.7 days after the last injection. Differences in the IMI cure rates between that study[10] and the present study are most likely attributable to differences in the drug formulations used and the higher dose (20 mg/kg vs 5 mg/kg) of tilmicosin that was administered in the present study, which resulted in higher drug concentrations in mammary gland secretions. Also, the cows enrolled in the present study were fairly young; 50% of the cows in both the tilmicosin-1 and tilmicosin-2 groups were in their first or second lactation, and the mean age of those cows was 3.4 and 3.6 years, respectively. The probability that an *S aureus* IMI will be successfully treated is greater for young cows than for older cows.[12] However, half of the cows in both the tilmicosin-1 and tilmicosin-2 groups were in their third or greater lactation, and the cure rate was 100% for all cows, regardless of age.

The pharmacokinetic parameters for tilmicosin in dairy cows following administration of 1 dose (10 mg/kg, SC) of the commercially available formulation of the drug ($t_{1/2\beta}$, 33.84 ± 4.60 hours; C_{max}, 8.21 ± 2.70 µg/mL; t_{max}, 24 hours; and MRT, 76.08 ± 7.31 hours)[16] were significantly less than the corresponding parameters ($t_{1/2\beta}$, 4.7 ± 2.3 days; C_{max}, 14.4 ± 2.1 µg/mL; t_{max}, 3.0 ± 0.5 days; and MRT, 6.8 ± 1.7 days; Table 2) for the cows of the tilmicosin-1 group of the present study following administration of 1 dose (20 mg/kg, SC) of an experimental preparation of the drug. Presumably, this was a reflection of differences in the drug preparations and the fact that the dose of tilmicosin administered in the present study was twice that administered in the other study.[16] Tilmicosin is a time-dependent antimicrobial,[36] and the pharmacokinetic parameters of the experimental preparation of tilmicosin in milk determined for the present study were consistent with that classification. When the pharmacokinetic parameters for the commercially available formulation of tilmicosin[16] were compared with those for the long-acting experimental preparation of tilmicosin used in the present study, the results suggested that the experimental preparation was a better choice than the commercial formulation for dry cow treatment.

Previous studies[10,14] have been conducted to investigate the cure rates for IMIs caused by *S aureus* following intramammary administration of tilmicosin. Infusion of *S aureus*–infected mammary glands with a sterile solution containing 1,500 mg of tilmicosin at dry off resulted in cure rates of 74.2%[10] and 72.5%.[14] Thus, it appears that administration of a high dose of tilmicosin is necessary to achieve good efficacy against *S aureus* IMIs. The total dose of tilmicosin administered SC to the cows of the present study was much higher than 1,500 mg; therefore, it should not be surprising that a 100% cure rate was achieved for IMIs caused by *S aureus*. However, it is important to note that a tilmicosin preparation designed for intramammary administration is not currently commercially available nor is the experimental preparation of tilmicosin that was injected SC in the present study.

Also, tilmicosin is not approved for the treatment of mastitis, and its extralabel use in dairy cows may or may not be legal depending on the country in which the extralabel use is being considered.

In the present study, the mean SCC did not differ significantly among the 3 treatment groups at dry off or 15 days after calving; the mean SCC for the tilmicosin-1 group was significantly lower than that for the ceftiofur and tilmicosin-2 groups at 30 days after calving. Numerically, the mean SCC for the cows in the tilmicosin-1 and tilmicosin-2 groups was consistently lower than that for the cows in the ceftiofur group. A similar finding was reported by investigators of another study[10] in which cows were treated at dry off with benzathine cephapirin or tilmicosin by intramammary infusion or tilmicosin by SC administration. However, in that study,[10] the mean SCC for cows that received an SC injection of tilmicosin was significantly lower than the mean SCC for cows that received an intramammary infusion of tilmicosin. The investigators of that study[10] postulated that direct contact of tilmicosin with the mammary gland epithelium following intramammary infusion caused irritation, which resulted in an increase in the SCC. Given the other environmental factors that affect SCC, further research is required to elucidate the effects of tilmicosin administered by an intramammary route on the SCC of dairy cows.

Qualitative analysis of milk samples for tilmicosin residues revealed that cows in the tilmicosin-2 group had drug residues in their milk for 18 days after calving, which suggested that protocol was not practical for routine use in a commercial field setting. Conversely, none of the cows in the tilmicosin-1 group had tilmicosin residues detected in their milk after calving. The manufacturer of the qualitative assay used in the present study certified that it was capable of detecting tilmicosin; however, the exact limit of detection was unknown, although 40 ppb appeared to be an acceptable value.[37]

In the present study, cows with *Staphylococcus* spp–IMIs that were treated with an experimental preparation of tilmicosin (20 mg/kg, SC) at dry off (tilmicosin-1 group) or at dry off and again 20 days later (tilmicosin-2 group) had a 100% cure rate. However, it is important to point out that similar cows treated with a long-acting intramammary preparation of ceftiofur at dry off also had a 100% cure rate, and that product is approved for such use and commercially available throughout the world. Thus, we are not recommending that the experimental preparation of tilmicosin be used instead of or in preference to the approved ceftiofur product. Further research is necessary to elucidate residue elimination patterns of the experimental preparation of tilmicosin following administration of various dry cow treatment protocols to dairy cows before it can be considered for commercial use. Also, its efficacy against other mammary gland pathogens such as *Streptococcus uberis*[10,26] and its effect on bacterial resistance patterns at the herd level warrant further investigation.

Acknowledgments

Supported by Project UNAM/PAPIIT IT200714.

The authors thank Dr. Rocío Angélica Ruiz-Romero for technical assistance.

Footnotes

a. Experimental preparation of tilmicosin, manufactured by Casal's Internacional S.A. de C.V. (Guadalajara, Mexico), under Patent MX/E/2014/011982 held by the National Autonomous University of Mexico (UNAM), Instituto Nacional de la Protección Industrial (INPI), Mexico City, Mexico.
b. Spectramast DC, Zoetis, Florham Park, NJ.
c. Ekomilk Scan somatic cell counter, Bulteh 2000 Ltd, Stara Zagora, Bulgaria.
d. Biofoam, DeLaval, Tumba, Sweden.
e. Valiant Triple Proteccion, ABS Global, Deforest, Wis.
f. Sigma-Aldrich Corp, Milwaukee, Wis.
g. JASCO Analytical Instruments, Easton, Md.
h. Symmetry C18 column and precolumn, Waters Corp, Milford, Mass.
i. EASYCHROM 2.0, Ampersand International Inc, Beachwood, Ohio.
j. Acrodisc, Pall Corp, New York, NY.
k. PKAnalyst, Micromath Scientific Software, St Louis, Mo.
l. Delvotest, DSM, Heerlen, The Netherlands.
m. SAS, version 9.1.3, SAS Institute Inc, Cary, NC.

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT: 800.176.089-2

COMPROBANTE DE RECAUDO SISTEMA SIPI

Radicado: 15270184
Recibo: 17-51992
Fecha: 04/07/2017

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Patente PCT
TILMICOSINA DE ACCIÓN PROLONGADA MEJORADA Y EL USO DE LA MISMA EN EL TRATAMIENTO DEL COMPLEJO RESPIRATORIO BOVINO (CRB) Y EN EL PERIODO DE LA VACA SECA

Detalle del pago

Id.	Rentístico	Concepto y Servicio	Cant.	Val. Unit.	Subtotal
1	50005-01-01	Solicitud examen de patentabilidad Patente de Invención (DESCUENTO INTERNET	1	$ 996,000	$ 996,000
		TOTAL			$ 996,000