DEPARTAMENTO DE QUÍMICA ANALÍTICA,
NUTRICIÓN E BROMATOLOXÍA

UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

FACULTAD DE VETERINARIA

TRATAMIENTOS ANTIMICROBIANOS EN MEDICINA VETERINARIA: EFECTOS SOBRE LA MICROBIOTA INTESTINAL DE POLLOS Y SU REPERCUSIÓN EN CARNES DE PRODUCCIÓN CONVENCIONAL Y ECOLÓGICA

Memoria que para optar
al grado de doctor presenta:
José Manuel Miranda López.
Lugo, 2007.

DEPARTAMENTO DE QUÍMICA ANALÍTICA,
NUTRICIÓN E BROMATOLOXÍA

D. Alberto Cepeda Sáez, catedrático de universidad, D. Carlos Manuel Franco Abuín, profesor titular de universidad y Dª Beatriz Isabel Vázquez Belda, profesora interina de universidad, pertenecientes al Departamento de Química Analítica, Nutrición y Bromatología de la Universidad de Santiago de Compostela,

AUTORIZAN a D. José Manuel Miranda López a la presentación del Trabajo titulado "Tratamientos antimicrobianos en medicina veterinaria: efectos sobre la microbiota intestinal de pollos y su repercusión en carnes de producción convencional y ecológica", realizado bajo su dirección en la Facultad de Veterinaria de la Universidad de Santiago de Compostela.

Y para que así conste, firman la presente en Lugo, en Mayo de 2007.

Fdo. D. Alberto Cepeda Sáez

Fdo. D Carlos Manuel Franco Abuín

Fdo. Dª Beatriz Isabel Vázquez Belda

Fdo. D. José Manuel Miranda López

DEPARTAMENTO DE QUÍMICA ANALÍTICA,
NUTRICIÓN E BROMATOLOXÍA

D. Alberto Cepeda Sáez, Catedrático de Universidad y Director del Departamento de Química Analítica, Nutrición y Bromatología de la Universidad de Santiago de Compostela,

INFORMA que D. José Manuel Miranda López ha realizado en este departamento y bajo la dirección de D. Alberto Cepeda Sáez, D. Carlos Manuel Franco Abuín y Dª Beatriz Isabel Vázquez Belda el trabajo titulado: "Tratamientos antimicrobianos en medicina veterinaria: efectos sobre la microbiota intestinal de pollos y su repercusión en carnes de producción convencional y ecológica", que presenta para optar al grado de doctor.

Y para que así conste donde proceda, firman la presente en Lugo, el 2 de Mayo de 2007.

Fdo. Alberto Cepeda Sáez

AGRADECIMIENTOS

En primer lugar me gustaría agradecer a mis directores, **D. Alberto Cepeda Sáez, D Carlos Manuel Franco Abuín y Dª Beatriz Isabel Vázquez Belda**, por haber confiado en mí y haberme brindado la oportunidad de trabajar en su laboratorio y llevar a cabo esta tesis doctoral. Muchas gracias por acordaros de mí cuando no estaba en mi mejor momento. Eso es algo que no se olvida.

A mi pequeñita, **Alicia**, por haber llenado mis días de alegría. A ti te debo muchos días de animo, de alegría, de comprensión, de saber escucharme, de darme fuerzas y motivos para seguir adelante y no rendirme. A ti te debo mucho más de lo que esta tesis significa y aunque sea con retraso, no dudes que te lo compensaré. No vayas a pensar que te vas a poder librar de mí. Has sido, eres y siempre serás mi pequeñita. Mi amor.

A **mis padres**, que tanto se han esforzado para que yo pudiese comenzar hace tantos años mi aventura universitaria. Siempre me han apoyado y puesto a mi disposición todo cuanto necesitaba, aunque para ello tuviesen que partirse el lomo trabajando. El día que sea padre me conformaría con llegar a ser la décima parte de lo bueno que vosotros habéis sido conmigo. A **mi hermano**, a quien seguro que también le hubiese gustado poder dedicarse a la ciencia, también este trabajo es en parte tuyo. A **mis abuelos**, que aunque no podrán verme en la presentación seguro que desde algún lugar lo estarán haciendo y se sentirán orgullosos.

A **mi nueva familia mexicana**. Ahora que esta tesis entra en su última etapa, llega el momento de poder pasar más tiempo juntos y conocernos mejor.

A la banda de los pocheros (**Eli, Tino, Ana, Manolo, Moncho, Marta, Julián, Encarna, Manolito.com, Richi, Dolores y Lúa**) que tan agradables hacen mis fines de semana. Mi vida no sería lo mismo sin vosotros.

A mis amigos **Pablo y Miguel**, que durante la carrera han sido mis mejores amigos y que todavía hoy en día, aunque desgraciadamente ya nos vemos poco, seguimos compartiendo una bonita amistad y los tres sabemos que podemos contar con los demás para lo que haga falta. Este trabajo, también en cierto modo es mérito vuestro porque vosotros fuisteis quienes más me animasteis para que empezase mi tesina y como consecuencia de ello terminase aficionándome tanto a la investigación.

Y ya por último, aunque no por eso menos importante, a todos los integrantes de esta gran familia que es el LHICA. A **Carlos y Bea (y Basi)**, que ya hace mucho que han dejado de ser unos compañeros para convertirse en buenos amigos. Muchas gracias por todos los momentos compartidos y por hacer que en vuestra casa me sienta como en la mía. A **Alberto, Cristina y Mr George**, por ser, además de excelentes profesionales, tan amables y haber creado este buen ambiente de trabajo. La verdad es que es admirable el equipo de trabajo que habéis formado, lástima que a veces no sea lo que más se valora en ciertas instancias. A **Sesé**, que aunque ya no esté aquí fue mi gran profesora cuando llegué por primera vez al laboratorio. A **Chelo** (A jefa), **Ana, Samuel, Pascual, Conchi, Bea, Mónica, Pili**

Calo, Alicia, y también a todos los que ya no están pero que durante la realización de este trabajo también han compartido momentos conmigo **(Lolo, Mónica, Tere, Anita, Begoña, Marta, Enka.....)** muchas gracias a todos por ser como sois. Un gran equipo. Quiero agradecer especialmente a **Carmen** por su amabilidad y por estar siempre dispuesta para hacer todo el trabajo que haga falta. Muchas gracias por tu inestimable ayuda y sigue así. Te estás convirtiendo (posiblemente) en la mejor técnico de laboratorio del mundo. A **Mr Sunday**, por haber intentado la misión imposible de enseñarme Inglés y por tenerme en tal alta estima, aunque la verdad es que no merezco ni la mitad.

Quiero dar las gracias también a todos los integrantes de la **Oficina Comarcal de Becerreá**, por haberme recibido tan bien.

Mucha más gente que ha compartido merecería sin duda formar parte de esta sección, pero el mencionarlos a todos sería casi imposible. De todos modos muchas gracias a todos por los momentos compartidos y perdonadme por no poder incluiros en esta sección.

ÍNDICE

ABREVIATURAS

ADN	Ácido desoxirribonucleico
ANOVA	Análisis de varianza
ARN	Ácido ribonucleico
ARNt	Ácido ribonucleico de transferencia
ATCC	*American Type Culture Collection*
BOE	Boletín Oficial del Estado
ºC	Grado centígrado
CE	Comunidad Europea
CFU g^{-1}	Unidades formadoras de colonias por gramo
CFU/g	Unidades formadoras de colonias por gramo
CLSI	Clinical Laboratory Standards Institute
CMB	Concentración Mínima Bactericida
CMI	Concentración Mínima Inhibitoria
Co	*Company*
col.	Colaboradores
DHFR	dihidrofolato reductasa
DHPS	Dihydropterato sintetasa
DHPPP	Dihidro-6-hidroximetilpterin-pirofosfato
DOUE	Diario Oficial de la Unión Europea
E.	Enterococcus
EC	*European Comunity*
et al	Y otros
g	Gramo
GABA	Ácido gamma-aminobutírico
g Kg^{-1}	Gramos por kilo
g l^{-1}	Gramos por litro
h	Hora
ISO	*International Organization for Standardization*
L	Litro
LAB	*Lactic acid bacteria*
L/Kg	Litros/kilogramo
LMR	Límite Máximo Residual
log	Logaritmo
m	Minuto
MAPA	Ministerio de Agricultura, Pesca y Alimentación
m^2	Metro cuadrado
MIC	*Minimum Inhibitory Concentration*
min	minuto
mL	Mililitro
ml l^{-1}	Mililitros por litro
mm	Milímetro
n	número de muestras
NCCLS	*Nacional Comitte of Clinical Laboratory Standrads*
NI	No identificado
no.	*number*

mg kg^{-1}	Miligramos por kilo
µg	Microgramo
µg/ml	Microgramo/mililitro
µg ml^{-1}	Microgramo /mililitro
P	Probabilidad
PABA	Ácido paraaminobenzoico
PAC	Política agraria común
RD	Real Decreto
SD	Desviación estándard
spp.	Especies
St	San
Tm	Toneladas métricas
ufc	Unidades formadoras de colonias
UK	*United Kingdon*
UNE	Una Norma Española
UNE-EN	Una Norma Española-Europe Normative
USA	Estados Unidos
UV	ultravioleta
X^2	Chi-cuadrado
VRBD	*Crystal-violet neutral-red bile glucose agar*
vs	*versus*
w/v	proporción peso/volumen

RESUMEN

En estos últimos años, el uso de antimicrobianos como promotores de crecimiento ha sido objeto de arduo debate a nivel mundial. Por una parte, numerosos científicos consideraban excesivo el riesgo para la salud pública debido a la posible aparición de cepas resistentes a estos medicamentos. Pero por la otra parte, otros especialistas afirmaban que dicho riesgo no estaba lo suficientemente demostrado y defendían este uso por los beneficios que genera tanto desde el punto de vista de la rentabilidad en la producción ganadera como en la salud pública, ya que su utilización también conlleva la eliminación de los microorganismos patógenos contaminantes de los alimentos de origen animal. Finalmente, las autoridades comunitarias de la Unión Europea dentro de los principios de precaución, optaron por prohibir desde el 1 de enero de 2006, la utilización de cualquier tipo de antimicrobiano como promotor del crecimiento.

Pero esta prohibición ha traído como consecuencia un notable aumento del empleo de los antimicrobianos más frecuentemente utilizados con finalidad terapéutica, lo que se sospecha que puede conllevar un aumento en las tasas de aparición de cepas bacterianas resistentes a los antimicrobianos.

En el trabajo que aquí se presenta, se aborda el estudio de la presencia de resistencias bacterianas a determinados grupos de antimicrobianos. Para abordar este problema, primero se ha procedido a la administración de un tratamiento terapéutico en animales de granja (pollos, dado que su consumo va cada vez más en aumento). Los antimicrobianos elegidos pertenecen a cada una de las tres familias más utilizadas en avicultura: enrofloxacina (quinolonas), doxiciclina (tetraciclinas) y una mezcla de sulfamidas (sulfamidas).

Complementariamente, se ha realizado un muestreo de alimentos de origen a animal (carne de pollo y de cerdo) procedentes de sistemas de producción "ecológica" y "convencional" (en intensivo, principalmente) en diferentes establecimientos de venta al público. Estas muestras fueron analizadas en lo referente al grado de contaminación por diversas poblaciones bacterianas. Paralelamente se estudió el grado de resistencia de las bacterias aisladas, a un amplio grupo de antimicrobianos.

Como parte de este trabajo de investigación, se ha llevado a cabo una evaluación independiente de un nuevo medio cromogénico selectivo para el cultivo de *Enterococcus* spp. (Chromocult® enterococci agar) utilizando para ello muestras de heces de pollo con una contaminación presuntamente elevada.

1. INTRODUCCIÓN

La avicultura es un sector muy importante dentro de la economía y dieta occidentales, por lo que su producción industrial a nivel mundial tiene cada vez más relevancia, habiéndose incrementado su producción en los últimos 50 años más de un 800% (Fairchild y col., 2005). La aparición de numerosas patologías infecciosas como consecuencia del hacinamiento al que estos animales son sometidos durante su cría y engorde en las explotaciones modernas, ha ocasionado enormes pérdidas para los agentes económicos relacionados. Para contrarrestar este problema, durante años se han utilizado antibióticos que habitualmente se adicionan en el pienso de los animales de engorde, bien como profilácticos o como promotores del crecimiento. Tras la prohibición a partir del 1 de Enero de 2006, por parte de las autoridades , de la utilización de cualquier tipo de antimicrobiano como promotor del crecimiento (Reglamento (CE) 1831/2003) se está produciendo un notable aumento en el empleo de los antimicrobianos utilizados con función terapéutica. Si bien, es un hecho aceptado que el cese de la administración de promotores del crecimiento contribuye de un modo muy notable a la disminución de las tasas de resistencia bacteriana frente a tales promotores, así como a otros antimicrobianos con los cuales presentan resistencia cruzada (Bager y col., 2002; Casewell y col., 2006), un uso exagerado de los antimicrobianos de primera elección podría conducir hacia un aumento en la resistencia que incluso podría llegar a invalidarlos como opción terapeútica. El tracto intestinal de las aves suele albergar niveles elevados de microorganismos patógenos como *Campylobacter* o *Salmonella* y patógenos oportunistas como ciertos *Escherichia coli* o *Enterococcus,* sin aparentes consecuencias patológicas para el animal portador. En el caso de *Campylobacter*, la cual es considerada hoy en día la bacteria enteropatógena más frecuente en seres humanos en los países desarrollados (Heuer y col., 2001; Avrain y col., 2003; Desmonts y col., 2004), numerosos estudios han identificado a la carne de pollo como la vía de infección más habitual para las personas (Ermel y col., 2000; Luber y col., 2003; Philips y col., 2003) lo cual ha adquirido una enorme relevancia clínica debido especialmente a su cada vez mayor grado de resistencia a ciertos antimicrobianos como las fluoroquinolonas. Del mismo modo, el uso de antimicrobianos como promotores del crecimiento en aves de corral ha sido ampliamente relacionado con la diseminación de cepas de *Enterococcus* y *E. coli*

resistentes (Aarestrup y col., 2000; Joseph y col., 2001; Van den Boogard y col., 2001; Van den Boogard y col., 2002; Tejedor-Junco y col., 2005; Von Baum y Marre, 2005).

1.1. Bases legales del uso de antimicrobianos en medicina veterinaria

La ley 25/1990 del medicamento, desarrollada en lo referente a la comercialización, prescripción y dispensación de los medicamentos veterinarios mediante el RD 109/1995, estableció como obligatoria la receta veterinaria (Figura 1) para un amplio abanico de fármacos, entre ellos, todos aquellos destinados a animales productores de alimentos para consumo humano que requieran "tiempo de supresión", y por lo tanto, para los antimicrobianos. Este hecho, unido a la obligatoriedad de conservar estas recetas por parte del centro dispensador del medicamento, así como por parte del facultativo veterinario y del propietario de los animales, ha representado conjuntamente con la creación de los libros de tratamientos de explotación (Figura 2) una importante medida de regulación en el uso de estos fármacos. Estas nuevas obligaciones facilitan enormemente por parte de las autoridades competentes un control hasta entonces muy difícil del uso de estas substancias. El Reglamento (CE) 1831/2003, el cual prohibió totalmente a partir del 1 de Enero de 2006 el uso de antimicrobianos como promotores del crecimiento, ha contribuido a reducir el consumo total de los mismos en medicina veterinaria, quedando por lo tanto únicamente autorizados para la administración a un animal o grupo de animales que presente síntomas de infección clínica (terapia), o bien para la administración de un antimicrobiano a un animal o grupo de animales en los cuales la morbilidad y/o mortalidad ha excedido la frecuencia habitual (control).

CONSELLO GALEGO DE COLEXIOS VETERINARIOS

Receita veterinaria normalizada / *Receta veterinaria normalizada*
11270407005001

Código de barras:

Identificación dos animais, se é o caso: / *Identificación de los animales, en su caso:*

Dps.

☐ É prescrición excepcional / *Es prescripción excepcional*

Doses e observacións: / *Dosis y observaciones:*

CEA de explotación / se é o caso *(en su caso)*

Propietario / Responsable:

DNI N.º:

Enderezo / *Dirección:*

Especie:

N.º de animais: / *N.º de animales:*

Data / *Fecha:*

Caduca ós dez días. Válido para un só tratamento. / *Caduca a los diez días. Válido para un solo tratamiento.*

EXEMPLAR PARA O CENTRO DISPENSADOR / *EJEMPLAR PARA EL CENTRO DISPENSADOR*

PARA CONSERVAR 3 ANOS / *A CONSERVAR 3 AÑOS*

Tempo de espera / *Tiempo de espera:* (se é o caso / *en su caso*)

CARNE	LEITE / *LECHE*	OVOS / *HUEVOS*

Facultativo veterinario prescritor / *Facultativo veterinario prescriptor*

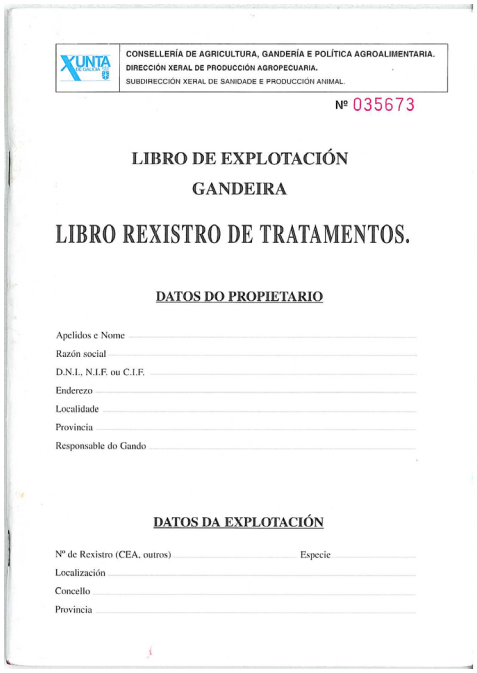
Sinatura e selo normalizado / *Firma y sello normalizado*

Sinatura e selo do dispensador: / *Firma y sello del dispensador:*

Data de dispensación: / *Fecha de dispensación:*

Figura 1. Receta veterinaria normalizada

Tras finalizar el empleo de un agente antimicrobiano en un animal productor de alimentos para consumo humano, es necesario que se espere un tiempo antes de su sacrificio. Este es el denominado "período de supresión", necesario para poder obtener alimentos con concentraciones de residuos, procedentes del uso de medicamentos, inferiores a los Límites Máximos residuales (LMR) establecidos en la legislación. Este tiempo de espera se establece mediante estudios de depleción fijados para cada formulación comercial, a partir de las vías de administración recomendadas y para todas las especies de destino. Se determina así el período tras el cual las concentraciones detectadas son inferiores al LMR y se añade un período de seguridad para compensar la variabilidad biológica, cuyos valores van desde un 10% a un 30%. De este modo, el grado de resistencia de la población bacteriana del animal adquirida durante estos tratamientos quizás requiera un control más exhaustivo que el que actualmente se lleva a cabo basado en el respeto de los LMR marcados en el Reglamento (CE) 2377/1990, por el cual se fija el procedimiento comunitario para el establecimiento de estos valores.



CONSELLERÍA DE AGRICULTURA, GANDERÍA E POLÍTICA AGROALIMENTARIA.
DIRECCIÓN XERAL DE PRODUCCIÓN AGROPECUARIA.
SUBDIRECCIÓN XERAL DE SANIDADE E PRODUCCIÓN ANIMAL.

Nº 035673

LIBRO DE EXPLOTACIÓN
GANDEIRA

LIBRO REXISTRO DE TRATAMENTOS.

DATOS DO PROPIETARIO

Apelidos e Nome
Razón social
D.N.I., N.I.F. ou C.I.F.
Enderezo
Localidade
Provincia
Responsable do Gando

DATOS DA EXPLOTACIÓN

Nº de Rexistro (CEA, outros) Especie
Localización
Concello
Provincia

Figura 2. Libro de tratamientos oficial en la Comunidad Autónoma de Galicia

1.2. Relación entre el uso de antibióticos en animales y la aparición de resistencias en humanos

Cuando se usan antibióticos en medicina veterinaria, se puede producir un fenómeno de selección de cepas resistentes de entre la población intestinal habitual de los animales y también de entre las bacterias patógenas objeto de dicho tratamiento. Al cesar la presión selectiva ejercida por el antimicrobiano, estas cepas seleccionadas en el intestino animal, comienzan a ser reemplazadas por bacterias susceptibles aportadas por el alimento y por el ambiente. Sin embargo, investigaciones recientes están demostrando que la resistencia adquirida durante el uso de antibióticos en ocasiones puede persistir más allá del período de supresión de dicho antibiótico (Pedersen y Wedderkopp, 2003; Griggs y col., 2005).

Por ejemplo, cuando aves que durante su producción han sido tratadas con antimicrobianos llegan al matadero para su carnización, portan en su piel y plumas una gran cantidad de bacterias que pueden haber adquirido resistencia a los antimicrobianos que fueron utilizados durante su producción. Estas bacterias pueden contaminar las superficies de trabajo del establecimiento en el cual son procesadas y pueden, a partir de las mismas, contaminar por contacto otras carnes. En el caso de las aves de corral, es especialmente frecuente además que durante su procesamiento en el matadero se produzcan roturas de vísceras intestinales que de este modo contaminan la canal del animal (Franco Abuín y col., 1994; Stern y Robach, 2003; Wiuff y col., 2003). De este modo, las cepas resistentes presentes en la carne podrían causar en el hombre la aparición de infecciones o la transferencia de resistencias a la microbiota presente en el consumidor, ya sea por reemplazo de su microbiota habitual o mediante la transferencia de determinantes genéticos portadores de resistencia a antimicrobianos.

No es esta la única vía por la cual estas bacterias pueden llegar a los humanos, dado que también pueden ser transferidas mediante el contacto directo con los animales, caso típico de granjeros y matarifes (Cornican y col., 2001; Van den Boogard y col., 2001). Del mismo modo, estas bacterias resistentes originadas durante los tratamientos antimicrobianos, pueden llegar por medio del estiércol al medio ambiente y así contaminar otros animales (domésticos y silvestres). Trabajos recientes han demostrado la presencia de bacterias del género *Enterococcus* resistentes a la vancomicina en

roedores silvestres y animales de compañía. Este fenómeno de resistencia a la vancomicina ha sido atribuido al amplio uso de avoparcina, antimicrobiano permitido como promotor del crecimiento hasta hace poco y que presenta resistencia cruzada con la vancomicina (Mallon y col., 2002; Philips y col., 2003).

Otra vía de llegada a las personas de bacterias resistentes a los antimicrobianos la constituyen los vegetales. Éstos pueden ser contaminados por las heces de animales utilizadas como abonos y así finalmente llegar a transmitir estas bacterias resistentes a los consumidores (Philips y col., 2003). Además aunque de forma mucho menos habitual que el caso de los animales, los vegetales son también objeto de tratamientos antimicrobianos, como es el caso de las tetraciclinas y los aminoglucósidos, utilizados de forma habitual para prevenir infecciones bacterianas en árboles frutales (Falkiner, 1998; Vidaver, 2002).

1.3. Antibioterapia

Los antimicrobianos son compuestos químicos producidos de forma natural por los seres vivos o fabricados mediante síntesis, capaces de interferir en el desarrollo de ciertos microorganismos (acción bacteriostática) o de provocar su destrucción (acción bactericida).

Que estas sustancias supriman el crecimiento u originen la destrucción de otros microorganismos, va a depender del tipo de sustancia, de su concentración en el medio en el que actúa y del tipo de microorganismo contra el que es empleado. De este modo, un mismo principio puede ser bacteriostático o bactericida en función de las variables anteriores.

La mayor parte de los antimicrobianos son producidos por microorganismos, y así de los más de 5000 existentes actualmente, la tercera parte proceden del género *Streptomyces*. También existen ciertas especies de hongos, líquenes y plantas superiores que producen sustancias con acción bactericida y bacteriostática (Botana y col., 2002).

1.3.1. Principios de antibioterapia

La primera cuestión que se debe de abordar antes del comienzo de la terapia es si ésta es realmente necesaria, ya que, por ejemplo, muchas veces el hecho de que haya presencia de fiebre o leucocitosis puede llevar a pensar en la presencia de una infección bacteriana cuando en realidad no la hay. Hay que tener en cuenta que la aplicación de una terapia inapropiada por la administración de fármacos antibióticos innecesarios, provoca gastos económicos superfluos, y lo que es más peligroso y objeto de este trabajo, puede conducir a la aparición de resistencias bacterianas y efectos secundarios asociados a la alteración de la flora normal del huésped. Así pues, el objetivo fundamental de la terapia es proveer una concentración de fármaco efectiva, en el sitio de infección, durante un tiempo suficiente para obtener una cura tanto clínica como bacteriológica, evitando al mismo tiempo, tanto como sea posible, la aparición de efectos indeseables, tales como:

- La toxicidad del fármaco en el animal huésped.
- El desarrollo de resistencia microbiana al fármaco administrado.
- En animales de consumo humano, la presencia de residuos de estos fármacos en tejidos comestibles.
- La selección del fármaco más apropiado para el tratamiento depende del microorganismo causal. El conocimiento de éste puede basarse o derivar de:
- La experiencia histórica o la clínica.
- De exámenes de frotis, mediante morfología de la bacteria, tinciones o diversas bioquímicas de resultados rápidos.
- Cultivos del microorganismo.
- Pruebas *in vitro* de la actividad antimicrobiana.

En resumen, la elección del fármaco y diseño del tratamiento debe depender del conocimiento del microorganismo causante de la enfermedad, de las acciones del fármaco sobre el microorganismo (farmacodinámica), de las acciones del fármaco sobre el huésped (toxicidad), y de la absorción, el destino y la eliminación del fármaco del huésped (farmacocinética), junto con las consideraciones de resistencia, residuos, bienestar animal y económicas (Kucers and Bennett, 1975; Schreiber y col., 1995;)

1.3.2. Concentración Mínima Inhibitoria (CMI) y Concentración Mínima Bactericida (CMB)

El diseño de regímenes de dosificación óptimos implica la selección de un agente con actividad inhibitoria en las concentraciones que se obtienen *in vitro* tras la administración de dosis terapéuticas. Sin embargo, la potencia de los fármacos antimicrobianos se determina normalmente por pruebas *in vitro*.

Esta potencia puede expresarse a través de una variedad de mediciones de actividad, siendo las más útiles y comúnmente utilizadas la Concentración Mínima Inhibitoria (CMI) y la Concentración Mínima Bactericida (CMB). Debe remarcarse que ambas medidas son marcadores teóricos de la actividad antibacteriana, utilizadas porque es imposible cuantificar la actividad antibacteriana en sus receptores dentro de la célula bacteriana.

La CMI se define como la concentración más baja del fármaco que inhibe el crecimiento bacteriano (bacteriostasis) cuando un inóculo bacteriano estandarizado se expone al antimicrobiano durante un período fijo de tiempo. La CMB se define como la concentración del fármaco más bajo que reduce el recuento bacteriano en un 99,9% cuando un inóculo estandarizado se expone al fármaco durante un período fijo de tiempo. Estos 2 marcadores de actividad pueden variar dependiendo del tamaño del inóculo, la duración del período de incubación y la composición del medio de cultivo, razón por la cual es de máxima importancia la más precisa estandarización de los procedimientos utilizados.

Asimismo, los antimicrobianos que son ácidos o bases débiles podrán variar su actividad dependiendo del pH del medio. La mayoría de los fármacos son activos en su forma no ionizada, siendo la ionización menor en medios alcalinos (para bases débiles) o ácidos (para ácidos débiles).

1.3.3. Administración y absorción de los antimicrobianos

Los fármacos antimicrobianos pueden ser administrados de diversas maneras.

a) Administración tópica, por ejemplo, en infecciones dérmicas, oculares u óticas.

b) Localmente en el interior de un compartimiento corporal como en el caso de las mamitis en rumiantes.

c) Por administración oral con absorción subsiguiente desde el tracto alimentario.

d) Por administración parenteral subcutánea o intramuscular con absorción desde los diferentes lugares de administración.

e) Por administración directa al torrente circulatorio.

Los fármacos con suficiente liposolubilidad se absorben bien tras la administración oral, con una biodisponibilidad cercana al 100%. Por otro lado, las moléculas con una liposolubilidad baja ofrecen una pobre distribución tras la administración oral, sin embargo, estas moléculas son eficaces tras la administración oral para el tratamiento de infecciones localizadas en el tracto gastrointestinal, con la ventaja de reducir los efectos indeseables del fármaco sobre el huésped.

La administración tópica o local de los antimicrobianos tiene el objetivo de provocar concentraciones importantes del fármaco para el tratamiento de infecciones localizadas en el sitio de aplicación o la cavidad corporal en la que se inyecta. Esto es preferible a la exposición de todo el organismo a concentraciones efectivas de los fármacos, especialmente los poco liposolubles, en el sitio de infección.

La administración intravenosa tiene la ventaja de conseguir concentraciones altas de forma instantánea, lo que es fundamental en el tratamiento de infecciones que ponen en riesgo la vida del animal. Una ventaja adicional de esta vía se observa para los antimicrobianos cuya actividad depende de la concentración, debido a que las altas concentraciones iniciales facilitan la penetración a los tejidos y sitios de infección.

En el caso de las aves de corral, la vía habitual de administración es oral a través del agua de bebida, exponiendo al tratamiento a la totalidad de las aves presentes en la misma nave de explotación (Prescott y Desmond, 1988; Botana y col., 2002).

1.3.4. Distribución de los antimicrobianos

Los antimicrobianos liposolubles tienen generalmente una amplia distribución en el organismo, alcanzando concentraciones significativas en el interior de las células, al igual que en el fluido transcelular, en cambio los compuestos de características

hidrosolubles, tienen una distribución restringida especialmente al liquido transcelular. Esta distribución restringida no es necesariamente una desventaja, ya que la mayoría de las infecciones bacterianas están confinadas al líquido extracelular.

La distribución de los fármacos en el organismo también depende de sus características ácidas o básicas, así los fármacos, ya sean ácidos o bases débiles, poseen dos propiedades clave. En primer lugar, comúnmente se encuentran parcialmente disociados a pH fisiológico y en segundo, la fracción no ionizada usualmente lipófila, atraviesa fácilmente las membranas biológicas.

Así por ejemplo, las fluoroquinolonas son altamente liposolubles y se comportan como bases débiles. Las sulfamidas, en cambio, son ácidos orgánicos débiles y penetran pobremente en aquellos fluidos de pH ácido en relación al plasma (líquido intracelular, leche y líquido prostático). En contraste, los fármacos que son bases débiles (excepto los aminoglucósidos) penetran fácilmente en estos fluidos, sufriendo secuestro iónico en medios ácidos.

Una vez que los antimicrobianos han llegado al lugar de la infección, hay una barrera más que deben atravesar, la pared bacteriana (Figura 3). Para la mayoría de los antimicrobianos la penetración es más fácil en bacterias aerobias grampositivas que en bacterias aerobias gramnegativas, debido a la diferente conformación de la pared bacteriana.

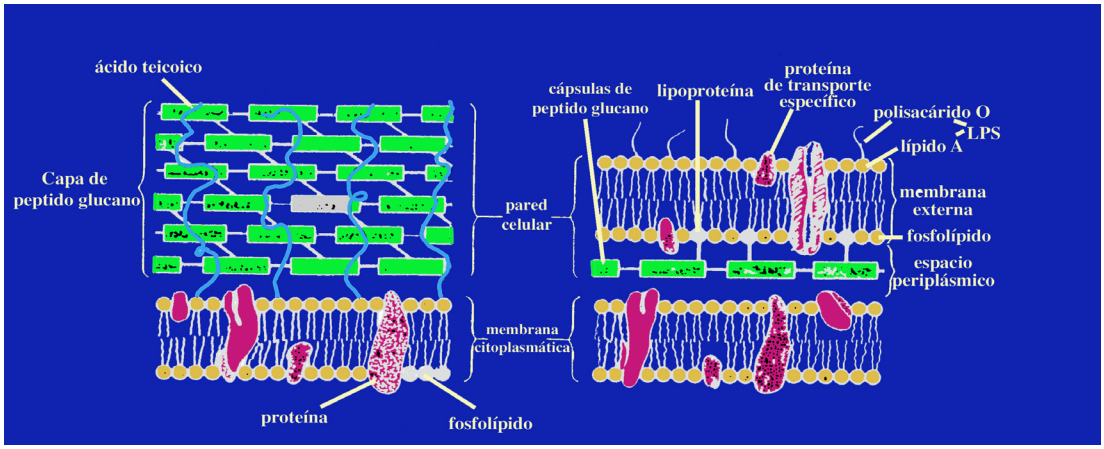


Figura 3. Estructura de la pared bacteriana de bacterias gram + (izquierda) y gram - (derecha)

Los microorganismos anaerobios estrictos poseen mecanismos bioquímicos diferentes de las bacterias aerobias, por lo cual algunos antimicrobianos muy efectivos frente a bacterias aerobias o anaerobias facultativas (consideradas difíciles de combatir),

como las fluoroquinolonas y los aminoglucósidos, son totalmente ineficaces frente a este tipo de microorganismos (kucers y col., 1975; Prescott y Desmond, 1988; Botana y col., 2002).

1.3.5. Eliminación de los antimicrobianos

Los antimicrobianos de características hidrófilas (y usualmente polares) se excretan en altas concentraciones por la orina. Considerando que alrededor del 99% del agua filtrada en el glomérulo se reabsorbe durante todo el proceso de formación de orina, la proporción orina/plasma para fármacos que no experimenten reabsorción a nivel tubular será de 100:1.

Clínicamente, la excreción rápida de los antimicrobianos en la orina tiene dos consecuencias importantes para su selección y dosificación:

a) en infecciones generalizadas implica dosificaciones frecuentes, especialmente si el antimicrobiano tiene cinética de muerte bacteriana dependiente del tiempo y se administra por vía endovenosa.

b) y por otra parte, las altas concentraciones en orina son una ventaja para combatir infecciones en el tracto urinario.

Otra vía importante de excreción para algunos antimicrobianos es la secreción activa en la bilis. En el caso de los antimicrobianos liposolubles, esta secreción puede estar seguida de una reabsorción intestinal, conformando la llamada circulación enterohepática, que disminuye el aclaramiento de los fármacos (Botana y col., 2002).

Otro de los mecanismos más importantes es la biotransformación. Esta puede tener lugar en las células de la mucosa del tracto gastrointestinal y en el riñón, aunque las enzimas más versátiles para el metabolismo de los fármacos se encuentran en los hepatocitos. Los antimicrobianos liposolubles se metabolizan rápidamente por esta vía dando lugar a compuestos inactivos de excreción rápida. Pero los compuestos hidrosolubles no pueden penetrar en las células, y por ende, en los sitios de biotransformación; en consecuencia, son excretados en orina (en algunos casos en la bilis) en altas concentraciones (Kucers y col., 1975; Prescott y Desmond, 1988; Botana y col., 2002).

1.3.6. Efectos secundarios de los antimicrobianos

En la selección del antimicrobiano a utilizar en cada caso debemos tener en cuenta que éstos también presentan efectos adversos para el huésped, como son (Thomas y col., 1974; Botana y col., 2002):

a) Efectos tóxicos dependientes de la dosis sobre las células y los tejidos del huésped.

b) La toxicidad idiosincrásica, que es impredecible.

c) Efectos adversos que surgen en situaciones especiales, como la interacción con otros fármacos, en animales viejos, hembras gestantes, o enfermedades preexistentes.

d) Posible aparición de fenómenos de tipo alérgico o de hipersensibilidad.

e) Promoción de resistencia a los antimicrobianos.

f) Interferencia en la microbiota normal del huésped.

g) Aparición de residuos en los tejidos comestibles para el ser humano.

1.3.6.1. Resistencia bacteriana

En principio y como norma general, el empleo de los agentes antimicrobianos no sería la principal causa de la aparición de resistencias, sino que más bien suprime las bacterias sensibles existentes en el hospedador y respeta las resistentes. (Prescott, 2000).

Hasta el momento, se conocen varios mecanismos por los que se producirían las bacterias resistentes:

a) Inactivación de los antibióticos por enzimas.

b) Impermeabilización de las bacterias.

c) Alteración de los receptores de la célula bacteriana a los que se unen los antibióticos.

d) Desarrollo de mecanismos de retroinhibición en las vías metabólicas.

e) Aparición de enzimas con escasa afinidad por los fármacos.

f) Expulsión del antibiótico al exterior de la célula bacteriana.

La posibilidad de que las bacterias intercambien su material genético, unido al hecho de que su tiempo de generación es corto, son factores que favorecen enormemente la posibilidad de aparición de esas resistencias.

Existe la posibilidad de que las bacterias sean resistentes a los antibióticos porque carezcan de los mecanismos celulares propios para que estos agentes ejerzan su acción (resistencia natural). Además, se da el caso de que bacterias que *in vitro* son sensibles a la vez resultan ser resistentes *in vivo*.

La resistencia adquirida, de base genética, puede aparecer como consecuencia de mutaciones cromosómicas, o lo que es más importante, por la adquisición de material genético transmisible (Figura 4), existiendo de este modo varios mecanismos mediante los cuales las bacterias pueden adquirir resistencia.

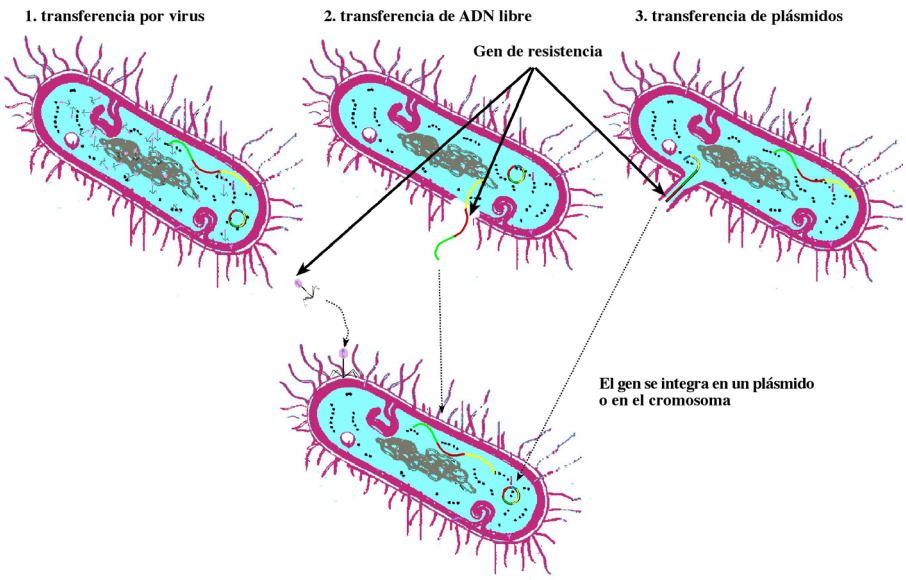


Figura 4. Mecanismos de captación de resistencias adquiridas mediante material genético

Las mutaciones cromosómicas que originan la resistencia contribuyen a producir cambios en la pared bacteriana, mientras que la resistencia transmisible contribuye a codificar enzimas que metabolizan los antibióticos (Prescott y Desmond, 1988). La resistencia cromosómica suele ser un fenómeno de aparición gradual, mientras que la resistencia transmisible es con frecuencia un fenómeno de aparición brusca.

Las mutaciones que originan la resistencia a los antibióticos suelen ser acontecimientos espontáneos que implican modificaciones de las secuencias de

nucleótidos de los cromosomas, que normalmente nada tienen que ver con la presencia de los antibióticos. Suelen además ir acompañadas de otras modificaciones que aparecen en células bacterianas, de forma que en numerosas ocasiones las bacterias que presentan resistencia de origen cromosómico a los antibióticos son menos viables que las células bacterianas de las cuales proceden, pudiendo desaparecer paulatinamente de la población bacteriana incluso sin que haya ningún antibiótico que las seleccione. No obstante, algunas mutantes son tan viables como las células bacterianas de las cuales proceden (Prescott y Desmond, 1988; Botana y col., 2002).

Los mecanismos por los cuales las mutaciones cromosómicas originan la resistencia a los antibióticos incluyen la modificación de los sitios de unión de los antibióticos a las células bacterianas, por ejemplo la modificación de ribosomas (estreptomicina), la modificación de la permeabilidad celular (Como en el caso de la penicilina y las tetraciclinas), la mayor producción de enzimas inactivantes, y la mayor producción de metabolitos competitivos (Figura 5).

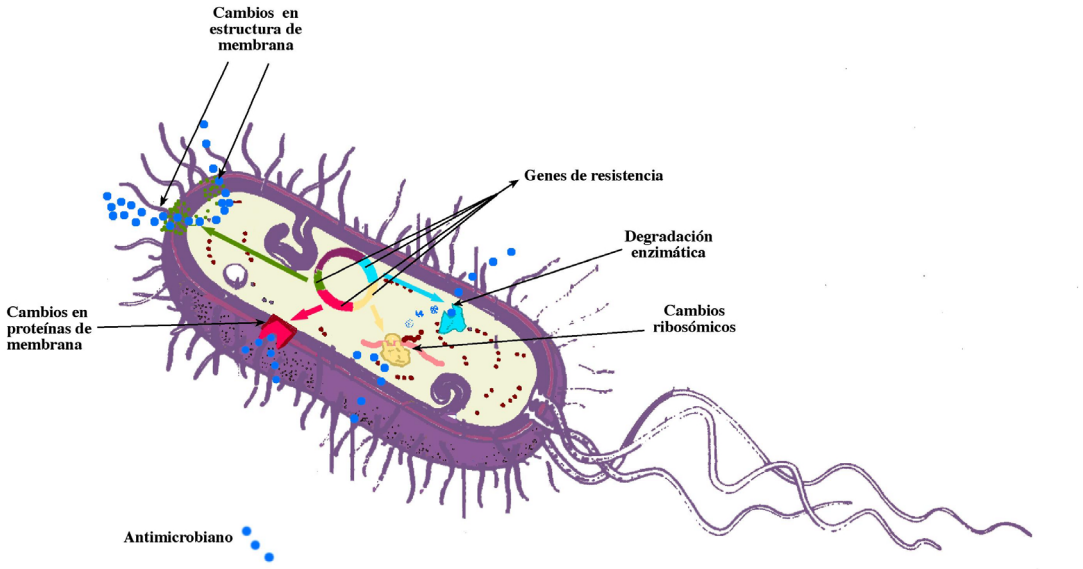


Figura 5: Principales mecanismos de resistencia bacteriana

Los antibióticos frente a los cuales aparecen resistencias de este tipo con mucha facilidad, como la eritromicina o la estreptomicina, se utilizan normalmente asociados con otros antibióticos, ya que la posibilidad de que en una misma bacteria tengan lugar dos mutaciones al mismo tiempo, es la suma de las posibilidades de que cada mutación

aparezca de forma aislada. Asimismo, las mutantes resistentes a antibióticos aparecen con menor frecuencia *in vivo* que *in vitro*, debido a que las propias defensas del hospedador elimina muchas de ellas (Botana y col., 2002).

1.3.6.2. Interferencia en la microbiota normal

La microbiota característica de la piel, aparato gastrointestinal, etc., coexiste de forma armónica y hasta sinérgica, con el hospedador. De todos los antimicrobianos son muy pocos los que no suprimen en grado alguno esta microbiota. Los efectos supresores pueden variar, desde efectos secundarios temporales y mínimos, como diarrea leve, hasta la proliferación de patógenos que ponen en peligro la vida del animal.

En general, se ha demostrado que los fármacos activos contra los microorganismos anaerobios como las penicilinas producen mayores efectos que por ejemplo las sulfamidas o fluoroquinolonas. También se ha observado que la aparición de este tipo de fenómenos es más frecuente tras la administración oral que en las administraciones parenterales (Prescott y Desmond, 1988).

1.3.6.3. Residuos de los antimicrobianos en productos alimenticios de origen animal

La Unión Europea, a través del comité para productos médicos y veterinarios, ha determinado un LMR para la mayoría de los fármacos autorizados en los diferentes tejidos diana. Estos límites han sido establecidos en función de estudios de toxicidad crónica a partir de los llamados "límite sin efecto observable" y "límite sin efecto microbiológico". La determinación del primero, exige mediciones de las más bajas concentraciones del fármaco que no provocan efecto farmacológico en los animales de laboratorio y en el segundo caso, consiste en determinar las concentraciones de antimicrobiano que no provocan ningún efecto sobre las bacterias más sensibles, en particular las del tracto gastrointestinal. Posteriormente, se determina el "nivel de ingesta diaria admisible", a través de la aplicación de factores de seguridad de 100 a 1000 veces en el primer caso y de 10 veces en el segundo (Botana y col., 2002).

El período de supresión o de retirada, es el tiempo que debe transcurrir desde la administración del fármaco en dosis terapéuticas, hasta el momento en que el alimento puede ser consumido sin riesgo durante períodos prolongados, es decir, que se cumpla con los valores de LMR en los diferentes tejidos animales.

1.3.7. Antimicrobianos utilizados en avicultura con mayor frecuencia

Actualmente, los antimicrobianos más utilizados en aves de corral son las tetraciclinas, las fluoroquinolonas y las sulfamidas potenciadas (Antunes y col., 2003; Avrain y col., 2003; Peters y col., 2003; Van den Boogard y col., 2001; Van den Boogard, 2002). Las tetraciclinas son en la actualidad los antimicrobianos más utilizados en pollos y pavos (Chopra y Roberts, 2001; Avrain y col., 2003; Philips y col., 2003), dadas las propiedades favorables de esta familia de fármacos como son su amplio espectro, la posibilidad de administración oral, que permite tratar fácilmente grandes cantidades de animales, la ausencia de efectos adversos importantes, y su bajo precio (Finch y col., 1997; Chopra y Roberts, 2001). A continuación y aunque económicamente resulta contraproducente, se destaca la utilización de fluoroquinolonas, cuando aún no se ha agotado la posibilidad del empleo de tetraciclinas y sulfamidas potenciadas, pero su uso se ha ido generalizando en medicina aviar desde que la enrofloxacina fue aprobada para su utilización en aves de corral. Actualmente, en los países europeos en los cuales su uso en animales de producción está permitido es una de las familias de antimicrobianos con un mayor consumo (Avrain y col., 2003; Philips y col., 2003).

El método habitual para la administración de estas tres familias de antimicrobianos es en el agua de bebida, medicando a todo el grupo de aves presente en la misma nave de producción, práctica que se lleva a cabo normalmente incluso aun cuando sólo una pequeña parte de los animales presentes están realmente enfermos. Un problema adicional podemos encontrarlo en el cálculo de la dosificación necesaria. Dado que el antimicrobiano es administrado en el agua *ad libitum*, esto puede provocar la submedicación de un buen número de animales lo cual conlleva que se incremente sustancialmente la probabilidad de selección de bacterias resistentes (McDermott y col.,

2002). Incluso esto puede provocar la sustitución de la microbiota normal del ave por otra microbiota en la cual abundan más los agentes patógenos.

1.3.7.1. Quinolonas

Las quinolonas son compuestos químicos que constituyen una amplia familia de agentes antibacterianos. Su desarrollo comenzó en la década de los 60, con la síntesis del ácido nalidíxico (ácido 1-etil-1,4-dihidro-7-metil-4-oxo-1,8-naftiridina-3-carboxílico) (Figura 6).

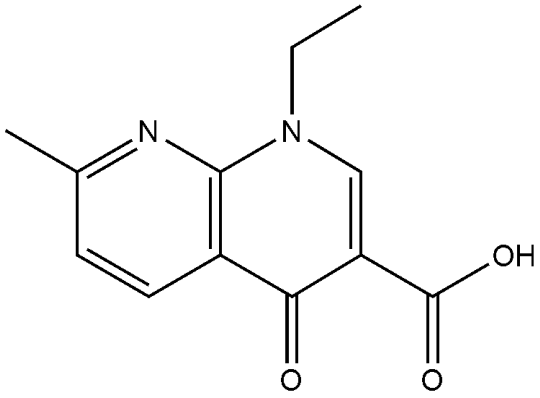


Figura 6. Ácido nalidíxico

Las primeras quinolonas sintetizadas, el ácido nalidíxico y el ácido oxolínico, ofrecían un estrecho margen de actividad antimicrobiana, poca biodisponibilidad oral, distribución limitada en el organismo y gran capacidad para inducir resistencias bacterianas. Sus usos clínicos se limitaban principalmente al tratamiento de infecciones provocadas por bacterias gramnegativas en el tracto urinario y de forunculosis en peces de granja.

Posteriormente se sintetizaron otras quinolonas que poseían una mayor actividad contra bacterias gram-negativas. Más tarde, en los años 80 la molécula sufrió tres modificaciones clave:

1.- sustitución del grupo CH por N en posición 8.

2.- Introducción de un anillo de piperazina en posición 7 (esta sustitución extendió el espectro a bacterias gramnegativas, incluyendo especies de *Pseudomona*).

3.- Introducción de un átomo de flúor en posición 6 (lo cual aumentó aún más el espectro del grupo y amplió la actividad a bacterias grampositivas), constituyendo así las fluoroquinolonas.

Estas modificaciones han ampliado sus aplicaciones clínicas, y además los cambios estructurales han conducido al aumento de la potencia y la mejora de la farmacocinética, especialmente en lo referente a su liposolubilidad (Botana y col., 2002).

1.3.7.1.1. Actividad antimicrobiana de las quinolonas

Su espectro es bastante amplio e incluye a bacterias gramnegativas y algunos microorganismos grampositivos, aunque tienen capacidad limitada frente a *Streptococcus* y *Enterococcus* y solo una actividad débil frente a bacterias anaerobias estrictas.

Este grupo tiene en particular una excelente actividad contra bacterias aerobias gramnegativas y una de sus ventajas es que no destruyen los enterococos intestinales comensales. Asimismo, tienen una actividad entre moderada y buena frente a *Chlamydia*, micobacterias, micoplasmas, ureaplasmas y rickettsias, siendo también activas frente a microrganismos intracelulares.

Su potencia es alta frente a algunos microorganismos sensibles con valores de CMI de 0,01 μg/ml o menores. La actividad frente a bacterias gramnegativas es mayor a pH alcalino, mientras que la actividad frente a bacterias grampositivas no se modifica con cambios en el pH (Ball, 1998; Poole, 2000a; Poole, 2002b).

1.3.7.1.2. Mecanismos de acción de las quinolonas

El cromosoma bacteriano comprende una doble cadena de ADN que es una molécula continua miles de veces más larga que la célula bacteriana. Esta cadena tiene una disposición de doble hélice.

Las fluoroquinolonas basan su mecanismo de acción en que inhiben la enzima ADN girasa (topoisomerasa de tipo II). Esta es una enzima compuesta de 4 subunidades cuya función incluye el desenrollamiento, corte y resellado del ADN. La ADN girasa también interviene en el plegamiento y enrollamiento del ADN bacteriano alrededor del

centro de ARN, otorgándole la apariencia de bucles o lazos. Cada lazo se enrolla después negativamente mellando ambas cadenas de ADN, pasando la cadena rota alrededor de la cadena acompañante y resellando la doble muesca. Estas funciones son inhibidas por las fluoroquinolonas, que se unen a la subunidad A de la ADN girasa. En los mamíferos, existe una enzima similar a la ADN girasa que interviene en los cortes dobles del ADN; sin embargo, ésta no superenrolla el ARN y no se ve afectada por las fluoroquinolonas (Ball, 1980).

Las fluoroquinolonas son bactericidas y tienen un inicio de acción rápido, lo cual es muy importante en el tratamiento de animales inmunodeprimidos. Los cationes bivalentes como el calcio y el magnesio antagonizan la acumulación de fluoroquinolonas en el interior de la bacteria, probablemente por un efecto quelante (Botana y col., 2002). Se ha descrito en algunas bacterias un sistema de transporte de salida (Poole, 2000a; Poole, 2002b).

Paradójicamente, a concentraciones muy altas (40 x CMI), la acción bactericida de algunas fluoroquinolonas disminuye. Esto puede resultar de la síntesis de ARN, lo que parece ser necesario para el efecto bactericida.

La resistencia se produce principalmente por la reducción de la penetración a través de la pared de la célula bacteriana. Sólo raramente aparecen formas mutantes de la ADN girasa, mientras que hasta la fecha no se han identificado enzimas bacterianas capaces de degradar las fluoroquinolonas.

La resistencia mediada por plásmidos es extremadamente rara. La resistencia cruzada entre fluoroquinolonas es posible, aunque mantienen la actividad frente a bacterias resistentes a otros grupos de fármacos, como aminoglucósidos, macrólidos y β-lactámicos (Ball, 1998).

1.3.7.1.3. Farmacocinética de las quinolonas

El grado de ionización de estas moléculas es bajo y todas ellas se comportan *in vivo* como moléculas altamente liposolubles. En solución, se comportan como bases débiles. Su carácter lipófilo determina sus propiedades farmacocinéticas. Se absorben bien por vía oral, intramuscular y subcutánea. La absorción oral en pavos y pollos es del 30-90%,

variando entre los diferentes compuestos y las características intestinales animales, como por ejemplo la presencia de iones bivalentes.

Las fluoroquinolonas se distribuyen ampliamente por el organismo. Esto indica que se distribuyen a través del líquido extracelular y pasan fácilmente al líquido transcelular, incluyendo la leche, el líquido sinovial, el líquido prostático, el semen, fluidos uterinos y el líquido cefalorraquídeo, alcanzando también altas dosis en el medio intracelular. Las concentraciones tisulares generalmente igualan o exceden a las concentraciones plasmáticas, y también se han descrito altas concentraciones en el hueso. La excelente penetración de las fluoroquinolonas al interior de las células y a los tejidos es consecuencia de su carácter lipófilo, pero también se ve facilitado por el bajo grado de unión a proteínas plasmáticas.

Las fluoroquinolonas se metabolizan en el hígado, donde el grado de biotransformación depende del compuesto y de la especie. Generalmente sufren reacciones de hidroxilación y de oxidación, que las transforma en oxoquinolonas, las cuales se someten posteriormente a reacciones de fase 2, consistentes en la conjugación con ácido glucurónico. Los glucuronoconjugados sintetizados son excretados en la orina y la bilis.

Algunas fluoroquinolonas sufren circulación enterohepática, probablemente tras la conjugación de betaglucuronidasa sobre los conjugados. Las concentraciones urinarias de las fluoroquinolonas se mantienen altas durante 24 horas o más. Algunos compuestos son secretados en el túbulo proximal renal a traves del sistema transportador de ácidos orgánicos.

La vida media de estos compuestos está en el intervalo 2-7 horas, dependiendo del compuesto y la especie. Algunos metabolitos tienen actividad antimicrobiana, asi, la enrofloxacina (Figura 7) se convierte en ciprofloxacina (Figura 8), y ésta ultima se utiliza en humanos como medicamento madre. La conversion de enrofloxacina en ciprofloxacina es muy importante en cánidos y muy escasa en équidos y aves (Ball, 1998; Köhler y Pechere, 1998; Talens-Visconti y col., 2002).

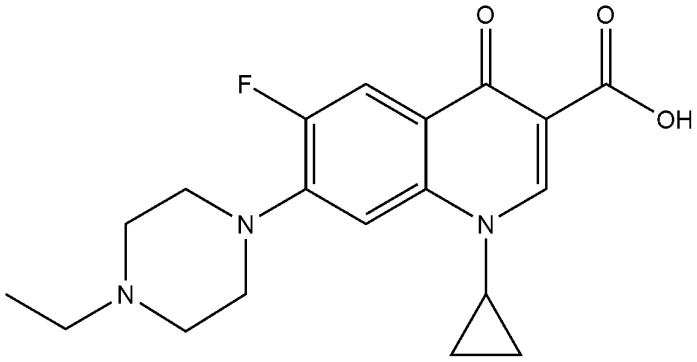


Figura 7. Enrofloxacina

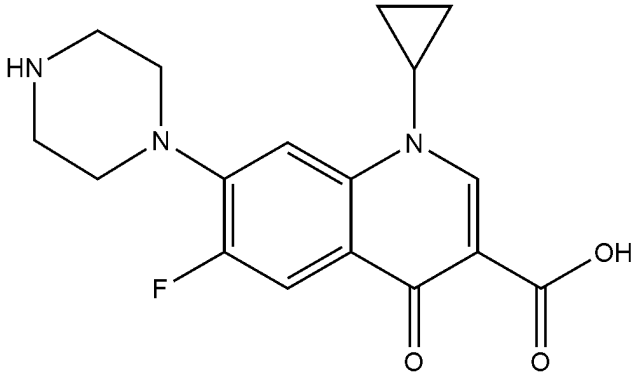


Figura 8. Ciprofloxacina

1.3.7.1.4. Toxicidad y efectos secundarios de las quinolonas

En general, las fluoroquinolonas tienen un amplio margen de seguridad en mamíferos y aves. Sin embargo, un inusual pero característico efecto es la acción erosiva sobre los cartílagos en crecimiento. Esta artropatía afecta especialmente a las articulaciones que soportan peso, aunque esta característica solo está documentada en cánidos, especie para la cual también se han descrito reacciones de fotosensibilización. Asimismo, se han descrito en otras especies reacciones adversas como mareos, cefaleas, nauseas y vómitos, estos últimos tan solo en la especie humana.

Altas dosis de fluoroquinolonas pueden causar convulsiones, por lo que se ha sugerido que actúan como antagonistas del GABA del sistema nervioso central (Botana y col., 2002).

1.3.7.1.5. Uso clínico de las quinolonas

Debido a su alta potencia, amplio espectro, actividad dependiente de la concentración, óptimas características farmacocinéticas y baja toxicidad, las fluoroquinolonas se utilizan ampliamente para un numero importante de infecciones, como las neumonías típicas de terneros y lechones, infecciones urinarias en animales de compañía, infecciones dérmicas en cánidos y félidos e infecciones intestinales en porcinos, bovinos y aves.

Entre las indicaciones más comunes se encuentran las salmonelosis agudas en terneros y la colibacilosis en aves y porcino. También es frecuente su uso para combatir infecciones en lugares de difícil acceso, como son las osteomielitis y las prostatitis.

Dado su mecanismo de actuación, la dosificación recomendada para su uso clínico debe apuntar a conseguir concentraciones máximas altas (con respecto a la CMI) en lugar de mantener las concentraciones plasmáticas por encima de la CMI. Las dosis altas administradas con intervalos prolongados producen mejores resultados que la administración frecuente de un régimen fraccionado. Para la medicación de aves y peces se recomienda la administración en intervalos prolongados en el agua o el alimento (Ball, 1998).

1.3.7.1.6. Desarrollo de resistencias bacterianas a quinolonas

Se ha detectado un aumento en la tasa de resistencia bacteriana en personas no expuestas a estos agentes, la cual podría ser causada por su uso en producción animal. Al mismo tiempo, se ha detectado un incremento en la aparición de cepas bacterianas con menor susceptibilidad e incluso resistentes en animales destinados al consumo. Se ha registrado resistencia a la enrofloxacina en *Escherichia coli* y distintas especies de *Campylobacter* y *Salmonella* (Endzt y col., 1991; Blanco y col., 1997; Gurbay y col., 2001; Kijima-Tanaka y col., 2003).

Para alcanzar su efecto bactericida, las quinolonas deben penetrar a través de la membrana celular y alcanzar la DNA girasa o la topoisomerasa IV, razón por la cual los mecanismos de resistencia a las quinolonas incluyen mutaciones en los genes que codifican la DNA girasa y la topoisomerasa IV, dando lugar a alteraciones en la pared

de la membrana que disminuyen la penetración intracelular del fármaco y actividad de transportadores activos endógenos que provocan la expulsión de los antimicrobianos desde la membrana celular al medio exterior. Estos mecanismos de resistencia pueden manifestarse solos o en combinación, si bien parece que *in vivo* el aumento en el grado de resistencia a las quinolonas es producto de varios mecanismos simultáneos. (Talens-Visconti y col., 2002).

Así, los mecanismos de resistencia bacteriana a las quinolonas pueden dividirse en 3 grupos:

1- Resistencias de tipo cromosómico que dan lugar a mutaciones en segmentos definidos de los genes que codifican la DNA girasa y la Topoisomesara IV (Nakamura, 1997).

2- Resistencias por alteraciones en la membrana externa bacteriana que disminuyen la penetración intracelular del fármaco. Estas modificaciones se originan en alteraciones de los genes que codifican los canales por los cuales los antimicrobianos penetran normalmente en la célula (porinas), lo que impide la entrada del quimioterápico en la bacteria.

3- Resistencias basadas en la expulsión del antibacteriano desde el medio intracelular al expulsar por acción de transportadores endógenos activos.

Sin embargo, no se han descrito enzimas bacterianos capaces de degradar o inactivar a las quinolonas en el medio intracelular (Gibreel y col., 1998; Poole, 2000a; Poole, 2002b).

1.3.7.2. Tetraciclinas

Las primeras tetraciclinas (clortetraciclina y oxitetraciclina) fueron descubiertas a finales de la década de los 40 a partir de *Streptomyces aureofaciens* y *S. rimosus*, respectivamente. Otras tetraciclinas fueron descubiertas posteriormente, tanto moléculas naturales producidas a partir de *Streptomyces*, o productos semisintéticos como los casos de la metaciclina, doxiciclina (6-desoxi-5-hidroxi-tetraciclina) (Figura 9) y minociclina.

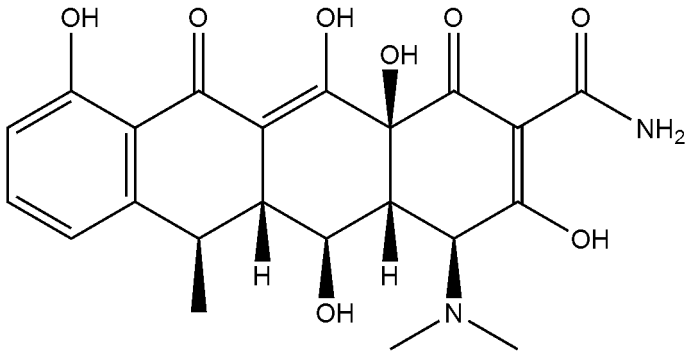


Figura 9. Doxiciclina

Todas las tetraciclinas tienen la misma estructura básica, 4 anillos unidos en línea, y a partir de ellos la unión de diferentes grupos funcionales en distintos carbonos origina los diferentes compuestos. El más sencillo con actividad antimicrobiana es la 6-desoxi-6-demetil-tetraciclina.

Otra característica importante derivada de la estructura de las tetraciclinas es su fuerte actividad como agentes quelantes de iones metálicos, lo que determina en gran parte su actividad antimicrobiana y sus propiedades farmacocinéticas.

La estabilidad de las tetraciclinas en disolución varía con el pH y el derivado específico del que se trate, aunque suelen ser estables frente a pH ácido. En su mayoría se comportan como anfóteros y por lo tanto pueden actuar como aniones, formando sales de sodio, o como cationes, obteniéndose los correspondientes clorhidratos, siendo estos últimos los preparados más comunes (Schappinger, 1996).

1.3.7.2.1. Actividad antimicrobiana de las tetraciclinas

Las tetraciclinas son antibióticos de amplio espectro por definición, ya que son efectivas contra bacterias gramnegativas, tanto aerobias como anaerobias, asi como contra grampositivas, microorganismos atípicos como clamydias, micoplasmas, ricketsias e incluso contra algunos protozoos. También son usadas en medicina humana como profiláctico en la prevención de la malaria causada por *Plasmodium falciparum* resistente a la mefloquina. Actualmente el aumento en las resistencias aparecido en los últimos años por la utilización de estos antibióticos como promotores del crecimiento, actividad prohibida actualmente en la UE pero de uso corriente en paises como USA, ha limitado en cierta medida su uso terapéutico (Bager y col., 2002).

1.3.7.2.2. Mecanismo de acción de las tetraciclinas

Su mecanismo de acción es un efecto bacteriostático basado en la inhibición de la síntesis de proteinas bacterianas. Dicha inhibición la llevan a cabo evitando la asociación entre el aminoacil-ARNt y el ribosoma, uniéndose las tetraciclinas específicamente a la subunidad 30 S del ribosoma. El resultado es que se impide la adición de aminoácidos a la cadena peptídica en formación, lo que impide la enlongación de la cadena. Algunas tetraciclinas inhiben también la síntesis de proteinas en células eucariotas, lo cual es de utilidad para combatir algunos protozoos. Se ha sugerido que el mecanismo de actuación en este caso estaría relacionado con la presencia de ribosomas de tipo bacteriano en las mitocondrias, aunque actualmente no existe una explicación molecular satisfactoria para este efecto (Edlind, 1991; Chopra and Roberts, 2001). Además, se ha demostrado que en altas dosis inhiben de igual forma la síntesis de proteínas de mamíferos (Botana y col., 2002), aunque esto ocurre a concentraciones mucho más elevadas que las utilizadas como antimicrobianos.

1.3.7.2.3. Farmacocinética de las tetraciclinas

Las tetraciclinas son normalmente administradas por vía oral, aunque en algunas está indicada la vía parenteral. La absorción tras la administración oral tiene lugar en el estómago y la porción proximal del intestino delgado y está muy influenciado por la presencia de comida, leche o cationes divalentes. En particular, es especialmente importante la presencia de calcio, el cual inhibe la absorción de las tetraciclinas mediante la formación de quelatos.

Los niveles alcanzados en suero sanguíneo después de una dosificación oral normal se encuentran normalmente en el rango 2-5 μg/ ml, y en la mayor parte de las tetraciclinas son necesarias 4 tomas diarias para poder mantener estos niveles de concentración en suero. No obstante, la larga vida media de la doxiciclina y la minociclina permite alcanzar estos niveles con una o dos dosis diarias, lo cual es de una gran aplicación especialmente en la clínica veterinaria de aves de corral, en donde el gran número de animales a tratar hace que la administración sea dificultosa.

Las tetraciclinas tienen en general una buena penetración en los diferentes fluidos y tejidos corporales y se excreta por la orina. Además, normalmente se encuentran en los esputos niveles en torno al 20% de las concentraciones alcanzadas en el suero sanguíneo, lo cual hace que esta familia de antimicrobianos sea particularmente útil en el tratamiento de las infecciones del tracto respiratorio (Kucers and Bennett, 1975; Willians, 1992).

1.3.7.2.4. Toxicidad y efectos secundarios de las tetraciclinas

Aunque las tetraciclinas son fármacos relativamente seguros, no están exentos de efectos adversos, estando en general relacionados con:

1. Efectos irritantes (vómitos después de su administración o daños tisulares en el punto de inyección).
2. Capacidad de alteración de la flora intestinal, con su capacidad para fijar el calcio (efectos cardiovasculares y depósito en el tejido óseo).
3. Nefro y hepatotoxicidad, siendo el efecto nefrotóxico especialmente peligroso cuando se administran productos caducados o inadecuadamente conservados.

Además de lo mencionado, en el caso de las tetraciclinas más lipófilas, como la doxiciclina, es posible la aparición de alteraciones digestivas, y están contraindicadas en hembras gestantes y durante el período reproductor de las aves (Botana y col., 2002).

Debido a su amplio espectro de acción, las tetraciclinas provocan inevitablemente una alteración importante de la microbiota intestinal, siendo frecuente la aparición de diarreas durante el tratamiento. Tampoco son infrecuentes las sobreinfecciones por levaduras, hongos, o bacterias resistentes (Prescott y Desmond, 1988; Botana y col., 2002).

1.3.7.2.5. Uso clínico de las tetraciclinas

En los últimos años, el uso de las tetraciclinas ha descendido en muchos países en clínica humana debido al desarrollo de nuevos fármacos con mayor vida media, más activos y con mejor tolerancia (Chopra y col., 1992; Freeman y col., 1994). No obstante,

las tetraciclinas siguen siendo utilizadas para combatir infecciones como las causadas por *Brucella* o las infecciones gastrointestinales causadas por *Helicobacter pylori*, además de ser utilizada como profiláctico contra la malaria gracias a su acción contra *Plasmodium falciparum* (Scharz y col., 1999).

En el caso de la medicina veterinaria son especialmente utilizadas para el tratamiento de diversas infecciones en aves de corral, rumiantes, suidos y animales de compañía (Kordick y col., 1997). Las tetraciclinas son una de las familias de antibióticos más baratas, y su coste de producción ha descendido enormemente debido a las mejoras en la tecnología de fabricación (Liss y Batchelor, 1987). Estas características las hacen especialmente atractivas para su uso en naciones en vías de desarrollo (Finch, 1997).

1.3.7.2.6. Desarrollo de resistencias

Dado que el uso de tetraciclinas tanto en medicina humana como veterinaria se remonta a más de 60 años, no es de extrañar que dicha presión selectiva haya provocado la aparición de numerosas cepas bacterianas resistentes a estos fármacos. A dicho efecto ha contribuido enormemente el hecho de que durante muchos años hayan sido utilizadas masivamente como promotores del crecimiento a dosis subterapéuticas, una práctica prohibida hoy en día en la UE pero vigente todavía en países en vías de desarrollo (Chopra y Roberts, 2001).

Hoy en día es frecuente encontrar resistencias en bacterias causantes de zoonosis aisladas de animales como *Salmonella*, *Campylobacter* spp. *Yersinia* spp. y en bacterias comensales como *Escherichia coli* o *Enterococccus* spp., las cuales tienen una gran importancia ya que además de poder provocar infecciones clínicas en los animales, pueden ocasionar también infecciones en el hombre ya sea por contacto directo con los animales vivos o por ingestión de los productos animales obtenidos a partir de los mismos.

La resistencia a las tetraciclinas es un ejemplo de resistencia transmitida por plásmidos portadores de genes capaces de transferir resistencia (llamados *tet* genes). En la actualidad se conocen más de 20 *tet* genes diferentes capaces de transferir resistencia

a tetraciclina u oxitetraciclina (Chopra y Roberts, 2001). Estos *tet* genes aportan a las bacterias receptoras capacidad de resistencia mediante diferentes mecanismos:

1- Resistencia por alteraciones en la membrana externa bacteriana que disminuyen la penetración intracelular del fármaco. Esta resistencia se basa en la producción por parte de la bacteria de proteínas capaces de asociarse a la membrana que participan en la expulsión de tetraciclinas de la bacteria, lo cual reduce la concentración intracelular de tetraciclinas y protege por tanto a los ribosomas de su acción.

2- Resistencia por protección de los ribosomas. Esta resistencia se basa en la producción por parte de las bacterias de proteínas citoplásmicas que protegen a los ribosomas de la acción de las tetraciclinas.

3- Resistencia por inactivación enzimática. Esta resistencia se basa en la producción por parte de las bacterias de enzimas capaces de inactivas las tetraciclinas dentro del citoplasma.

4- Mecanismos desconocidos. Hoy en día todavía se desconoce el mecanismo por el cual ciertos genes como el tet(U) o el tet(M) confieren resistencia a las células receptoras. No obstante, su estructura no es similar a los que aportan los tres mecanismos anteriormente descritos, motivo por el cual se sospecha que pueden aportar resistencia mediante mecanismos diferentes (Willians, 1992; Ross y col., 1998; Chopra y Roberts, 2001).

1.3.7.3. Sulfamidas

Las sulfamidas o sulfonamidas son fármacos antimicrobianos de amplio espectro, que se utilizan solos o en combinación con otros fármacos en el tratamiento de infecciones por organismos aerobios grampositivos y gramnegativos en casi todas las especies domésticas. Son el primer ejemplo de sustancia química con acción bactericida sintetizada por el hombre.

La primera sulfamida fue descubierta por Paul Gelmo en 1908 de forma accidental cuando intentaba conseguir mejores colorantes para teñir la lana. Tras él, en 1935 un patólogo alemán llamado Gerhard Domagk publicó el primer informe sobre la actividad antimicrobiana de dicho tinte, que se habia patentado en 1932 bajo el nombre de Prontosil (Domagk y col., 1935). Domagk demostró que dicho tinte era capaz de

eliminar estreptococos de ratones infectados. La posterior investigación concluyó que dicho producto se metabolizaba en el organismo produciendo sulfanilamida y que este metabolito era el responsable de la acción antibacteriana. Fue durante la segunda guerra mundial donde científicos británicos realizaron numerosos estudios para contrarrestar la ventaja que suponía para Alemania la utilización de un antimicrobiano eficaz en tiempos de guerra (Domagk y col., 1935; Sköld, 2000).

1.3.7.3.1. Actividad antimicrobiana de las sulfamidas

Tienen un amplio espectro que inhiben el crecimiento de bacterias grampositivas, gramnegativas y ciertos protozoos, como los coccidos. Se consideran ineficaces frente a la mayoría de anaerobios obligados, por lo que no se recomienda su uso para este tipo de infecciones. Entre los microorganismos habitualmente susceptibles se incluye algunos miembros de la familia Enterobacteriaceae, así como otros agentes como *Streptococcus, Bacillus, Brucella, Cryptosporidium, Listeria, Erysipelothrix, Chlamydia, Toxoplasma, coccidios* y *Pneumocystis carinii*. Otros microorganismos como *Pseudomonas*, *Enterococcus*, *Mycoplasma*, *Mycobacterium* y *Bacteroides*, son habitualmente resistentes tanto a la acción de las sulfamidas solas como a la combinación de sulfamidas con diaminopirimidinas (Huovinen, 2001).

Las sulfamidas han sido muy empleadas hasta la aparición de numerosas resistencias durante los años cincuenta y sesenta, a partir de los cuales su utilización por si sola ha caído drásticamente pasando a ser utilizada de forma conjunta con diaminopirimidinas. El efecto sinérgico que las sulfamidas presentan junto con estas substancias hace que hoy en día suelan ser utilizadas junto con trimetoprina (Figura 10), ormetoprina o aditoprina (Huovinen, 2001).

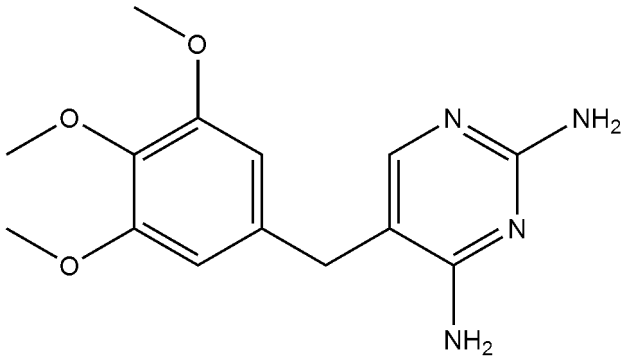


Figura 10. Trimetoprim

Todas las sulfamidas comparten en su estructura el grupo p-aminobenceno sulfonamida. Este grupo es esencial en la acción antimicrobiana de las mismas, ya que tiene enormes similitudes con el acido p-aminobenzoico, precursor del ácido fólico y de los ácidos nucleicos en las bacterias. Las modificaciones de este grupo fueron encaminadas a reducir su toxicidad y mejorar su farmacocinética. La actividad antibacteriana de las sulfamidas es similar, por lo que el resultado de un análisis de susceptibilidad en un miembro de este grupo suele ser válido para todas las sulfamidas (Kucers and Bennett, 1975; Botana y col., 2002).

Aunque las sulfamidas son sustancias anfotéricas, las formulaciones comerciales en sales de sodio se comportan como ácidos débiles, por lo cual la alcalinización de la orina aumenta la solubilidad de las sulfamidas en la misma y ayuda a prevenir el problema de cristalización de las antiguas sulfamidas en los túbulos renales (Botana y col., 2002).

La trimetoprina o trimetoprim, la diaminopirimidina más frecuentemente utilizada en combinación con las sulfamidas es la 2,4-diamino-5-(3,4,5-trimetoxibencil)-pirimidina. Se comporta en solución como una base débil, tiene un gran carácter lipófilo y en general se distribuye de forma más rápida que las sulfamidas. Las combinaciones más frecuentes en veterinaria son las de trimetropina con sulfadiazina (Figua 11) o con sulfametoxazol (Figura 12) (Huovinen, 2001; Kucers and Bennett, 1975).

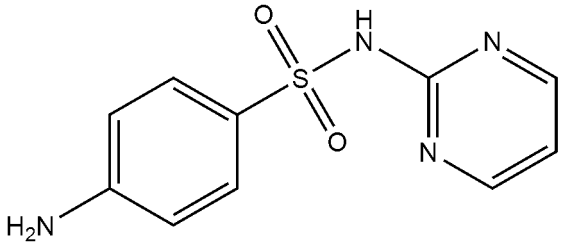


Figura 11. Sulfadiazina

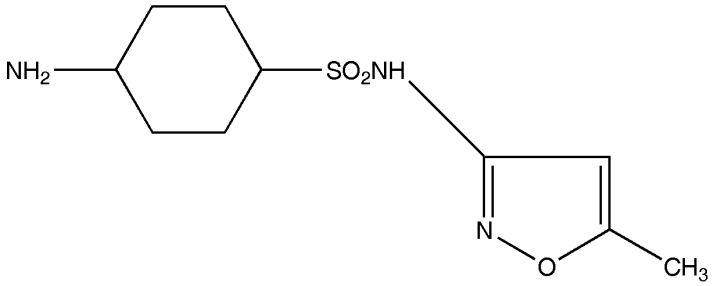


Figura 12. Sulfametoxazol

1.3.7.3.2. Mecanismo de acción de las sulfamidas

El mecanismo de acción de las sulfamidas se basa en la inhibición de la enzima dihydropterato sintetasa (DHPS), la cual cataliza la formación de dihidrofolato a partir del ácido p-aminobenzoico (PABA) y del dihidro-6-hidroximetilpterin-pirofosfato (DHPPP) (Huovinen, 2001; Sköld, 2000), los cuales son utilizados por las bacterias para la síntesis del ácido fólico. A través de una serie de reacciones, el PABA se convierte en ácido dihidrofólico (ácido fólico) con la participación secuencial de las enzimas DHPS y dihidrofolato reductasa (DHFR). Es en este nivel donde actúan las sulfamidas, ya que debido a su gran similitud engañan literalmente a la enzima DHPS, que incorpora la sulfamida en lugar del PABA (Rolan y col., 1979; Sköld, 2000). Esta acción de bloqueo del ácido fólico es competitiva, por lo que se puede invertir aumentando la concentración del PABA en el medio interno bacteriano (Botana y col., 2002).

La carencia de toxicidad para los mamíferos se debe a que éstos utilizan el ácido fólico preformado procedente de la dieta, y de este modo, al no necesitar metabolizar el PABA, las sulfamidas tienen poca actividad. En el siguiente paso, el ácido fólico se transforma en ácido tetrahidrofólico por medio de la enzima DHFR, sobre la que si actúan las pirimidinas utilizadas de forma conjunta con las sulfamidas. No obstante, la afinidad de las pirimidinas por la enzima de los mamíferos es mucho menor que por la de las bacterias. La concentración necesaria para inhibir la enzima de los mamíferos es unas 60.000 veces mayor que la necesaria para inhibir la enzima bacteriana (Rolan y col., 1979; Sköld, 2000; Botana y col., 2002).

1.3.7.3.3. Farmacocinética de las sulfamidas

Las sulfamidas se comportan como ácidos débiles y dado sus elevados valores de pKa suelen existir en los líquidos biológicos de forma molecular neutra. Esto les permite, por norma general, atravesar membranas biológicas con facilidad, por lo que su absorción oral generalmente es extensa y rápida y su distribución en el organismo amplia. La excepción la constituyen las sulfamidas locales, como la sulfaquinixalina, cuya acción es local.

La biodisponibilidad de las sulfamidas es generalmente alta. Su eliminación es una combinación de excreción renal y biotransformación hepática aunque también pueden ser metabolizadas en otros tejidos, por lo que las diferencias entre especies son muy amplias. Su biotransformación es saturable y tiene lugar por acetilación, glucuronización e hidroxilación aromáticas (Prescott y Desmond, 1988).

Aunque las características anteriores son aplicables a un gran porcentaje de sulfamidas, conviene recordar que las diferencias entre fármacos de este grupo pueden ser extremas, con niveles de unión a proteinas plasmáticas que oscilan entre el 20 y el 95% y fracciones ionizadas en sangre que oscilan entre el 1 y el 99%. Estas diferencias se hacen todavía más exacerbadas cuando se consideran diferentes especies, por lo que es necesario tener muy en cuenta el compuesto en particular y la especie en la que se va a utilizar para diseñar una dosificación terapéutica adecuada (Botana y col., 2002).

1.3.7.3.4. Toxicidad y efectos secundarios de las sulfamidas

Las sulfamidas pueden producir una serie de efectos secundarios típicamente reversibles, algunos de los cuales son de naturaleza tóxica, y otros, de carácter alérgico. Con la posología habitual la toxicidad es mínima para los animales domésticos con todas las sulfamidas excepto la sulfaquinoxalina. Cuando existe toxicidad, ésta consiste habitualmente en alteraciones del tracto urinario, de la hematopoyesis o reacciones de hipersensibilidad, aunque de forma mucho más casual se han observado otras alteraciones como hepatitis, alteraciones tiroideas, alteraciones causadas por su antagonismo con el ácido fólico (muy raro a las dosis habituales) o queratoconjuntivitis seca (Prescott y Desmond, 1988).

1.3.7.3.5. Uso clínico de las sulfamidas

La aparición de resistencias después de tantos años de utilización ha llevado a un progresivo abandono de su uso que actualmente es prácticamente total en medicina humana y muy acusado en medicina veterinaria, aunque dentro de ésta última sigue utilizándose sobre todo en forma de sulfamidas potenciadas. De forma general se utilizan para combatir infecciones en el sistema genitourinario y en menor medida en infecciones del aparato digestivo.

En aves se utilizan diversas combinaciones de sulfamidas y diaminopirimidinas y en menor medida sulfamidas solas en el tratamiento y profilaxis de coccidiosis, pasteurelosis y colibacilosis en gallináceas. Las combinaciones de pirimetamina y sulfaquinoxalina se utilizan también en el tratamiento de la coccidiosis tanto en pollos como en pavos (Sköld, 2000; Botana y col., 2002).

1.3.7.3.6. Desarrollo de resistencias

La resistencia a las sulfamidas es bastante frecuente, y debido a la similitud de acción entre los diversos miembros de este grupo, esta resistencia suele ser cruzada, por lo que no es recomendable cambiar de una sulfamida a otra para evitar la aparición de resistencias, sino para mejorar la toxicidad y la farmacocinética. La resistencia puede ser cromosómica o transmitida por plásmidos o transposones, siendo esta última la más frecuente e importante desde el punto de vista clínico. Los mecanismos por los cuales puede estar causada esta resistencia son:

1. Alteraciones en la permeabilidad de la pared celular que impide la entrada de las sulfamidas al interior de la célula.
2. Expulsión del antibacteriano desde el medio intracelular al expulsar por acción de transportadores endógenos activos.
3. Cambios en el grupo p-aminobenceno sulfonamida por la enzima sobre la que actúa como falso sustrato, la dihidropteroato sintetasa.

Un aumento en la concentración del PABA en el medio bacteriano o la presencia de purinas y timidina en lesiones purulentas también disminuye la acción antibacteriana de las sulfamidas. La resistencia a la combinación sulfamida-diaminipirimidina es menos frecuente que cada fármaco por separado, pero también se puede producir (Prescott y Desmond 1988; Sköld, 2000; Botana y col., 2002).

1.3.8. Perspectivas futuras de la antibioterapia veterinaria

Después de la prohibición de los antimicrobianos como aditivos en la alimentación animal llevada a cabo por el Reglamento (CE) 2821/1998 en 1997 (avoparcina) y en 1999 (bacitracina, espiramicina, tylosina y virginiamicina), se constató que su uso descendió notablemente, arrastrando de este modo el consumo total de antimicrobianos. En Dinamarca, por ejemplo, el uso de antimicrobianos en animales cayó más de un 50% desde 206 Tm en 1994 hasta 94 Tm en 2001 (Bager y col., 2002; Philips y col., 2003), debido al abandono del uso de estos antibióticos como promotores del crecimiento. Esto ha llevado paralelo un marcado descenso en la tasa de resistencia a estos agentes (Monnet y col., 2000; Bager y col., 2002; Philips y col., 2003; Mayrhofer y col., 2004) como la práctica desaparición de la resistencia a la avoparcina en los enterococos aislados de carne de pollo y cerdo. En el caso de la resistencia a vancomicina, ésta disminuyó desde un 75% en enterococos aislados de heces de pollo en 1995 hasta un 5% en 2001, y en carne de pollo desde un 20% hasta un 5% en el mismo período. Del mismo modo, la resistencia a virginiamicina en *E. faecium* descendió desde un 60% en 1997-1998 hasta un 30% en heces de pollo y un 5% en carne de pollo en 2001 (Bager y col., 2002).

No obstante, un potencial efecto negativo de la prohibición del uso de antimicrobianos como promotores del crecimiento lo encontramos en el simultáneo incremento de las cantidades consumidas de los antibióticos utilizados como terapeuticos, los cuales pasaron en Dinamarca de 48 Tm en 1996 hasta 94 Tm en 2001 (Bager y col., 2002). Dentro de éstos destacan las tetraciclinas, cuyo consumo aumentó más de un 100%, penicilinas de amplio espectro, sulfamidas potenciadas, macrólidos y aminoglucósidos. Así, aunque hubo más de un 50% de descenso en las cantidades totales consumidas, se ha producido un marcado incremento en el consumo de

antibióticos más comúnmente utilizados en la medicina veterinaria (hay que recordar que en Dinamarca una de las familias más comúnmente utilizadas, las fluoroquinolonas, están prohibidas para uso en animales productores de alimentos). Teniendo en cuenta que ya actualmente la aparición de cepas resistentes a estas familias de antimicrobianos es cada vez más frecuente y que cabe esperar un marcado aumento de su utilización a raíz de a entrada en vigor de la prohibición impuesta por el Reglamento (CE) 1831/2003, parece necesaria la llamada a la prudencia en la práctica clínica para evitar incluso que estos antimicrobianos queden en un futuro próximo invalidados como opción terapéutica.

1.4. Avicultura

En avicultura industrial, al hablar de pollo de carne (broiler), se define a un tipo de ave, de ambos sexos, que se caracteriza fundamentalmente por su rápida ganancia de peso, grandes masas musculares (sobre todo en pechuga y patas), con corto período de crecimiento y engorde (aproximadamente 6-7 semanas). Estas características han convertido al broiler en la base de la producción masiva de carne de consumo habitual (Yagüe, 2005). La popularización del broiler como ave de consumo, se podría achacar a una serie de motivos:

1.- Es una carne muy nutritiva y apta para todas las edades.

2.- Es relativamente barata de producir.

3.- No existe contraindicación en su consumo por motivos religiosos.

Los primeros intentos para la crianza masiva de pollos, se llevaron a cabo en Estados Unidos a finales del siglo XIX, pero no fue hasta la década de los años 1920-1930, cuando empezó a tomarse en serio la explotación de granjas dedicadas a ellos.

En España, la industria del broiler como tal, no comenzó hasta finales de los años 50, con la entrada de las primeras estirpes de aves pesadas procedentes de Estados Unidos; hasta entonces, no se disponía ni de medios técnicos ni de instalaciones. En el último medio siglo, el sector español ha evolucionado de forma semejante al de los países más desarrollados, pero con el inconveniente de la dependencia del exterior. Esto es así, sobre todo en cuanto a genética, alimentación (por tener que importar buena parte

de los cereales y casi la totalidad de la soja) y sector farmacéutico (por la necesidad de los aditivos y productos farmacológicos necesarios para llevar a buen fin la crianza).

1.4.1. Producción mundial de carne de ave y /o de pollo

En primer lugar, hay que distinguir entre "carne de ave" y "carne de pollo", pues la primera, abarca además del pollo desde gallinas de desvieje hasta pavo, pato, perdiz o avestruz.

Así, en relación con el consumo total de carne de ave, la de carne de pollo es de:

- 61% en Francia e Italia.
- 81% en Países Bajos y Reino Unido.
- 90% en España.
- 85% en el resto del mundo.

A nivel mundial, los principales productores son EE. UU, China y Brasil, seguidos en cuarto lugar a nivel mundial por la Unión Europea (Tabla 1). Dentro de ésta, destacan especialmente Reino Unido (17%), España (13%) y Francia (12%) (Figura 6).

PRINCIPALES PAÍSES PRODUCTORES DE CARNE DE POLLO (BROILER)

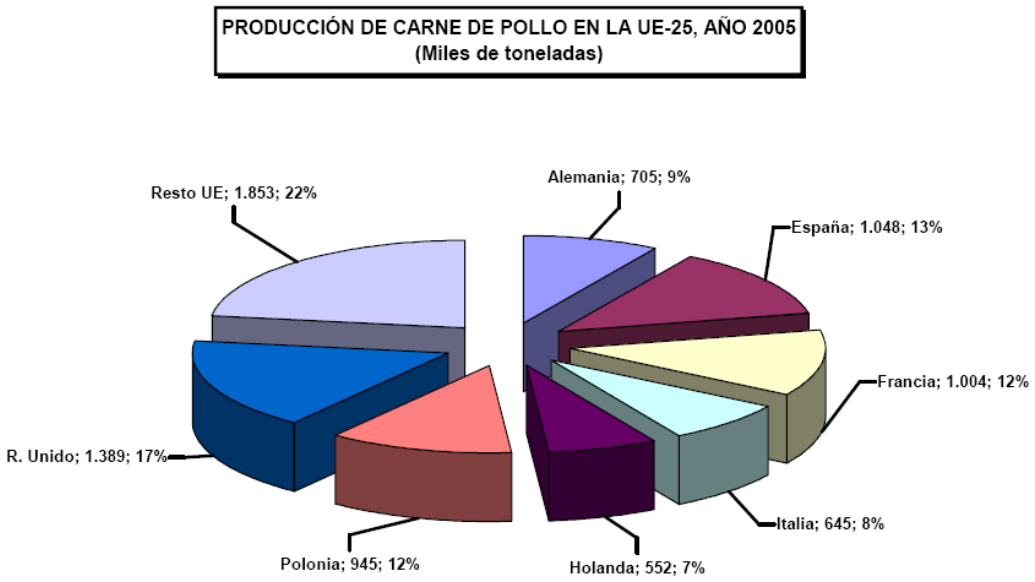
(miles de Tm)

PAISES	1999	2000	2001	2002	2003	2004	2005	%
EE. UU.	13.367	13.703	14.033	14.467	14.696	15.286	15.870	26,8
R.P. CHINA	8.550	9.269	9.278	9.558	9.898	9.998	10.200	17,2
BRASIL	5.526	5.980	6.567	7.449	7.645	8.408	9.360	15,8
U.E. *	**6.286**	**6.181**	**6.486**	**6.434**	**6.294**	**8.021**	**8.141**	**13,7**
MÉJICO	1.784	1.936	2.067	2.157	2.290	2.389	2.510	4,2
INDIA	820	1.080	1.250	1.400	1.500	1.650	1.900	3,2
TAILANDIA	980	1.070	1.230	1.275	1.340	900	950	1,6
JAPÓN	1.078	1.091	1.074	1.107	1.127	1.124	1.165	2,0
CANADÁ	847	877	927	932	929	946	1.000	1,7
MALASIA	684	786	813	784	835	862	896	1,5
ARGENTINA		870	870	640	750	910	1.080	1,8
OTROS	7.304	6.866	6.311	6.598	5.760	5.852	6.165	10,4
TOTAL	**47.226**	**49.709**	**50.906**	**52.801**	**53.064**	**56.346**	**59.237**	**100,0**

Fuente: MAPA 2005

Tabla 1. Principales países productores de carne de pollo

Dentro de las más de 8 millones de Tm. producidas por la Unión Europea, Reino Unido es actualmente el primer productor, con un 17% del total producido, seguido de España, con un 13% o lo que es lo mismo, algo más de 1 millón de Tm (Figura 13).



Fuente: MAPA 2005

Figura 13. Principales países productores de pollo en la CE 2005

1.4.2. Producción y consumo en España de carne de ave y/o pollo

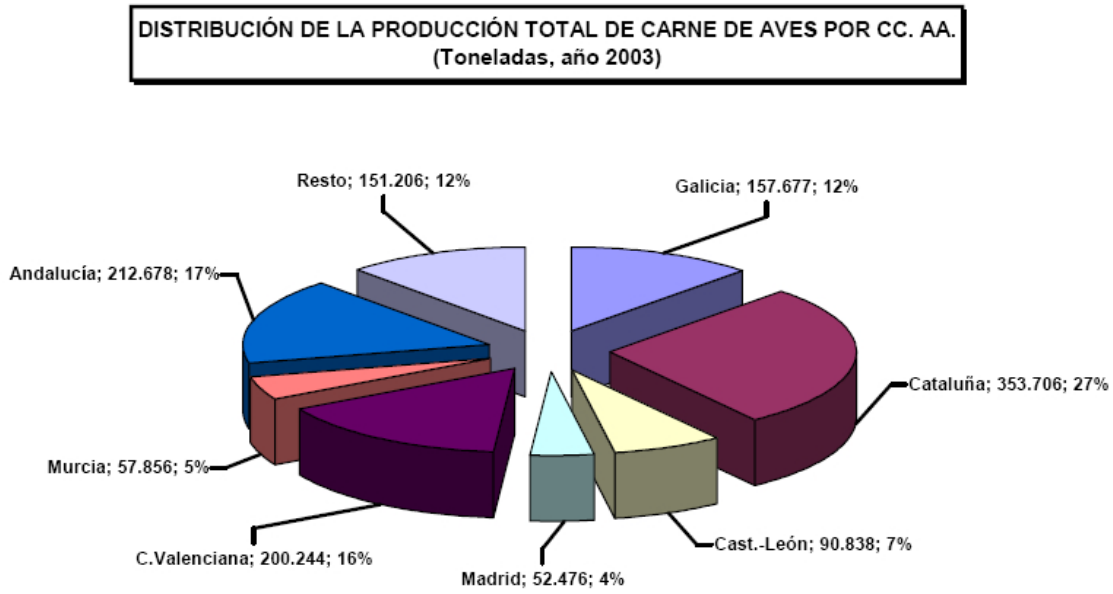
El sector avícola de carne de pollo, representa en España más del 10% de la producción final ganadera, siendo más del 90% cebaderos de pollos de integración (reciben los pollitos con un día de edad y los alimentan hasta el sacrificio).

Existen unos 6.500 cebaderos, en los que se ceban, entre todos, unos 550 millones de pollos; el tamaño de estas plantas, oscila normalmente entre 10.000-100.000 plazas.

Por otro lado, más del 80% del pollo nacional es "pollo blanco", sin pigmentar y sólo un 0,4% de esta ganadería está dedicada a la cría de pollo ecológico.

La producción española de carne de pollo desde los años 60 hasta el año 2005, según MAPA ha aumentado desde 13.000 Tm hasta más de 1 millón de Tm anuales, lo cual nos da idea de la importancia alcanzada por este sector productor dentro de nuestro sector ganadero.

Dentro de nuestro país la producción se centra principalmente en la mitad este de la península. Actualmente, Galicia con una producción anual superior a las 150.000 Tm ocupa la cuarta posición tras Cataluña, la Comunidad Valenciana y Andalucía (Figura 14).



Fuente: MAPA 2005

Figura 14: Principales Comunidades Autónomas productoras de carne de pollo

El consumo real de carne de pollo en España, es difícil de establecer, sobre todo por la lenta pero continua progresión del consumo de productos elaborados en detrimento de las canales enteras. No obstante, según el MAPA el consumo medio alcanzó los 31,6 Kg/ persona en el año 2005. Es la carne más consumida en fresco, y la segunda en consumo total tras la carne de porcino. A pesar de que España se encuentra entre los primeros productores europeos de carne de ave, el balance comercial es habitualmente importador. No obstante, este consumo ha experimentado un cierto declive en los últimos años, posiblemente causado por la mayor accesibilidad de los consumidores a carnes de otras especies tradicionalmente consideradas como de mayor categoría, como el caso de la ternera.

1.4.3. Cría de pollo ecológico

La agricultura ecológica (Figura 15) engloba una serie de mecanismos de producción que pretende compatibilizar la obtención de alimentos para el hombre con la conservación del medio natural. Este tipo de producción nace como respuesta a la agricultura intensiva e industrial (Figura 16) practicada ampliamente en los países desarrollados. Este modelo industrial genera una serie de problemas como pérdida de la calidad intrínseca de los alimentos, la contaminación por residuos (tanto en los alimentos como en el medio ambiente e, incluso, en los propios agricultores), la pérdida de fertilidad de los suelos, la carencia de confort animal y la producción de una gran cantidad de subproductos de difícil eliminación.

Figura 15. Pollos productores de carne criados de forma ecológica

Figura 16. Pollos productores de carne criados de forma convencional

La sensibilidad creciente de la sociedad, así como un mayor grado de comprensión de los procesos vitales por parte de las ciencias agrícolas, ha llevado a la aparición de "nuevos métodos", que persiguen armonizar la consecución de una producción de alimentos rentable y competitiva, con la protección del entorno y la obtención de productos libres de residuos y de elevada calidad nutritiva.

Todo esto ha llevado a establecer una serie de normas y reglamentos que actualmente quedan supeditadas al Reglamento (CE) 1804/1999, por el que se completa, para incluir las producciones animales, el Reglamento (CE) 2092/91 sobre la producción agrícola ecológica y su indicación en los productos agrarios y alimenticios.

1.4.3.1. Requisitos para la producción ecológica de pollo en la Unión Europea

El pollo ecológico es también un pollo de campo, pero sujeto a unas exigencias de producción superiores. Por tanto, se trata de un producto de mayor calidad no sólo organoléptica sino también físico-química, pues en su obtención entra en juego la naturaleza ecológica de los diferentes elementos necesarios para la crianza: las aves, la alimentación, los alojamientos, los terrenos y la sanidad. De este modo, son requisitos fundamentales en este tipo de cría:

- Los terrenos donde vayan a construirse los alojamientos y los parques para las aves han de ser "vírgenes", es decir, ha de poder demostrarse que los mismos no se han utilizado para producciones agrícolas o ganaderas convencionales durante los últimos años.

- Los alojamientos tendrán una capacidad máxima de 4.800 pollos y la superficie total de gallineros en una granja no podrá superar los 1.600 m^2.

- En las construcciones deben evitarse aquellos materiales que potencialmente puedan ser nocivos para la salud de las aves o que puedan ocasionar residuos en su carne.

- Las aves deben tener un origen ecológico, es decir, deben proceder de granjas de multiplicación ecológicas. Al menos a partir del tercer día de edad, los pollos para engorde deben haber sido criados bajo normas ecológicas.

- La alimentación de las aves ha de basarse en los productos agrícolas adecuados, obtenidos por métodos ecológicos en la propia explotación agraria. Pero esto es, en muchos casos, prácticamente imposible, por lo que la reglamentación admite que la alimentación basal –los piensos- puedan provenir de otras explotaciones agrarias ecológicas o de fabricantes que preparen piensos ecológicos con el correspondiente certificado o aval gubernativo. Está permitido el empleo de alimentos para animales convencionales, de forma limitada, en aquellos casos en los que sea imposible obtener alimentos de producción ecológica. El porcentaje máximo permitido es de un 10% para herbívoros y de un 20% para otras especies.

- Se prohíbe en la alimentación de los animales el uso de antibióticos, coccidiostáticos, medicamentos, promotores de crecimiento o cualquier otra sustancia que estimule el crecimiento o la producción.

- Se permite el uso de distintos aditivos en los alimentos que consumen los animales como enzimas, microorganismos, agentes ligantes, antiaglomerantes, coagulantes y auxiliares tecnológicos para ensilar, como por ejemplo sal marina, suero lácteo, azúcar, melaza o harina de cereales.

- En esencia, la alimentación debe estar constituida en su totalidad por productos provenientes de la agricultura ecológica y de productos naturales tales como los minerales y las algas marinas. Se prohíbe el uso de harina de carne y de proteínas del petróleo y las harinas de pescado solamente están toleradas e cantidades limitadas- 3%

para pollos hasta 4 semanas antes del sacrificio y 2% para ponedoras- aunque deben evitarse en lo posible (Castelló Llobet y col., 2002; Reglamento (CE) 1804/1999).

- La fórmula del pienso debe contener, como mínimo, un 70% de cereales y productos proteaginosos u oleaginosos en granos enteros o habiendo sufrido tan solo tratamientos físicos y sin adición de productos químicos se síntesis. El complemento hasta un máximo de 30% debe provenir de subproductos de la agricultura ecológica.

- En lo que respecta a la sanidad, la reglamentación señala que "*debe procurarse que todas las prácticas de manejo se dirijan a conseguir la máxima resistencia a las enfermedades y a prevenir las infecciones*". Si es necesario realizar tratamientos, se recurrirá a la fitoterapia, aromaterapia, homeopatía y otras técnicas naturales (Castelló Llobet y col., 2002).

- Se prohíbe la utilización de medicamentos veterinarios de síntesis química como tratamientos preventivos. Sólo se podrán utilizar los medicamentos de síntesis química cuando se demuestre la ineficacia de los tratamientos descritos en el apartado anterior y bajo la responsabilidad de un veterinario.

- La prevención de enfermedades se basará en la selección de razas adecuadas para los ambientes a donde van a residir, se favorecerá el uso de piensos de alta calidad combinados con el pasto, y se evitará el hacinamiento de los animales.

Estos Reglamentos comunitarios regulan también aspectos relacionados con la importación de productos derivados de la agricultura ecológica procedentes de terceros países, en los que los criterios de producción ecológica y los sistemas de control fueron reconocidos como equivalentes a los vigentes en la Unión Europea.

1.5.3.2. Consumo de alimentos ecológicos

En el marco de las reformas de la Politica Agraria Común (PAC) emprendidas a finales de los años 80, ya se señalaba el reconocimiento del papel fundamental que podía desempeñar la agricultura ecológica en el cumplimientos de algunos de los objetivos planteados en la PAC como la reducción de excedentes, la promoción de productos de alta calidad y la integración en la agricultura de prácticas más respetuosas con el medio ambiente.

En la actualidad, el consumo de alimentos ecológicos está alcanzando tasas de crecimiento en torno al 20% en la Unión Europea. Estamos pues, ante un enorme desarrollo de este tipo de productos, a lo que contribuye la toma de conciencia por parte de los consumidores de las cuestiones relacionadas con la seguridad alimentaria y los problemas medioambientales derivados de los sistemas de cría convencionales. Dichos productos deben presentarse en los puntos de venta al público bajo la denominación "ecológico" o los prefijos "bio" o "eco", y deben llevar el sello que los distingan como certificados por el correspondiente consejo regulador (Figura 17).

Figura 17. Distintos logotipos de etiquetado de alimentos como "ecológicos"

1.5. Importancia de los medios de cultivo cromogénicos

La reciente introducción de los medios de cultivo bacteriano cromogénicos ha permitido mejorar espectacularmente la especificad y rapidez en la detección de los microorganismos diana (Manafi, 1996; Manafi, 2000). Estos medios utilizan la habilidad para detectar la presencia de enzimas específicos y exclusivos de ciertos tipos de microorganismos utilizando sustratos específicos que revelan la presencia de esas enzimas mediante cambios de color. Esta tecnología ha permitido el desarrollo de medios de cultivo capaces de establecer una preidentificación de los microorganismos en su aislamiento primario. La incorporación de esos sustratos a medios de cultivo selectivos puede llegar a conseguir que podamos prescindir de un reaislamiento y de pruebas bioquímicas para establecer la identidad de ciertos microorganismos.

Estos medios de cultivo resultan especialmente útiles para el análisis microbiológico de muestras que presentan una alta contaminación y que por lo tanto presentan una gran cantidad de otros microorganismos diferentes de los buscados que pueden originar confusión en el resultado. Para estos efectos, un nuevo medio selectivo

cromogénico ha sido recientemente diseñado por Merck (Chromocult® enterococi agar) (Figura 18) para el aislamiento y recuento de *Enterococcus* spp. utilizando una mezcla cromogénica destinada a producir una coloración rojiza en presencia de la β-D-glucosidasa propia de los enterococos. Otros microorganismos productores de β-D-glucosidasa, o bien producen coloraciones claramente diferentes de los enterococos, o bien es inhibido su crecimiento mediante la presencia de azida de sodio, un potente inhibidor de sistemas enzimáticos bacterianos implicados en el transporte de electrones (Hartman y col. 1966).

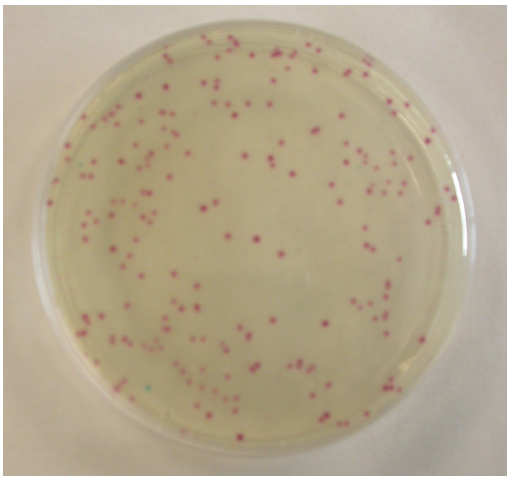


Figura 18. Colonias de *Enterococcus* spp. en Chromocult® enterococi agar

REFERENCIAS

Aarestrup, F.M., Agerso, Y., Gerner-Smiddt, P., Madsen, M., Jensen, L.B. 2000. Comparison of antimicrobial resistance phenotypes and resistance genes in Enterococcus faecalis and Enterococcus faecium from humans in the community, broilers, and pigs in Denmark. Diagn. Microbiol. Infect. Dis. 37, 127-137

Antunes, P., Réu, C., Sousa, J.C., Peixe, L., Pestana, N. 2003. Incidence of *Salmonella* from poultry products and their susceptibility to antimicrobial agents. Int. J. Food Microbiol. 82, 97-103.

Avrain, L., Humbert, F., L´Hospitalier, R.L., Sanders, P., Vernozy-Rozand, C. and Kempf, I. 2003. Antimicrobial resistance in *Campylobacter* from broilers: association with production type and antimicrobial use. Vet. Microbiol., 96, 267-276.

Ball, P. 1998. The Quinolones: history and overview, 2nd ed. Academic Press, San Diego, USA.

Bager, F., Emborg, H.D., Heder, O.E. 2002. DANMAP 2001- Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Demmark. Statens Serum institute, Danish Veterinary and Food Administration, Danish Medicines Agency, Danish Veterinary Institute, Copenhagen, Denmark. ISSN 1600-2032.

Blanco, J.E., Blanco, M., Mora, A., Blanco, J. 1997. Prevalence of bacterial resistance to quinolones and other antimicrobials among avian *Escherichia coli* isolates from septicemic and healthy chickens in Spain. J. Clin. Microbiol., 35, 2184-2185.

Botana López, L.M., Landoni, M.F., Martín-Jiménez, T. 2002. Farmacología y terapéutica veterinaria. McGraw-Hill Interamericana, Madrid.

Casewell, M., C. Friis, E. Marco, P. McMullin, Philips, I. 2003. The European ban of growth-promothing antibiotics and emerging consequences for human and animal health. J. Antimicrob. Chemother. 52, 159-161.

Castelló Llobet, J.A., Cedo Benet, R., Cepero Briz, R., García Martín, E., Pontes Pontes, M., Vaquerizo Florez, J.Mª. 2002. Producción de carne de pollo (2ª edic.). Real Escuela de Avicultura, Barcelona, España.

Chopra, I., Hawkey, P.M., Hinton, M. 1992. Tetraciclines, molecular and clinical aspects. J. Antimicrob. Chemother. 29, 245-277.

Chopra, I., Roberts, M. 2001. Tetracycline antibiotics: Mode of action, applications, molecular biology and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev. 65, 232-260.

Cornican, M., Buckley, V., Corbett-Feeney, G., Sheridan, F. 2001. Antimicrobial resistance in *Escherichia coli* isolates from turkeys and hens in Ireland. J. Antimicrob. Chemother. 48, 587-595.

Desmonts, M.H., Dufour-Gesbert, F., Avrain, L., Kempf, I. 2004. Antimicrobial resistance in *Campylobacter* strains from French broilers before and after antimicrobial growth promoter bans. J. Antimicrob. Chemother. 54, 1024-1030.

Domagk, G. 1935. Beitrag zur Chemotherapie der bakteriellen infektionen. Dtsch. Med. Wschr. 7, 250-253.

Edlind, T.D. 1991. Protein synthesis as a target for antiprotozoal drugs, p 569-586 In G. Coombs and M. North (ed.), Biochemical protozoology. Taylor & Francis, London, U.K.

Endzt, H.P., Ruijs, G.J., Van Klingeren, B., Jansen, W.H., Van der Reyden T., Mouton, R.P. 1991. Quinolone resistance in *Campylobacter* isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. J. Antimicrob. Chemother. 27, 199-208.

Ermel, G., Laisney, M.J., Rivoal, K., Ragimbeau, C., Gorin, S., Salvat, G., Colin, P. 2000. Les *Campylobacter* et la filière avicole. Sci. Techn. Avicoles. 20, 3–6.

Fairchild, A.S., Smith, J.L., Idris, U., Lu, J., Sanchez, S, Purvis, L.B., Hofacre, C., Lee, M.D. 2005. Effects of orally administered Tetracycline on the intestinal community structure of chickens and on *tet* determinat carriage by commensal bacteria and *Campylobacter jejuni*. Appl. Environ. Microbiol. 71, 5865-5872.

Falkiner, F.R. 1998. The consequences of antibiotic usage in horticulture. J. Antimicrob. Chemother. 41, 429-431.

Finch, R.G. 1997. Tetracyclines, p. 469-484. In O`Grady HP, Lambert RG. Finch, Greenwood D. eds. Antibiotic and chemotherapy, 7th ed. Edingurgh: Churchill Livingstone Ltd., Edinburgh.

Franco Abuín, C.M., Quinto Fernández, E.J., Fente Sampayo, C., Rodríguez Otero, J.L., Domínguez Rodríguez, L., Cepeda Sáez, A. 1994. Susceptibilities of

Listeria species isolated from food to nine antimicrobial agents. Antimicrob. Agents Chemother. 38, 1655-1657.

Freeman, C.D., Nightingale, C.C., Quintiliani, R. 1994. Minocycline: old and new therapeutic uses. Int. J. Antimicrob. Agents. 4, 325-335.

Gibreel, A., Sjögren, E., Kaijser, B., Wretlind, B., and Sköld, O. 1998. Rapid emergence of high-level resistance to quinolones in *Campylobacter jejuni* associated with mutational changes in *gyrA* and *par*C. Antimicrob. Agents Chemother. 42, 3276-3278.

Griggs, D.J., Johnson, M.M., Frost, J.A., Humphrey, T., Jorgensen, F., Piddock, L.J.V. 2005. Incidence and mechanism of ciprofloxacin resistance in *Campylobacter* spp. Isolated from commercial poultry flocks in the United Kingdom before, during and after fluoroquinolone treatment. Antimicrob. Agents Chemother. 49, 699-707.

Gurbay, A., Garrel, C., Osman, M., Richard, M.J., Favier, A., Hincal, F. 2001. Microsomal metabolism of ciprofloxacin generates free radicals. Frre Radical Bio. Med. 30, 1118-1121.

Heuer, O.E., Pedersen, K., Andersen, J.S., Madsen, M. 2001. Prevalence and antimicrobial susceptibility of thermophilic *Campylobacter* in organic and conventional broiler flocks. Lett. Appl. Microbiol. 33, 269-274.

Huovinen, P. 2001. Resistance to trimethoprim-sulfamethoxazole. Clin. Infec. Dis. 32, 1608-1614.

Joseph, S.W., Hayes, J.R., English, L.L., Carr, L.E., Wagner, D.D. 2001. Implication of multiple antimicrobial-resistant associated with the poultry environment. Food Add. Contaminants. 18, 1118-1123.

Kijima-Tanaka, M., K. Ishihara, A. Morioka, A. Kojima, T. Ozono, K. Ogikubo, T. Takahashi, and Y. Tamura. 2003. A national surveillance of antimicrobial resistance in *Escherichia coli* isolated from food-producing animals in Japan. J. Antimicrob. Chemother. 51, 447-451.

Köhler, T., Pechere, J.C. 1998. Bacterial resistance to quinolones: mechanisms and clinical implications. In V.T. Andriole (ed.), The quinolones. Academic Press, London, United Kingdom.

Kordick, D.L., Papich, M.G., Breitschwerdt, E.B. 1997. Efficacy of enrofloxacin or doxycycline for treatment of *Bartonella henselae* or *Bartonella clarridgeiae* infection in cats. Antimicrob. Agents Chemother. 41, 2448-2455.

Kucers, A., Bennett, N.M. 1975. The use of antibiotics: a comprehemsive review with clinical emphasis. William Heinemann Medical Books, London, United Kingdom.

Ley 25/1990, de 20 de Diciembre, del medicamento. BOE 22 Diciembre 1990 Nº 306, 38228-38279.

Liss, R.H., Batchelor, F.R. 1987. Economic evaluations of antibiotic use and resistance-a perspective: report of task force 6. Rev. Infect. Dis. 9(Suppl. 3): S297-S312.

Luber, P., Wagner, J., Hahn, H., Bartelt, E. 2003. Antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter coli* strains isolated in 1991 and 2001-2002 from poultry and humans in Berlin, Germany. Antimicrob. Agents Chemother. 47, 3825-3830.

Magkos, F., Arvaniti, F., Zampelas, A. 2006. Organic food: buying more safety or just peace of mind? A critical review of the literature. Crit. Rev. Food Sci. 46, 23-56.

Mallon, D.J.P., Corkill, J.E., Hazel, S.M, Sian Wilson, J., French, N.P., Bennett, M., Hart, C.A. 2002. Excretion of vancomycin-resistant enterococci by wild animals. Emerg. Infect. Dis. 8, 636-638.

Manafi, M. 1996. Fluorogenic and chromogenic substrates in culture media and identification test. Int. J. Food Microbiol. 31, 45-48.

Manafi, M. 2000. New developments in chromogenic and fluorogenic cultura media. Int. J. Food Microbiol. 60, 205-218.

MAPA,2005:
http://www.mapa.es/notas/documentos/AVICALT_PRESENTACI%C3%93N_DGG_30-06-05.ppt#22. Accedido el 20-3-2007.
www.mapa.es/app/SCH/indicadores/IndicadoresCA.aspx?lng=es. Accedido el 20-3-2007.

Mayrhofer, S., Paulsen, P., Smulders, J.M., Hilbert, F. 2004. Antimicrobial resistance profile of five major food-borne pathogens isolated from beef, pork and poultry. Int. J. Food Microbiol. 97, 23-29.

McDermott, P.F., Bodeis, S.M., English, L.L., White, D.G., Walker, R.D., Zhao, S., Simjee, S. and Wagner, D. 2002. Ciprofloxacin resistance in *Campylobacter*

jejuni envolves rapidly in chickens treated with fluoroquinolones. J. Infect. Dis. 185, 837-840.

Monnet, D.L. 2000. Toward multinational antimicrobial resistance surveillance systems in Europe. Int. J. Antimicrob. Agents. 15, 91-101.

Pedersen, K., Wedderkopp, A. 2003. Resistance to quinolones in *Campylobacter jejuni* and *Campylobacter coli* from Danish broilers at farm level. J. Appl. Microbiol. 94, 111-119.

Peters, J., Mac, K., Wichman-Schaue, H., Klein, G., Ellerbroek, L. 2003. Species distribution and antibiotic resistance patterns of enterococci isolated from food of animal origin in Germany. Int. J. Food Microbiol. 88, 311-314.

Philips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preston, R., Waddell, J. 2003. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. J. Antimicrob. Chemother. 53, 28-52.

Poole, K. 2000a. Efflux-mediated resistance to fluoroquinolones in Gram-negative bacteria. Antimicrob. Agents Chemother. 44, 2233-2241.

Poole, K. 2000b. Efflux-mediated resistance to fluoroquinolones in Gram-positive bacteria and the mycobacteria. Antimicrob. Agents Chemother. 44, 2595-2599.

Prescott, J.F., Baggot, J.D. 1988. Antimicrobial therapy in veterinary medicine. Blackwell scientific cop., Boston, USA.

Prescott, J.F., Dowling, P.M. 2000. Agriculture`s role in managing antimicrobial resistance: conference report. Canad. Vet. Jour. 41, 191-197.

Real Decreto 109/1995, de 27 de Enero Medicamentos. Regula los medicamentos veterinarios. B.O.E. 3 Marzo 1995, Nº 53, 15660-15664.

Reglamento (CE) 1831/2003 de 15 de febrero de 2005, relativo a la autorización de determinados aditivos en la alimentación animal. D.O.U.E. Nº 268, 29-40.

Reglamento (CE) 2377/1990 de 26 de Junio, por el que se establece un procedimiento comunitario de fijación de los límites máximos de residuos de medicamentos veterinarios en los alimentos de origen animal. D.O.L. 18 Agosto 1990 Nº 224, 1-8.

Reglamento (CE) 2821/1998 de 17 de Diciembre, por el que se modifica la Directiva 70/524/CEE sobre los aditivos en la alimentación animal, en lo que respecta a

la revocación de la autorización de determinados antibióticos. D.O.L. 31 Diciembre 2002 Nº 351,1-142.

Reglamento 1804/1999 de 19 de Julio. Producción Agrícola Ecológica. Completa, para incluir las producciones animales, el reglamento (CEE) núm 2092/91, de 24 de Junio de 1991, sobre producción agrícola ecológica y su indicación en los productos agrarios y alimenticios. D.O.L. Nº 222, 1-28

Reglamento 2092/1991 de 24 de Junio, sobre la producción agrícola ecológica y su indicación en los productos alimenticios. D.O.L. Nº 198, 1-15.

Roland, S., Ferone, R., Harvey, R.J., Styles, V.L., Morrison, R.W. 1979. The characteristics and significance of sulfonamides as substrates for *Escherichia coli* dihydropterase synthase. J. Biol. Chem. 254, 10337-10345.

Ross, J.I., Eady, E.A., Cove, J.H., Cunliffe, W.J. 1998. 16S rRNA mutation associated with tetracycline resistance in a gram-positive bacterium. Antimicrob. Agents Chemother. 42, 1702-1705.

Schnappinger, D., Hillen, W. 1996. Tetracyclines: antibiotic action, uptake, and resistance mechanism. Arch. Microbiol. 165, 359-369.

Sköld, O. 2000. Sulfonamide resistance: mechanisms and trends. Drug Resist. Upd. 3, 155-160.

Stern, N.J., Robach, M.C. 2003. Enumeration of *Campylobacter* spp. in broiler faeces and in corresponding processed carcasses. J. Food Prot. 66, 1557-1563.

Talens-visconti, R., Garrigues, T.M., Cantón, E. 2002. Mecanismos de resistencia bacteriana a las quinolonas. Revista española de Quimioterapia. 15, 25-31.

Tejedor-Junco, M.T., Afonso-Rodriguez, O., Martín-Barrasa, J.L., González-Martín, M. 2005. Antimicrobial susceptibility of *Enterococcus* strains isolated from poultry faeces. Res. Vet. Sci. 78, 33-38.

Uyttendaele, M., Detroy, P., Debevere, J. 1999. Incidence of *Salmonella*, *Campylobacter jejuni*, *Campylobacter coli*, and *Listeria monocytogenes* in poultry carcasses and different types of poultry products for sale on the Belgian retail market. J. Food Prot. 62, 735–740.

Van den Boogaard, A.E., Willens, R., London, N., Top, J., Stobbering, E.E. 2002. Antibiotic resistance of faecal enterococci in poultry, poultry farmers and poultry slaughterers. J. Antimicrob. Chemother. 49, 497-505.

Van den Bogaard, A.E., London, N., Driessen, C. and Stobberingh, E.E. 2001. Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. J. Antimicrob. Chemother. 47, 763-771.

Vidaver, A.K. 2002. Uses of antimicrobials in plant agriculture. Clin. Infect. Dis. 34, Suppl. 3, 107-110.

Von Baum, H., Marre, R. 2005. Antimicrobial resistance of *Escherichia coli* and therapeutic implications. Int. J. Med. Microbiol. 295, 503-511.

Willians, D.N. 1992. Tetracyclines. *In* Gorbach, S.L. y Blacklow, N.R. (ed.), Infectous Diseases. The W. B. Saunders Co., Philadelphia, USA.

Wiuff, C., Lykkesfeldt, J., Svendsen, O., Aarestrup, F.M. 2003. The effects of oral and intramuscular administration and dose escalation of enrofloxacin on the selection of quinolone resistance among *Salmonella* and coliforms in pigs. Res. Vet. Sci., 75, 185-193.

Yagüe, A. 2005. Carcaterística de la demanda de pollo en España y otros países. Jornadas profesionales de avicultura de carne, Valladolid, España. 25-27 Abril.

2. OBJETIVOS

Los objetivos principales de este trabajo son:

1. Determinar la evolución de la microbiota intestinal durante un tratamiento terapéutico normal con los grupos de antibióticos más frecuentemente utilizados en avicultura (tetraciclinas, quinolonas y sulfamidas).

2. Determinar el grado de resistencia bacteriana a estos antimicrobianos alcanzado durante el correspondiente tratamiento terapéutico.

3. Comprobar la eficacia del período de supresión para la recuperación de las tasas de resistencia de las diferentes poblaciones bacterianas intestinales estudiadas hasta los niveles previos al inicio del tratamiento antimicrobiano.

4. Determinar las posibles diferencias en el nivel de contaminación bacteriana, así como en la resistencia de éstas bacterias a diversos tipos de antimicrobianos, en la carne de animales de producción "ecológica" y "convencional".

5. Evaluar la utilidad de la monitorización de la resistencia a antimicrobianos de las bacterias contaminantes de la carne como herramienta para la detección de tratamientos antimicrobianos no permitidos en los sistemas de producción "ecológicos".

6. Evaluación del medio de cultivo selectivo Chromocult® enterococci agar para la cuantificación y aislamiento selectivo de *Enterococcus* spp. en muestras altamente contaminadas por este género bacteriano.

3. RESULTADOS Y DISCUSIÓN

3.1. Evolución de *Escherichia coli* intestinal durante tres tratamientos antimicrobianos comúnmente utilizados en pollo.

Evolution of resistance of poultry intestinal *Escherichia coli* along three commonly used antimicrobial therapeutic treatments in poultry.

Miranda, J.M., Vázquez, B.I., Fente, C.A., Barros-Velázquez, J., Franco, C.M., Cepeda A.

Artículo enviado para su publicación en el Journal of the Science of Food and Agriculture.
Fecha de envío: Octubre 2006.

Evolution of resistance of poultry intestinal *Escherichia coli* along three commonly used antimicrobial therapeutic treatments in poultry

[a]Miranda, J.M., [a]Vázquez, B.I., [a]Fente, C.A., [b]Barros-Velázquez, J., *[a]Franco, C.M., and [a]Cepeda A.

[a]Laboratorio de Higiene Inspección y Control de Alimentos. Dpto de Química Analítica, Nutrición y Bromatología. Facultad de Veterinaria. Universidad de Santiago de Compostela. 27002-Lugo. Spain.
[b]Area de Tecnología de los Alimentos. Dpto de Química Analítica, Nutrición y Bromatología. Facultad de Veterinaria. Universidad de Santiago de Compostela. 27002-Lugo. Spain.

Running title: E. coli resistance evolution in antimicrobial treatments in poultry.

ABSTRACT

The resistance rates of intestinal Escherichia coli populations in poultry were determined during treatment and withdrawal period with three antimicrobial agents commonly used in poultry medicine. A total of 108 chickens were considered: 18 were treated orally with enrofloxacin, 18 with doxycycline and 18 with sulfonamides, while another 18 chickens were maintained as controls for each antimicrobial group. Faecal samples were taken during the treatment and after the withdrawal period. A total of 648 E. coli strains (216 per antimicrobial agent tested) were isolated, identified and the Minimal Inhibitory Concentrations (MICs) to the antimicrobials used were determined using a standardized broth microdilution method. The resistance rates of intestinal E. coli to all antimicrobials tested significant increased during the course of treatment. Significant (P=0.0136) differences in resistance rates persisted until the end of the withdrawal period between the treated and control batches for enrofloxacin, but not for doxycycline or the sulfonamides.

Keywords: E. coli, poultry, resistance, enrofloxacin, doxycycline, sulfonamides

1. Introduction

The risks associated to the use of antimicrobial agents for the therapeutic treatment of sick animals has been subject of debate in the recent years. Thus, on one hand, some scientists are very concerned about the potential consequences, such as the development and spread of bacterial resistance, while others state that there is not enough evidence that might point towards such potential risk, or even consider potential human health benefits derived from antibiotic use in food animals.[1-3]

In poultry farming, as well as with other intensively reared animals, antibiotics may be administered to whole flocks rather than individual animals. In the EC, the administration of antimicrobials with the water or feed to animals at smaller doses than those employed for therapeutic purposes in order to enhance animal growth is totally banned since January 2006. After the ban of the use of avoparcin, bacitracin, tylosin, espiramicin and virginiamicin as growth promoters in the 1990s, the level of bacterial resistance to such antimicrobials decreased considerably. Nevertheless, increasing amounts of antimicrobial agents have been used with therapeutic purposes in veterinary medicine since then[2,4]

In poultry medicine, the antimicrobials used as therapeutics are generally administered by feed or drinking water. Together with tetraciclines, quinolones form one of the most widely used antimicrobial families in poultry therapy.[5] Sulfonamides are also an important class of antibacterial compounds used in veterinary practice, as well as in human medicine for the treatment of some bacterial, protozoal or fungal infections. [6-7]

E. coli is commonly found in the intestinal tract of humans and animals. Its use as an indicator bacterium is useful because changes in the resistance of this species may serve as a good indicator of resistance in potentially pathogenic bacteria, since E. coli readily acquires the antimicrobial resistance commonly found in different animal species.[9,10]

Poultry food products are an important source of E. coli since at the time of slaughter faecal contamination from the gut readily pollutes poultry carcasses and as result poultry meat may be contaminated with E. coli.[11,15] Other authors have reported that vegetables may also be contaminated from animals via sewage and manure, which

may act as a resistant bacterial source.[2] These resistant bacteria may colonize the human intestinal tract and may also be a source of resistance genes to human endogenous microflora.[15] Hence, resistant E. coli from poultry selected during the veterinary antimicrobial treatments can infect humans both directly and via food.

The aims of the present work were to determine the degree of antimicrobial resistance of E. coli during the most commonly used antimicrobial treatments and after their withdrawal period. Such period is determined as a function of the persistence of antimicrobial residues in animal tissues. Thus, it is of crucial interest the investigation of the resistance rates of the bacterial populations present in the animals intestine, since this may provide valuable information to establish the safety of these animal foods when the withdrawal period has just finished.

2. Materials and methods

2.1. Animals and treatments employed

A total of 108 healthy "label" chickens aged 4-6 weeks obtained from the same commercial hatchery were used. They were fed with the same antibiotic-free feed before starting antimicrobial treatment. These birds were split into three groups of thirty-six per group. In each of these three groups, 18 broilers were treated with a therapeutic antimicrobial dosage and another 18 were maintained as controls. None of the chickens had direct physical contact with any of the other birds during the assays since they were housed in individual cages in the animal facility. Contamination between the treated and untreated batches was prevented by housing them in different rooms. The poultry were fed twice daily with antibiotic-free commercial poultry feed and had free access to the medicated water, in the case of the treated poultry, or to antimicrobial-free water in the case of the control batches. Determination of water intake was performed at 12 h intervals.

All parts of this study were carried out according to EC Council Directives concerning the laws, regulations and administrative provisions of the member states regarding the protection of animals used for experimental and other scientific purposes.

2.2. Enrofloxacin treatment

Eighteen birds were weighed and treated with a therapeutic dose of enrofloxacin in water (0.05 g l^{-1}) of Colmyc-E® (S.P. Veterinaria, Tarragona, Spain) over 5 days, in accordance with the manufacturer instructions, and another 18 were weighed and kept untreated as controls. Each group of chickens was sampled immediately before starting treatment (day 0); on the first day of treatment (day 1); on the third day of treatment (day 3); on the last day of treatment (day 5); 6 days after the end of the treatment (day 11), and after the withdrawal time had ended (day 17). Faecal samples were taken by swabbing the cloacae of each bird with sterile swabs to obtain a minimum of 0.5 g of faecal content, which was placed aseptically in a sterile tube.

2.3. Doxycycline treatment

Eighteen birds were weighed and treated with a therapeutic dose of doxycycline in water (1 g l^{-1}) of Doxidol® (Fatro Uriach Veterinaria, Barcelona, Spain) over 5 days, in accordance with the manufacturer instructions, and another 18 were weighted and kept as controls. Each group of chickens was sampled immediately before starting treatment (day 0), on the first day of treatment (day 1); on the third day of treatment (day 3); on the last day of treatment (day 5); 3 days after the end of the treatment (day 8), and after the withdrawal time had ended (day 12). Faecal samples were taken by swabbing the cloacae of each bird with sterile swabs to obtain a minimum of 0.5 g of faecal content, which was placed aseptically in a sterile tube.

2.4. Sulfonamides treatment

Eighteen birds were weighed and treated with a therapeutic dose of a sulfonamides mixture (1.33 g Sulfaquinoxaline + 1.66 g Sulfamethazine + 1.66 g Sulfameracine + 3.33 g sulfisoxazole /100 ml) in water (15 ml l^{-1}) from Cunisan Aviar® (Arimany,

Barcelona, Spain) over 4 days. This was followed 2 days of repose and another 3 days of treatment, in accordance with the instructions on the package insert. A further 18 were weighed and kept as controls. Each group of chickens was sampled immediately before starting treatment (day 0); on the first day on treatment (day 1); on the fourth day of treatment (day 4); on the last day of treatment (day 9); seven days after the end of the treatment (day 16), and after the withdrawal time had ended (day 24). Faecal samples were taken by swabbing the cloacae of each bird with sterile swabs to obtain a minimum of 0.5 g of faecal content, which was placed aseptically in a sterile tube.

2.5. Isolation and identification of E. coli

Faecal samples were taken to the laboratory in an ice chest in less than half an hour for immediate processing. The faecal samples of 3 animals belonging to the same group were placed together in a sterile masticator bag with an appropriate volume (1/9) (w/v) of sterile buffered peptone water (Merck, Darmstadt, Germany) and subsequently homogenized with a masticator (Aes, Combourg, France) for 2 m. After homogenisation, samples were tested for isolation and identification of E. coli. 1 ml of 10^{-3} to 10^{-7} dilutions of homogenates were tested in poured plates of Fluorocult® agar prepared as specified by the manufacturer (Merck). After the agar had solidified, the plates were overlaid with 3-4 ml of melted Fluorocult® and incubated at 44 ºC for 24 h. After incubation, pink to red colonies showing blue florescence after exposure to a 365 nm ultraviolet lamp were considered as E. coli.

After incubation, 3 typical colonies were harvested and transferred onto Columbia agar with 5 g Kg^{-1} Sheep blood (BioMérieux, Marcy l`Etoile, France) and incubated at 44º C for 24 h in order to obtain pure cultures. This was done for each batch of chickens (three birds) on each sampling day (18 strains per group and day), a total of 648 strains being obtained (216 strains per antimicrobial tested).

These pure cultures were characterized by colony and cell morphology, Gram stain, methyl red, citrate test, oxidase and catalase activity and indole production. Positive strains were confirmed by API 20E (BioMérieux).

All isolates were stored at -80 °C until further analysis using Maintenance Freeze Medium units (Oxoid, Basingstoke, UK).

2.6. Antibiotic susceptibility testing of bacteria

Antibiotic susceptibility testing was performed using a broth microdilution susceptibility test on micro-titre plates to determine Minimum Inhibitory Concentrations (MICs). MICs and levels of resistance were determined according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS).[16] Standard antimicrobial dilutions were obtained with an enrofloxacin reference standard (Bayer AG, Leverkusen, Germany), doxycycline (Fluka, St Gallen, Switzerland) and sulfisoxazole (Sigma Chemical Co, St. Louis, USA). The final concentration ranges were 0.008 to 64 μg ml^{-1} for enrofloxacin, 0.25 to 128 μg ml^{-1} for doxycycline and 4 to 2048 μg ml^{-1} for sulfisoxazole. Each tray also contained a positive and a negative growth control well.

The microtitre plates were incubated for 18 to 24 h at 37ºC with a source of moisture to prevent dehydration. MICs were determined by visual observation of the lowest concentration yielding no visible growth for enrofloxacin and doxycycline in the wells. For sulfisoxazole, the MIC was defined as the concentration of the drug in the well that elicited approximately 80% inhibition of growth as compared to the growth in the control wells with no drug added. The MICs used were those recommended by the CLSI for veterinary pathogens: ≥ 2 μg ml^{-1} for enrofloxacin, ≥ 16 μg ml^{-1} for doxycycline, and ≥ 512 μg ml^{-1} for sulfisoxazole. The MICs that inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of total strains were calculated from the MIC values.

Target MIC ranges were verified with E. coli ATCC 25922 reference strain as quality control. Quality control was considered acceptable if the results obtained were within ranges recommended by the CLSI.[16]

2.7. Statistical analysis

The amounts of water ingested by the treated and control birds were compared using an unpaired Student's t test. The distributions of resistant strains were compared by means of the X^2 test and Fisher's exact test. Differences were considered significant when probabilities were lower than 0.05. All statistical analyses were carried out using Statgraphics version 5.0.1. (SAS Institute, North Carolina, USA).

3. Results

3.1 Enrofloxacin treatment

For enrofloxacin treatment, the average amount of water ingested by the treated animals during the 5 days of treatment was 621 mL (SD=269.93) and for the untreated birds was 682.83 mL (SD=168.59), *P*=0.643. These results clearly indicate that enrofloxacin treatment had no effect on the final amount of water ingested. Furthermore, total consumption of the antimicrobial during the 5 days of treatment was 31.07 mg of enrofloxacin/bird (SD=13.136) (6.21 mg per bird and day). Since the average weights of the chickens employed on day 0 was 552 g (SD=114.32), the real dose ingested was 11.25 mg kg^{-1} of body weight and day.

The results obtained for the MIC_{50}, MIC_{90}, MIC ranges and resistance rates are shown in Table 1. There were no significant differences between the control and treated batches as regards the MIC profile on day 0. Likewise, as expected there were no significant differences in the control batches during the assay.

Immediately after starting the treatment, pre-existing enrofloxacin resistant E. coli populations were rapidly selected and this resistance was maintained until the end of withdrawal period (88.9% *vs* 22.2% in the control batches, respectively). Thus, significant differences were obtained from day 1 to day 17 with respect to the resistance rates of the treated and control chickens. Such resistance rates reached their higher value for treated poultry on day 5 (88.9%), thereafter remaining at this level until day 17. For the control batches, the resistance rates ranged from 16.7% to 50%.

3.2. Doxycycline treatment

In the case of doxycycline treatment, the average amount of water ingested by the treated animals during the 5 days of treatment was 429.67 mL (SD=113.28) and, in the case of the untreated birds was 524.17 mL (SD=173.30), *P*=0.290. Such results indicate that the doxycycline treatment had no effect on the final amount of water ingested. Thus, the average antimicrobial consumption per bird during the 5 days of treatment was 42.97 mg of doxycycline/bird (8.59 mg per bird and day) (SD=13.28). Since the average weight of the chickens used on day 0 was 638 g (SD=109.26), the real dose ingested was 13.47 mg kg^{-1} of body weight and day.

The results obtained for the MIC_{50}, $MIC_{90,}$ MIC ranges and resistance rates are shown in Table 2. No significant differences were observed between the control and treated batches as regards the MIC profile on day 0. Likewise, as expected there were no significant differences for the MIC profile in the control batches during the assay.

During the treatment, pre-existing doxycycline resistant E. coli populations were rapidly selected and this resistance was maintained until the end of the withdrawal period (44.4% *vs* 16.7% in the control batches, respectively). Significant differences were obtained on days 3, 5 and 8 with respect to the resistance rates of the treated and control chickens, respectively. These resistance rates reached their higher value for treated poultry at the end of the withdrawal period (44.4%). For the control batches, resistance rates ranged from 0% to 16.7%.

3.3. Sulfonamide treatment

For the sulfonamides, the average amount of water ingested by the treated animals was 623 mL (SD=182.18) and for untreated birds was 596 mL (SD=109.86). This result shows that sulfonamides treatment had no effect on the final amount of water ingested. Thus, total consumption of the antimicrobials during the 9 days of treatment was 870.02 mg sulfonamides/bird (SD=254.42) (124.29 mg per bird and day). Since the average weights of the chicken employed on day 0 was 678 g (SD=175.26), the real dose ingested was 183.32 mg kg^{-1} of body weight and day.

The results obtained for the MIC_{50}, MIC_{90}, MIC ranges and resistance rates are shown in Table 3. There were no significant differences between the control and treated batches as regards the MIC profile on day 0. Likewise, as expected there were no significant differences for the MIC profile in the control batches during the assay.

As in the cases reported above, immediately after starting the treatment, pre-existing sulfonamides resistant E. coli populations were rapidly selected and this resistance was maintained until the end of withdrawal period (94.4% *vs* 61.1% in the control batches, respectively). One more time, significant differences were obtained from day 1 to day 16 with respect to the resistance rates of the treated and control chickens. These resistance rates reached their higher value for the treated poultry on day 4 and 9 (100%). For the control batches, the resistance rates ranged from 11.1% to 61.1%.

4. Discussion

In the case of enrofloxacin, the E. coli resistance rates obtained in this work before starting treatment resulted to be higher than those reported by the European surveillance programmes, although the rates observed in this study are in agreement with previous data reported by other authors.[18-20] Thus, the E. coli quinolone resistance reported in recent years, such as the 10% of ciprofloxacin resistance described in the Netherlands or the 88% of nalidixic acid resistance, and 38% of ciprofloxacin resistance in E. coli isolated from Spanish supermarket poultry products, shows that this resistance is increasing in the recent years.[11,15]

This increase in the resistance of E. coli to quinolones seems to be caused by the wide use of these antimicrobials for veterinary purposes in recent years. It has been documented that ciprofloxacin resistance in E. coli isolated from broilers is higher than when the microorganism is isolated from other sources such as pigs and humans. This seems be due to the higher use of quinolones in chickens than in pigs or humans.[9,11] Also, a recent work has shown that quinolone resistance in E. coli isolated from broilers previously dosed with quinolones was significantly higher than the resistance of E. coli isolated from poultry without exposure to quinolones (49.5% *vs* 33.7%, respectively).[21]

In the case of doxycycline, in recent years bacterial resistance has been widely documented in the case of certain respiratory pathogens, but few authors have addressed its resistance in pathogens of animal origin, such as E. coli.[22-24]

This is very important since doxycycline is widely used in veterinary medicine, especially in chickens and turkeys and especially in developing nations.[25] The resistance rates obtained in this work before the start of treatment were relatively lower as compared to those established by other authors for tetracyclines in E. coli isolated from poultry faeces as 75% or the 43.8% of resistance to tetracycline obtained by other authors in E. coli isolated from broilers in Spain. [10,19] Nevertheless, this may be compatible with the resistance rates obtained in the present work since it has been documented that some tetracycline-resistant bacteria may be sensitive to doxycycline.[16]

In the case of sulfisoxazole, the resistance rates obtained in this study before starting treatment and in the control batches (55.6%-61.1%, respectively) were in agreement with previously reported data, such as the 69.7% of sulfadimethoxine-resistant strains obtained in E. coli strains isolated from poultry in Japan. Or the 52.1% of resistance obtained for the trimethoprim-sulfamethoxazole in E. coli isolated from broilers in Spain.[9,20]

In order to these results, during the three treatments administered, pre-existing resistant E. coli populations were rapidly selected and this resistance was maintained until the end of the withdrawal period. Taking into account that at the time of slaughter poultry meat may be contaminated with faecal E. coli, and the fact that treated chickens are often sent to the slaughterhouse immediately after the withdrawal period, these resistant bacteria selected by the antimicrobial treatments could be a risk for public health after poultry slaughter and processing.

Moreover, the resistant E. coli strains selected by the antimicrobial treatments might reach humans *via* other animals, sewage, or other humans such as farmers or slaughterers.[2]

According to the results obtained in the present work, these indirect ways of transmission could be also an important way of transmission of resistant bacteria to human, since the three antimicrobials evaluated in this work effectively selected antimicrobial resistant populations during the corresponding treatments.

Taking into account that after the ban of the use of antimicrobials as grown promoters in the EC is expected an increment of quantities consumed of antimicrobials used as therapeutics, an undesirable consequence of this prohibition would be able to be the loss of efficacy of these important antimicrobials.

Acknowledgments

The authors wish to thank the Xunta de Galicia for granting research project (PGIDIT05TAL003E). The authors also thank Carmen Carreira for her technical assistance.

Table 1. MIC_{50}, MIC_{90}, Range and Resistance rates for E. coli strains obtained from treated and control birds during and after enrofloxacin application in their drinking water

	Sampling Day					
	Day 0	**Day 1**	**Day 3**	**Day 5**	**Day 11**	**Day 17**
Treated Birds						
no. strains	18	18	18	18	18	18
MIC_{50}	0.5	4	4	8	4	4
MIC_{90}	8	16	16	8	8	16
Range	<0.125-8	<0.125-16	<0.125-16	<0.125-8	0.5-32	<0.125-8
no. resistant (%)	6(33.3)	13(72.2)	15(83.3)	16(88.9)	16(88.9)	16(88.9)
Control Birds						
no. strains	18	18	18	18	18	18
MIC_{50}	<0.125	<0.125	<0.125	<0.125	<0.125	<0.125
MIC_{90}	8	8	8	8	4	4
Range	<0.125-8	<0.125-8	<0.125-8	<0.125-8	<0.125-8	<0.125-8
no. resistant (%)	4 (22.2)	6 (33.3)	9 (50)	6 (33.3)	3(16.7)	4 (22.2)
P	0.4049	0.0450	0.2051	0.0368	0.0001	0.0136

MIC_{50}, MIC_{90} and Range expressed in µg ml^{-1}

Table 2. MIC_{50}, MIC_{90}, Range and Resistance rates for E. coli strains obtained from treated and control birds during and after doxycycline application in their drinking water

	Sampling Day					
	Day 0	**Day 1**	**Day 3**	**Day 5**	**Day 8**	**Day 12**
Treated Birds						
no. strains	17	18	16	18	18	18
MIC_{50}	2	4	8	4	8	8
MIC_{90}	16	8	16	16	16	16
Range	<0.25-16	<0.25-16	<0.125-16	0.5-16	<0.25-16	<0.25-32
no. resistant (%)	3(17.6)	2(11.1)	6(37.5)	4(22.2)	6(33.3)	8(44.4)
Control Birds						
no. strains	15	17	18	18	18	18
MIC_{50}	2	1	1	2	4	4
MIC_{90}	4	8	8	4	8	16
Range	<0.25-4	<0.25-16	<0.25-8	<0.25-8	0.5-32	<0.25-16
no. resistant (%)	0 (0)	2 (11.8)	1 (5.6)	0 (0)	1(5.6)	3 (16.7)
P	0.1773	0.4410	0.0214	0.0354	0.0272	0.1648

MIC_{50}, MIC_{90} and Range expressed in µg ml^{-1}

Table 3. MIC_{50}, MIC_{90}, Range and Resistance rates for E. coli strains obtained from treated and control birds during and after sulphonamides application in their drinking water

	Sampling Day					
	Day 0	**Day 1**	**Day 4**	**Day 9**	**Day 16**	**Day 24**
Treated Birds						
no. strains	18	18	18	18	18	18
MIC_{50}	512	512	1024	1024	1024	1024
MIC_{90}	2048	1024	2048	1024	2048	2048
Range	<4-2048	<4-2048	512-2048	512->2048	<4-2048	<4->2048
no. resistant (%)	11(61.1)	13(72.2)	18(100)	18(100)	16(88.8)	17(94.4)
Control Birds						
no. strains	18	18	18	17	18	18
MIC_{50}	512	<4	<4	<4	<4	512
MIC_{90}	1024	<4	1024	1024	1024	1024
Range	<4-2048	<4-1024	<4-1024	<4-2048	<4-2048	<4-2048
no. resistant (%)	10 (55.6)	2 (11.1)	9 (50)	7 (41.2)	9 (50)	11(61.1)
P	0.0663	0.0009	0.0018	0.0019	0.0153	0.0959

MIC_{50}, MIC_{90} and Range expressed in µg ml^{-1}

5. References

1. White W, Medical consequences of antibiotic use in agriculture. *Science* **279:** 996-997 (1998).

2. Philips I, Casewell M, Cox T, De Groot B, Friis C, Jones R, Nightingale C, Preston R, and Waddell J, Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J Antimicrob Chemother* **53:** 28-52 (2003).

3. Doyle M, Dealing with antimicrobial resistance. Food Technology **8:** 22-29 (2006).

4. Monnet DL, Toward multinational antimicrobial resistance surveillance systems in Europe. *Int J Antimicrob Agents* **15:** 91-101 (2000).

5**.** Avrain L, Humbert F, L´Hospitalier RL, Sanders P, Vernozy-Rozand C and Kempf I, Antimicrobial resistance in Campylobacter from broilers: association with production type and antimicrobial use. *Vet Microbiol* **96:** 267-276 (2003).

6. Posyniak A, Zmudzki J and Mitrowska K, Dispersive solid-phase extraction for the determination of sulfonamides in chicken muscle by liquid chromatography. *J Chomatogr A* **1087:** 259-264 (2005).

7. Meneau I, Sanglard D, Bille J and Hauser PM, Pneumocystis jiroveci Dihidropterase synthase polymorphisms confer resistance to sulfadoxine and sulfanilamide in Saccharomyces cerevisiae. *Antimicrob Agents Chemother* **48:** 2610-2616 (2004).

8. Patel OG, Mberu EK, Nzila AM and Macreadie IG, Sulfa drugs strike more than once. *Trends Parasitol* **20:** 1-3 (2004).

9. Kijima-Tanaka M, Ishihara K, Morioka A, Kojima A, Ozono T, Ogikubo K, Takahashi T and Tamura Y, A national surveillance of antimicrobial resistance in *Escherichia coli* isolated from food-producing animals in Japan. *J Antimicrob Chemother* **51:** 447-451 (2003).

10. Von Baum H and Marre R, Antimicrobial resistance of Escherichia coli and therapeutic implications. *Int J Med Microbiol* **295:** 503-511 (2005).

11. Sáenz Y, Zarazaga M, Briñas L, Lantero M, Ruiz-Larrea F and Torres C, Antibiotic resistance in Escherichia coli isolates obtained from animals, foods and humans in Spain. *Int J Antimicrob Agents* **18:** 353-358 (2001).

12. Threlfall EJ, Cheasty T, Graham A and Rowe B, Antibiotic resistance in Escherichia coli isolated from blood and cerebrospinal fluid: a 6-year study of isolates from patients in England and Wales. *Int J Antimicrob Agents* **9:** 201-205 (1998).

13. Charteston B, Gate JJ, Aitken A, Stephan B and Froyman R, Comparison of the efficacies of three fluoroquinolone antimicrobial agents, given as continuous or pulsed-water medication, against E. coli infection in chickens. *Antimicrob Agents Chemother* **42:** 83-87 (1998).

14. Guerra B, Junker E, Schoeter A, Malorny B, Lehmann S and Helmuth R, Phenotypic and genotypic characterization of antimicrobial resistance in German Escherichia coli isolates from cattle, swine and poultry. *J Antimicrob Chemother* **52:** 489-492 (2003).

15. Van den Bogaard AE, London N, Driessen C and Stobbering EE, Antibiotic resistance of faecal Escherichia coli in poultry, poultry farmers and poultry slaughterers. *J Antimicrob Chemother* **47:** 763-771 (2001).

16. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk and dilution susceptibility test for bacteria isolated from animals: approved standard M31-A2, 2nd ed. Wayne, PA: NCCLS; (2002).

17. Aarestrup FM, Jensen NE, Orsal SE and Nielsen TK, Emergence of resistance to fluoroquinolones among bacteria causing infections in food animals in Demmark. *Vet Rec* **146:** 76-78 (2000).

18. Barrow PA, Novell MA, Szmolleny G and Murphy CK, Effect of enrofloxacin administration on excretion of Salmonella enteritidis by experimentally infacted chickens and on quinolone resistance of their Escherichia coli flora. *Avian Pathol* **27:** 586-590 (1998).

19. Bywater R, Deluyker H, Deroover E, Jong A, Marion H, McConville M, Rowan T, Shryock T, Shuster D, Thomas V, Vallé M and Walters J, A European survey of antimicrobial susceptibility among zoonotic and commensal bacteria isolated from food-producing animals. *J Antimicob Chemother* **54:** 744-754 (2004).

20. Moniri R and Dastehgoli K, Fluoquinolone-resistant E. coli isolated from healthy broilers with previous exposure to fluoroquinolones: Is there a link?. *Microb Ecol Health Dis* **17:** 69-74 (2005).

21. Cunha BA, Doxycycline for community-acquired pneumonia. *Clin Infect Dis* **35:** 632 (2003).

22. Jones RN, Sader HS and Fritsche TR, Doxycycline use for community-acquired pneumonia: Comtemporary in vitro spectrum of activity against Streptococcus pneumoniae (1999-2002). *Diagn Microbiol Infect Dis* **49:** 147-149 (2004).

23. Koeth L, Felmingham MD, Jacobs MR and Rossi F, Antimicrobial resistance of Streptococcus pneumonia*e* and Haemophilus influenzae in Sao Paulo, Brazil from 1996 to 2000. *Int J Antimicrob Agents* **23:** 356-361 (2004).

24. Chopra I and Roberts M, Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* **65:** 232-260 (2001).

25. Cornican M, Buckley V, Corbett-Feeney G and Sheridan F, Antimicrobial resistance in Escherichia coli isolates from turkeys and hens in Ireland. *J Antimicrob Chemother* **48:** 587-95 (2001).

RESUMEN

Las tasas de resistencia de las poblaciones intestinales de *Escherichia coli* en pollos fueron medidas a lo largo de sendos tratamientos terapéuticos con enrofloxacina, doxiciclina y sulfamidas. Tanto los tratamientos como sus correspondientes períodos de supresión fueron efectuados atendiendo a las instrucciones del fabricante de cada uno de las tres presentaciones comerciales utilizadas.

Para este ensayo fueron utilizados un total de 108 pollos, de los cuales 18 fueron tratados con enrofloxacina, 18 con doxiciclina y 18 con sulfamidas. Por cada uno de los antimicrobianos, otros 18 pollos fueron mantenidos sin tratamiento como controles.

A cada uno de ellos se les extrajo periódicamente durante el tratamiento y período de supresión un mínimo de 0,5 g de heces mediante torundado de la cloaca. Estas heces fueron procesadas para conseguir el aislamiento de cepas de *E. coli* (un total de 648; 216 por antimicrobiano incluyendo pollos tratados y controles). Tras el correspondiente aislamiento e identificación bioquímica, estas cepas fueron utilizadas para determinar la CMI correspondiente al antimicrobiano utilizado mediante el método de microdilución en caldo.

Los resultados obtenidos mostraron que las tasas de resistencia de las poblaciones intestinales de *E. coli* de los pollos tratados aumentaron significativamente respecto de las poblaciones correspondientes de los pollos controles durante los tres tratamientos antimicrobianos. En el caso de la enrofloxacina, la tasa de resistencia de los *E. coli* intestinales de los pollos tratados continuó siendo significativamente superior (P=0.0136) a la de los pollos controles al finalizar el período de supresión.

En consecuencia, la resistencia a antimicrobianos de los *E. coli* intestinales es un factor muy a tener en cuenta en los tratamientos antimicrobianos a animales destinados al consumo humano. Dado que el período de supresión en ocasiones no es suficiente para la recuperación de las tasas de resistencia previas al tratamiento, y que en el faenado de este tipo de animales en el matadero es frecuente la contaminación de la carne debido a roturas en la pared intestinal de los pollos, podría producirse una contaminación por *E. coli* resistentes de la carne de los animales sometidos a tratamientos antimicrobianos.

3.2. Evolución de la microbiota intestinal en pollos y selección de resistencia durante el tratamiento terapéutico con enrofloxacina y su período de supresión

Study of the dynamics of faecal bacteria in poultry and the selection of resistant strains during therapeutic treatment with enrofloxacin and after the withdrawal period

Miranda, J.M., Franco, C.M., Vázquez, B.I., Fente, C.A., Barros-Velázquez, J., Cepeda A.

Artículo enviado para su publicación al Journal of Applied Microbiogy.
Fecha de envío: Septiembre 2006.

Study of the dynamics of faecal bacteria in poultry and the selection of resistant strains during therapeutic treatment with enrofloxacin and after the withdrawal period

[a]Miranda, J.M., [a]Franco, C.M.*, [a]Vázquez, B.I., [a]Fente, C.A., [b]Barros-Velázquez, J. and [a]Cepeda A.

[a]Laboratorio de Higiene Inspección y Control de Alimentos. Dpto de Química Analítica, Nutrición y Bromatología. Facultad de Veterinaria. Universidad de Santiago de Compostela. 27002-Lugo. Spain.
[b]Area de Tecnología de los Alimentos. Dpto de Química Analítica, Nutrición y Bromatología. Facultad de Veterinaria. Universidad de Santiago de Compostela. 27002-Lugo. Spain.

Abbreviated running headline: Enrofloxacin treatment in poultry.

ABSTRACT

Aims: To investigate the ability of enrofloxacin to select resistant populations in poultry intestinal bacteria during therapeutic treatment.
Methods and Results: Eighteen birds were treated over 5 days with enrofloxacin, and another 18 birds were maintained as controls. Faecal samples were obtained from the chickens by cloacae swabbing before starting treatment (day 0), on days 1, 3 and 5 of treatment and on days 11 and 17 of the withdrawal period. Populations of Enterobacteriacae*, Enterococcus* spp*,* Lactic Acid Bacteria and *Campylobacter* spp were estimated by plating on selective media. A total of 432 strains from these media were isolated in days 0, 5 and 17. These strains were characterised and Minimal Inhibitory Concentrations to enrofloxacin were determined.
Enterobacteriaceae showed the lowest counts on the third day of treatment while *Enterococcus* spp, LAB and *Campylobacter* spp counts were minimum at the end of treatment. Significant differences in populations during the treatment were found for

Enterobacteriaceae (*P*=0.003) and *Enterococcus* (*P*=0.0469). Resistance rates in bacteria isolated from treated poultry after the withdrawal period were significantly higher than those of control chickens for Enterobacteriaceae (*P*=0.001) and *Campylobacter* spp *(P*=0.0207).

Conclusions: Enrofloxacin resistance increased in all bacterial groups tested except enterococci. In the case of Enterobacteriaceae and *Campylobacter*, resistance rates remained significantly higher than those of control chickens after the withdrawal period.

Significance and Impact of the Study: Antimicrobial resistance of animal-origin bacteria is a factor to consider to establish a withdrawal period prior to antimicrobial usage for veterinary use.

Keywords: poultry, enrofloxacin, faecal, resistance, quinolone, antimicrobial.

INTRODUCTION

Antibiotic use is considered the most important factor promoting the emergence, selection, and dissemination of antibiotic-resistant microorganisms in both veterinary and human medicine (White 1998). Antibiotics are used in animals, as in humans, for therapy and the control of bacterial infections. In addition to therapeutic treatments, in poultry farming, as well as other intensively reared animals, antimicrobial agents may be administered continuously with the water or feed to animals at smaller doses than those employed for therapeutic purposes in order to enhance animal growth (Van den Boogard *et al.* 2001). Epidemiological research suggests that several antimicrobial-resistant bacteria isolated from humans could have primarily originated from animals raised for human consumption and that they have been selected during the animal production stage rather than in the practice of human medicine (Bager and Helmuth 2001). The occurrence of resistant bacteria in the intestine of animals is an important factor, because meat products can be readily contaminated with intestinal bacteria during slaughter and processing (Wiuff *et al*. 2003).

The fluoroquinolone enrofloxacin was initially used for therapeutic purposes through its addition to drinking water for the control of mortality in chickens associated with *E. coli* and *P. multocida* infections. This therapeutic use was recently banned in

some countries such as Denmark, and its conditions of use have been restricted in some countries, including the U.S. (DANMAP 2003; Davidson 2004).

It has been reported that quinolones, together with tetracyclines, are widely used in poultry, especially in turkeys and broilers (Van den Boogard *et al.* 2001; Avrain *et al.* 2003). The current method for medicating these animals is by treating the entire house via water, even though relatively few birds may be ill. This practise exposes more organisms to the antimicrobial agent and therefore is more likely to result in the emergence of resistant strains. An additional problem arises because the dose received by the chickens varies, since the antimicrobial is administered in water *ad libitum*. This may result in suboptimal dosing and may increase the probability of selecting for resistant strains in both healthy and diseased birds (McDermott *et al.* 2002). Thus, in the poultry intestines, the natural populations of bacteria probably contain small numbers of spontaneously resistant strains, which may then become the majority population under the selective pressure of fluoroquinolone use (Gupta *et al.* 2004). This will initially occur only within that population, but as the numbers of these resistant bacteria within the population (degree of resistance) increase, the risk of transmission to other populations increases.

The first reports of quinolone resistance appeared in the late 1980s (Piddock 1995): This resistance usually results from target site mutations (*gyrA* [*DNA gyrase*] and *parC* or *grlA* [*topoisomerase IV*]) or active export of the agents via efflux pumps, although other resistance mechanisms have been described (Poole 2000a; Poole 2000b; Peters *et al.* 2003). Ten years ago, we found a relationship between the use of quinolones in veterinary medicine and the development of quinolone resistance in *Campylobacter jejuni* and *Campylobacter coli* (Velazquez *et al.* 1995). Furthermore, other authors have found increased resistance rates to first-generation quinolones as well as reduced susceptibility to other more potent fluoroquinolones. This latter aspect has been reported for *Salmonella* spp, *Campylobacter* spp, *E. coli* and other zoonotic bacteria isolated from several food animals and in several countries after the introduction of fluoroquinolones into veterinary medicine (Barrow *et al.* 1998; McDermott *et al.* 2002; Wiuff *et al.* 2003; Pedersen and Wedderkopp 2003).

Several authors believed that the emergence and increase in frequency of fluoroquinolone-resistant strains was the result, at least in part, of the use of

fluoroquinolones in the poultry industry (Barrow *et al.* 1998; Van Boven *et al.* 2003), since despite the restrictions on the use of enrofloxacin, the emergence of a large number of fluoroquinolone-resistant bacterial species such as *Campylobacter* spp, or *E. coli*, with poultry meat as an important source, has been documented (Van Boven *et al.* 2003; Gupta *et al.* 2004).

Thus, since the withdrawal period is determined as a function of the persistence of antimicrobial residues in animal tissues, it is important to know the resistance rates of the intestinal bacterial population when the withdrawal period has just finished, since it is legal to send the birds to slaughter after the withdrawal period has ended.

The present study was undertaken in order to determine the population evolution and the degree of enrofloxacin resistance by intestinal bacteria along with therapeutic treatment and during the withdrawal period.

MATERIALS AND METHODS

Animals employed

A total of thirty-six healthy crossbreed chickens aged 4-6 weeks were obtained from the same commercial hatchery. All birds were fed with the same antibiotic-free feed before starting antimicrobial treatment. The birds were weighed and allocated into twelve groups of three birds each: treated chickens (groups 1-6) were treated with a therapeutic dose of enrofloxacin in water (0.05 g l^{-1}) of Colmyc-E® (S.P. Veterinaria, Tarragona, Spain) over 5 days, in accordance with the instructions on the package insert, and the other groups (groups 7-12) remained untreated, acting as controls. None of the chickens had direct physical contact with one another during the assays since they were housed in individual cages of the animal facility. Contamination between the treated and untreated batches was prevented by housing them in different rooms. The poultry were fed twice daily with antibiotic-free commercial poultry feed and had free access to the medicated water, in the case of the treated poultry, or to antimicrobial-free water in the case of the control batches. Determination of water intake was performed at 12 h intervals.

All parts of this study were carried out according to EC Council Directives concerning the laws, regulations and administrative provisions of the member states regarding the protection of animals used for experimental and other scientific purposes.

Experimental procedures

Each group of chickens was sampled immediately before starting treatment (day 0), on the first day on treatment (day 1), on third day of treatment (day 3), on the last day of treatment (day 5), 6 days after the end of the treatment (day 11), and after the withdrawal time had ended according with the insert package (day 17). Faecal samples were taken by swabbing the cloacae of each bird with sterile swabs to obtain a minimum of 0.5 g of faecal content and were placed aseptically in a sterile tube, weighed before and after swabbing to calculate faecal sample weight. Samples were taken to the laboratory in an ice chest in less than half an hour for immediate processing. Faecal samples of 3 animals belonging to each group were placed in a sterile masticator bag with an appropriate volume (1/9) (w/v) of sterile buffered peptone water (Merck, Darmstadt, Germany) and subsequently homogenised with a masticator (Aes, Combourg, France) for 2 minutes. After homogenisation, samples were tested for quantification, isolation and identification of Enterobacteriaceae*, Enterococcus spp*, Lactic acid bacteria (LAB) and *Campylobacter* spp .

Enterobacteriaceae: Appropriate dilutions of homogenates were tested in poured plates of Crystal-violet neutral-red bile glucose agar (VRBD) prepared as specified by the manufacturer (Merck). After the agar had solidified, the plates were overlaid with 3-4 ml of melted VRBD, and immediately incubated at 37 ºC for 24 h. After incubation, all pink to red colonies were counted.

Enterococcus spp*:* Appropriate dilutions of homogenates were tested by surface plating in Chromocult® enterococci agar, prepared as specified by the manufacturer (Merck). Plates were incubated at 37 ºC for 48 h and all red colonies were counted.

LAB: Appropriate dilutions were tested by surface plating in De Man, Rogosa and Sharpe agar (MRS), prepared as specified by the manufacturer (Merck). Plates were

incubated at 31 ºC for 72 h under a micro-aerobic atmosphere generated by Anaerocult C® system (Merck) in anaerobic jars (Oxoid, Basingstoke, UK). White circular colonies were counted.

Campylobacter spp*.*: Appropriate dilutions of homogenates were tested by surface plating in Campylossel® agar (BioMérieux, Marcy l`Etoile, France). Plates were incubated at 42 ºC for 48 h under a micro-aerobic atmosphere generated with the Campygen® system (Oxoid) in anaerobic jars. White colonies without haemolytic character were counted.

Only plates containing 20 to 250 colonies with the typical morphology of target bacteria were counted. Target bacteria count numbers were converted to $\log_{10}$ values and expressed as $\log_{10}$ CFU g^{-1} faeces after calculating the bacterial population obtained from triplicate assays for each culture media.

The sampling and processing procedures of poultry faeces described in this work were always carried out by the same laboratory personnel. Agar media types were prepared by the same research assistant throughout the study.

Isolation and identification of bacteria

After incubation and counting, 3 typical colonies for each bacterial group were picked and transferred onto Columbia agar with 5% Sheep blood (BioMérieux) and incubated under the same conditions of temperature, atmosphere and time as specified above in order to obtain a pure culture. This was done for each batch of chickens (three birds) on days 0, 5 and 17, obtaining a total of 432 strains (18 strains per bacterial group and treatment day).

These pure cultures were characterised as follows:

Enterobacteriaceae: Presumptive colonies were characterised by colony and cell morphology, Gram stain, oxidase, and catalase activity.

Enterococcus spp*.*: Presumptive colonies were characterised by colony and cell morphology, haemolytic character, Gram stain, oxidase, and catalase activity.

LAB: Presumptive colonies were characterized by colony and cell morphology, Gram stain, and catalase activity.

Campylobacter spp*:* Presumptive colonies were characterised by colony and cell morphology, haemolytic character, Gram stain, oxidase and catalase activity and Dyspot Campylobacter test (Oxoid).

All isolates were stored at -80 $^{\circ}$C until further analysis using Maintenance Freeze Medium units (Oxoid).

Antibiotic susceptibility testing of bacteria

Antibiotic susceptibility testing for Enterobacteriaceae, *Enterococcus* spp and LAB was performed using a broth microdilution susceptibility test on microtitre plates to determine the Minimum Inhibitory Concentration (MIC). MICs and levels of resistance were determined according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS 2002).

The microtitre plates were incubated for 18 to 24 h at 37ºC in the atmosphere in which the microbial group had been grown, and with a source of moisture to prevent dehydration of the outer well. Enrofloxacin was tested in two-fold increases from 0.016 to 32 μg ml^{-1} on the microtitre plates. Target MIC ranges were verified with *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 reference strains as quality control.

For *Campylobacter,* the susceptibility testing was done by agar dilution, in accordance with the guidelines from the NCCLS (2002), using *C. jejuni* ATCC 33560 as quality control. Test medium consisted of Mueller-Hinton agar containing 5% defibrinated sheep blood in a range of enrofloxacin from 0.016 to 32 μg ml^{-1}. Plates were incubated for 48 h at 37 ºC under a micro-aerobic atmosphere generated with the Campygen® system (Oxoid) .

The endpoint or MIC used was that recommended by the NCCLS (2002) for the Enterobacteriacae family: ≥ 2 μg ml^{-1}.The MICs that inhibited 50% (MIC50) and 90% (MIC90) of total strains were calculated from the MIC values obtained from each bacterial group. The enrofloxacin reference standard was a kind gift from Bayer AG (Leverkusen, Germany).

Statistical analyses

The amounts of water ingested by the treated and control birds were compared using an unpaired Student's t test. The mean log CFU g^{-1} values in faeces were analysed by analysis of variance (ANOVA), with treatment and day as models. The distributions of resistant strains were compared by means of the X^2 test and Fisher's exact test. Differences were considered significant when probabilities were lower than 0.05. All statistical analyses were carried out using Statgraphics version 5.0.1. (SAS Institute, North Carolina, USA).

RESULTS

The amount of water ingested by the treated animals during the 5 days of treatment was 621 mL (SD =269.93), and in the case of the untreated birds 682.83 ml (SD =168.59; *P*=0.643). This shows that enrofloxacin treatment has no effect on the final amount of water ingested. Furthermore, total antimicrobial consumption during the 5 days of treatment was 31.07 mg enrofloxacin per bird (SD = 13.136) (6.21 mg per bird and day). Since the mean weights of the employed chicken in day 0 was 552 g (SD= 114.32), the mean real dose ingested was 11.25 mg kg^{-1} of body weight and day.

The results for the evolution of the microbial population in faeces during treatment as well as during the withdrawal period for each microbial group considered are shown in figures 1 to 4 for Enterobacteriaceae, *Enterococcus* spp, LAB and *Campylobacter* spp, respectively.

The variations in bacterial counts were considerable, enclosure in the control batches. Immediately after the start of treatment, all the microbial populations underwent a decrease. These losses in viable counts differed, depending on the bacterial group considered. No significant differences were obtained with day as the designated model for any of the bacterial groups. Significant differences were obtained with treatment as the designated model for Enterobacteriaceae (*P*=0.003) and *Enterococcus* (*P*=0.0469). No significant differences were observed for the LAB and *Campylobacter* populations. In the case of Enterobacteriaceae, after 3 days of treatment the bacterial counts reached the lowest numbers after which they began to increase, although these

populations were always lower than those of the control batches up to the end of the withdrawal period. In the case of enterococci, LAB and *Campylobacter*, the lowest counts were observed on the fifth day of treatment, after which the populations began to increase and rose to values higher than those of the control birds on day 11 after the start of treatment.

The characterisations performed revealed the following specificities for the media employed: Enterobacteriaceae 91.7%, *Enterococcus* 98.11%, LAB 91.09% and *Campylobacter* 62.11%.

The results obtained for the MIC_{50}, MIC_{90}, MIC ranges and resistance rates are shown in Tables 1-4. There were no significant differences between the control and treated batches regarding the MIC profile for each bacterial group on day 0. Likewise, as expected, there were no significant differences in the control batches during the assay in any of the microbial groups tested.

During the course of the treatment, enrofloxacin efficiently selected resistant strains in Enterobacteriaceae population (Table 1), with a resistance rate of 94.4 % of the strains tested at the end of day 5. Thus, comparison of the resistance rates between treated and control chickens during the treatment revealed statistically significant differences for Enterobacteriaceae (P<0.001). For Enterococcus (Table 2) and LAB (Table 3), the resistance rates observed at the end of treatment were lower than obtained for Enterobacteriaceae: 11.1% for enterococci and 27.8% in the case of LAB. No significant differences were found in resistance rates for either enterococci or the LAB populations.

In the case of *Campylobacter* (Table 4), although this was the bacterial group showing the highest intrinsic resistance, the resistance rates only showed significant differences between isolates obtained by treated and untreated chickens at the end of withdrawal period (P=0.0207).

DISCUSSION

The mean real dose intake by the chickens was 11.25 mg kg^{-1} of body weight per day. This amount was slightly higher than the therapeutic dose recommended for enrofloxacin (10 mg kg^{-1} of body weight per day). This amount of antimicrobial intake

is an important factor in oral antimicrobial treatments carried by feed or water, since inadequate dosage could cause a high rate of resistant strains to originate, since it has been demonstrated that concentrations near or below the MIC are more apt to select for fluoroquinolone-resistant bacteria (McDermott *et al.* 2002).

The faeces obtained from poultry before the start of enrofloxacin treatment contained a highly variable population average for the bacterial groups studied, but always in concordance with the data reported by other authors (Devriese *et al.* 1991; Netherwood *et al.* 1999; Knarreborg *et al.* 2002; Stern and Robach 2003; Van Boven *et al.* 2003;).

The mean counts of Enterobacteriaceae (Figure 1) reported in the present work underwent a reduction until the third day of treatment. Since, in day 5, 94.4% of collected strains were resistant to enrofloxacin, it seems to suggest that when Enterobacteriaceae population began to increase, most intestinal Enterobacteriacea were already resistant to enrofloxacin. This reduction in counts was less dramatic than those obtained by other authors, who reported that after enrofloxacin administration in pigs, coliform bacterial counts decreased from 10^6-10^7 CFU g^{-1} to 10^3-10^5 CFU g^{-1} after 1 day of treatment, and that after 2 or 3 days of treatment, coliforms fully recovered in numbers (due to their replacement by enrofloxacin-resistant bacteria) and reached the same initial counts of 10^6-10^7 CFU g^{-1} coliforms (Wiuff *et al.* 2003). In our experiments, we observed that the initial number of Enterobacteriaceae counts only returned to the initial values at the end of the assay, when in fact these populations must have been mainly composed of resistant bacteria, as demonstrated by the 94% of enrofloxacin-resistant Enterobacteriaceae encountered on day 17.

The widely different evolution in the mean counts of intestinal Enterobacteriaceae during the enrofloxacin treatment, with data previously reported by Van Boven *et al.* (2003) about the wide decrease in poultry *E. coli* intestinal population after enrofloxacin administration, could be caused by the different resistance statement between the *E. coli* populations of the control chickens tested in the cited work (MICs varied from 0.06 to 0.125 μg ml^{-1}) and the Enterobacteriaceae populations tested in the present investigation, in which 27.8 % of Enterobacteriaceae population were enrofloxacin resistant before starting the treatment and the MIC_{90} = 8 μg ml^{-1}.

The frequent use of this antimicrobial for the treatment of *E. coli* infections in chickens seems to be responsible for the gradual increase in *E. coli* enrofloxacin resistance observed in recent years. In this sense, Aarestrup *et al.* (2000) reported that resistance to enrofloxacin had increased from 1993 to 1998 among a number of bacterial pathogens in pigs, such as *E. coli* and *Mannheimia* spp., while Barrow *et al.* (1998) reported that the use of fluoroquinolones to eliminate *Salmonella* from chickens, a procedure that has been implemented in some countries, rapidly leads to the development and selection of fluoroquinolone-resistant *E. coli*.

The resistance rates obtained for Enterobacteriaceae before the start of treatment were lower than, but compatible with, those established by other authors, such as Van den Boogard *et al.* (2001), who obtained a measure of 50% of *E. coli* isolated from poultry resistant to ciprofloxacin, and Sáenz *et al.* (2002), who obtained in spanish supermarket poultry products, a measurement of 38% of ciprofloxacin resistance and 88% of nalidixic acid resistance. The data obtained in the present work shows that enrofloxacin resistance in *E. coli* is happening frequently, especially in countries with a high level of antimicrobial outpatient consumption, such as Spain (Von Baum and Marre 2005).

The higher mutation frequencies of *E. coli* over normal strains makes it expected for Enterobacteriaceae to easily and rapidly develop enrofloxacin resistance, since this resistance is mainly caused by mutations (Poole 2000a; Poole 2000b; Von Baum and Marre 2005). The results obtained in the present study show that enrofloxacin use have a strong ability to select resistant strains onto Enterobacteriaceae population and to maintain such resistant population until the end of the withdrawal period. This is in accordance with data reported by Wiuff *et al.* (2003) concerning persistent resistance among the coliform flora for at least 2 weeks after treatment with this antimicrobial.

Bacterial mean counts corresponding to both enterococci and LAB underwent a reduction until the last day of treatment, when populations stopped decreasing and began to increase. This is in agreement with the less marked selection of enrofloxacin resistant strains in this group. In the case of LAB, although there was only one bacterial group that exhibited a decrease in counts greater than 1 $\log_{10}$ CFU g^{-1}, no significant differences were observed for these population counts during the assay. This could be

due to the higher variations in counts for this bacterial group (10^5-10^9 CFU g^{-1}), which hinder statistical analysis.

Less work has been devoted to the effect of enrofloxacin against enterococci and LAB in comparison with Enterobacteriaceae and *Campylobacter,* since fluoroquinolones are traditionally less active against gram-positive pathogens. Nevertheless, these antimicrobials are clinically useful against infections caused by *Enterococcus*, especially *E. faecalis* (Poole et al. 2000b). The resistance rates obtained for *Enterococcus* spp in the present work are compatible with the data reported by other authors like Peters *et al.* (2003), who noted that this bacterial group often shows resistance or intermediate susceptibility to quinolones, especially in *E. faecium*. Nevertheless, in the case of enterococci populations, in the present work resistance rates did not increase along the assay period in the treated poultry. This latter aspect should be taken into account in light of the ability of enterococci to develop resistance to other antimicrobials (Klare *et al.* 2003; Mannu *et al.* 2003).

For LAB, a small increase in the resistance rates was observed in the treated poultry, but this was not statistically significant. Although little scientific literature has been published about quinolone resistance for LAB, the development of antimicrobial resistance in this group could be important because LAB is strongly represented within the faecal bacterial population and could act as a reservoir of resistance, since the quinolone resistance is mainly due to point mutations in the genes coding for topoisomerases which cannot spill over to other populations, other resistance mechanisms mediated by plasmids that produce the Qnr protein or multidrug transport systems can probably spill over to other bacteria (Poole 2000a; Tran *et al.* 2005).

The *Campylobacter* counts obtained in the present work before starting treatment were similar to those obtained by other authors (Stern and Robach 2003; Van Boven *et al.* 2003). No significant differences were found among the assays. This is probably due to the higher intrinsic resistance rates in this bacterial group, which leads to the lower effect of enrofloxacin on these bacteria.

The resistance rates, MIC_{50} and MIC_{90}, obtained for *Campylobacter* in the control birds were compatible with the data reported by other authors (Avrain *et al.* 2003; Pedersen and Wedderkopp 2003; Desmonts *et al.* 2004). Enrofloxacin resistance in this microbial group has been widely documented, since fluoroquinolones are fist-choice

antimicrobials for serious *Campylobacter* infections, and poultry origin *Campylobacter* is the main source of human campylobacteriosis (McDermott *et al.* 2002; Desmonts *et al.* 2004). Over the years a gradual increase in Campylobacter resistance to this antimicrobial has been reported. Thus, among *C. jejuni* isolated from humans and broilers Aarestrup *et al.* (2000) found enrofloxacin resistance in 12% and 1% respectively. Other work in Demmark (DANMAP 1999) found in 1998 that enrofloxacin resistance in *C.jejuni* isolated from broilers to be 3%. Two years later it was found enrofloxacin resistances for *C.jejuni* isolated from broilers of 7% and 10% for *C. coli.* In Sweden, quinolone resistance among clinical isolates increased from 1% to 2% at the beginning of the 1990s to almost 25% a few years later (Gibreel *et al.* 1998). Desmonts *et al.* (2004) obtained enrofloxacin resistance rates in *Campylobacter* obtained from French broilers, especially for *C. Coli,* an important increase from the period 1992-1996 (2.1%) to the period 2001-2002 (38.6%).

Finally, in keeping with the results reported for Enterobacteriaceae, we detected significant differences in the resistance rates of *Campylobacter* after the end of the withdrawal period on day 17 as compared with the controls. This results supports the finding of McDermott *et al.* (2002) that enrofloxacin treatment does not eliminate *Campylobacter* species from the intestinal tract of chickens, but can select fluoroquinolone-resistant strains that can become the majority in the intestinal track of treated broilers. Other authors (Pedersen and Wedderkopp 2003; Griggs *et al.* 2005) reported that an increased proportion of quinolone-resistance occurs during therapeutic ciprofloxacin treatment, and these resistant strains persisted posttreatment on farms over several rotations, despite cleaning and disinfection of the land between rotations, and even in the absence of selective antimicrobial pressure.

One of the important tasks in the present work was to determine whether important resistance rates might persist at the end of the withdrawal period. Studies aimed at approving antimicrobials for specific use in animal medicine include specific drug depletion assays in order to determine when the maximum residual limit has been attained and hence to establish the corresponding withdrawal period. However, although the recovery of the bacterial population not resistant to the drug was expected to occur during the withdrawal period, in our hands this was not the case, either for Enterobacteriaceae or *Campylobacter*, although it did occur in the case of *Enterococcus*

and LAB. These findings indicate that the withdrawal period is not adequate to reduce the prevalence of enrofloxacin resistant bacteria.

Taking into account the faecal contamination of future poultry carcasses as well as the fact that treated chickens are often sent to the slaughterhouse immediately after the withdrawal period, it is expected that many enteric pathogens responsible for food-borne disease could develop resistance to enrofloxacin (Pedersen and Wedderkopp 2003; Wiuff *et al.* 2003; Berrang *et al.* 2004).

Table 1. MIC_{50} and MIC_{90}, MIC Range (μg ml^{-1}) and Resistance rates for Enterobacteriaceae strains obtained from treated and control birds during and after enrofloxacin treatment in their drinking water.

	Sampling Day*		
	Day 0	**Day 5**	**Day 17**
Treated Birds			
no. strains	18	18	18
MIC_{50} (μg ml^{-1})	0,5	8	2
MIC_{90} (μg ml^{-1})	8	16	8
Range	<0.016-8	<0.016-16	<0.016-8
no. resistant strains (%)	5(27.8)	17(94.4)	17(94.4)
Control Birds			
no. strains	18	18	18
MIC_{50}	<0.016	<0.016	<0.016
MIC_{90}	2	2	2
Range	<0.016-8	<0.016-4	<0.016-8
no. resistant strains (%)	4(22.2)	6(33.3)	4(22.2)

***Day 0: Before start of treatment. Day 5: End of treatment. Day 17: End of withdrawal period.**

Table 2. MIC_{50} and MIC_{90}, MIC Range (µg ml^{-1}) and Resistance rates for *Enterococcus* spp. strains obtained from treated and control birds during and after enrofloxacin application in their drinking water.

	Sampling Day*		
	Day 0	**Day 5**	**Day 17**
Treated Birds			
no. strains	17	18	18
MIC_{50}	0.5	0.5	0.5
MIC_{90}	2	1	2
Range	<0.016-16	<0.016-16	0.032-8
no. resistant strains (%)	5 (29.4)	2 (11.1)	5 (27.8)
Control Birds			
no. strains	18	17	18
MIC_{50}	0.5	0.5	<0.016
MIC_{90}	1	1	0.5
Range	<0.016-16	<0.016-2	<0.016-16
no. resistant strains (%)	5 (27.8)	6(33.3)	5(27.8)

***Day 0: Before start of treatment. Day 5: End of treatment. Day 17: End of withdrawal period.**

Table 3. MIC_{50} and MIC_{90}, MIC Range (µg ml^{-1}) and Resistance rates for Lactic Acid Bacteria (LAB) strains obtained from treated and control birds during and after enrofloxacin application in their drinking water.

	Sampling Day*		
	Day 0	**Day 5**	**Day 17**
Treated Birds			
no. strains	16	18	18
MIC_{50}	<0.016	0.5	<0.016
MIC_{90}	1	4	4
Range	<0.016-16	<0.016-8	<0.016-8
no. resistant strains (%)	1(6.3)	5 (27.8)	3 (16.7)
Control Birds			
no. strains	18	16	15
MIC_{50}	<0.016	<0.016	<0.016
MIC_{90}	1	0.064	1
Range	<0.016-8	<0.016-1	<0.016-4
no. resistant strains (%)	2 (11.1)	0 (0)	1 (6.7)

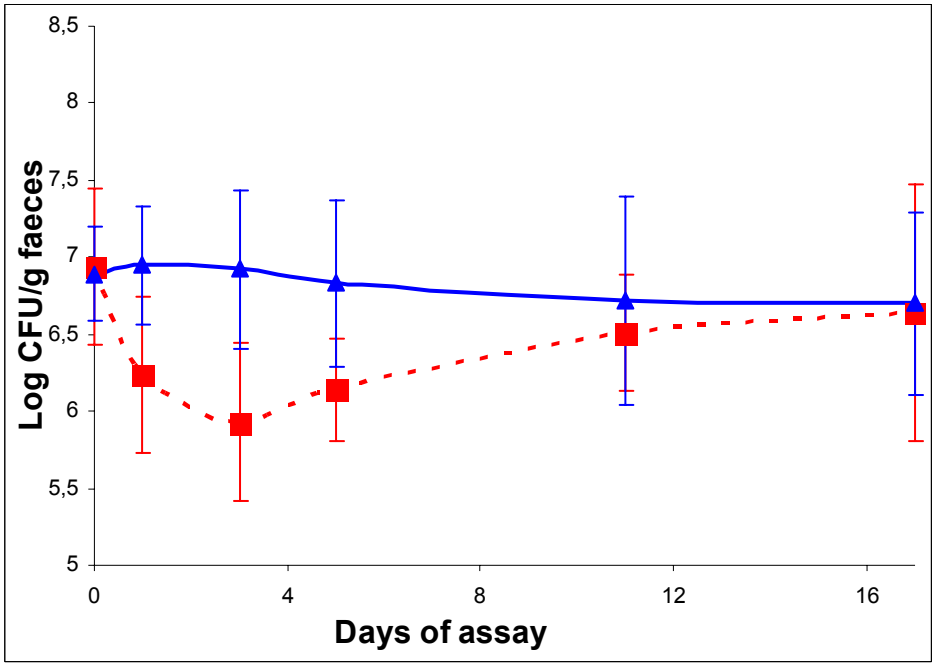
***Day 0: Before start of treatment. Day 5: End of treatment. Day 17: End of withdrawal period.**

Table 4. MIC_{50} and MIC_{90}, MIC Range (µg ml^{-1}) and Resistance rates for *Campylobacter* spp. strains obtained from treated and control birds along and after enrofloxacin application in their drinking water.

	Sampling Day*		
	Day 0	**Day 5**	**Day 17**
Treated Birds			
no. strains	15	14	17
MIC_{50}	1	1	4
MIC_{90}	4	32	32
Range	0.032-16	0.064-32	0.064-32
no. resistant strains (%)	5 (33.3)	5 (35.7)	11 (64.7)
Control Birds			
no. strains	18	16	15
MIC_{50}	0.5	0.125	1
MIC_{90}	32	4	4
Range	0.032-32	<0.016-16	0.032-16
no. resistant strains (%)	6 (33.3)	6 (37.5)	8 (53.3)

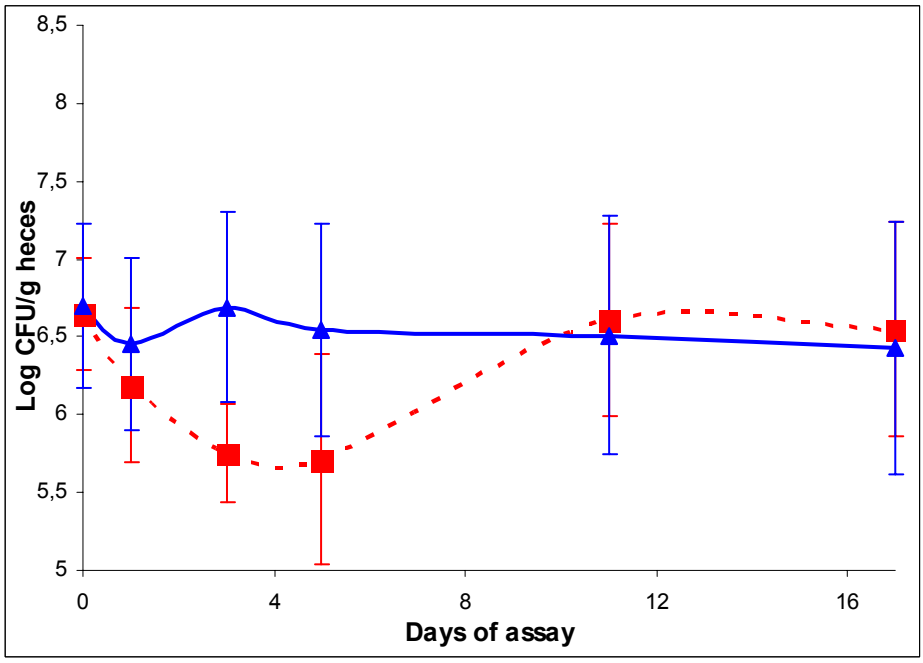
***Day 0: Before start of treatment. Day 5: End of treatment. Day 17: End of withdrawal period.**

Figure 1. Evolution of intestinal Enterobacteriaceae populations during enrofloxacin treatment and in the withdrawal period.



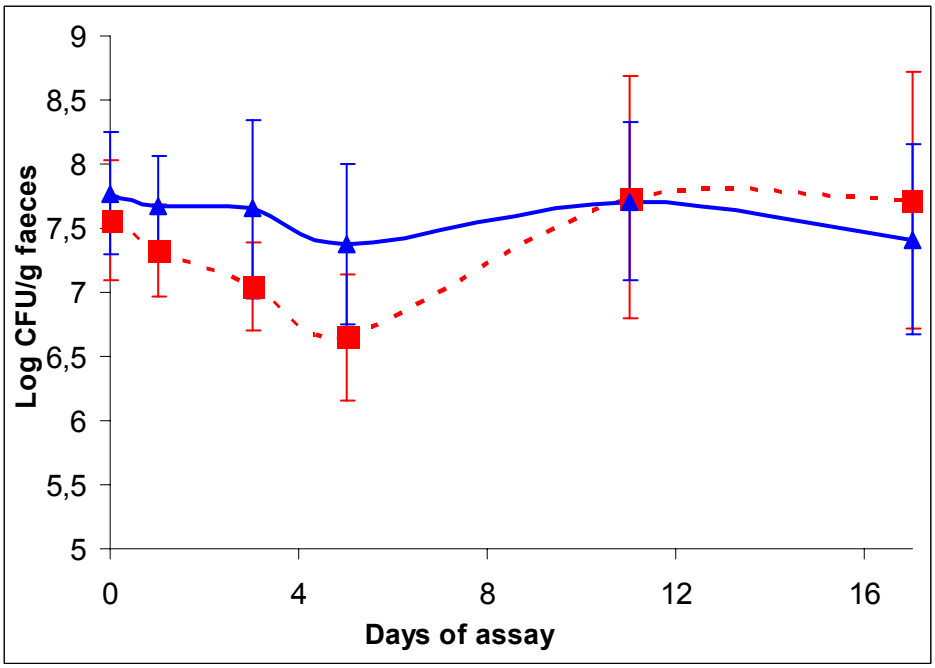
Squares denote treated chickens and triangles denote control chickens.

Figure 2. Evolution of intestinal *Enterococcus* spp. during enrofloxacin treatment and in the withdrawal period.



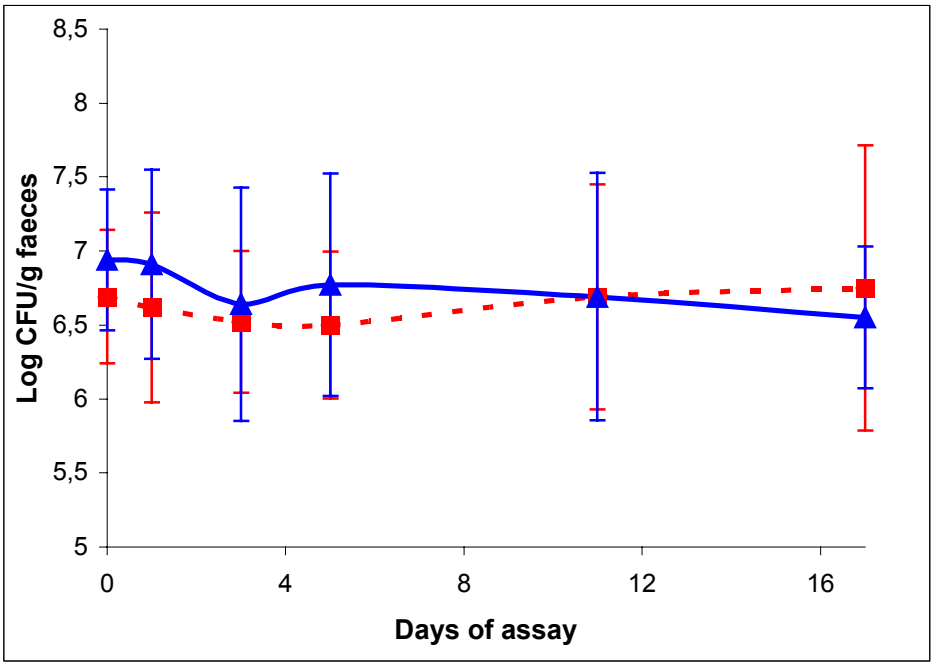
Squares denote treated chickens and triangles denote control chickens.

Figure 3. Evolution of intestinal Lactic Acid Bacteria (LAB) during enrofloxacin treatmentand in the withdrawal period.



Squares denote treated chickens and triangles denote control chickens.

Figure 4. Evolution of intestinal *Campylobacter* spp. during enrofloxacin treatment and in the withdrawal period.



Squares denote treated chickens and triangles denote control chickens.

REFERENCES

1. Aarestrup, F.M., Jensen, N.E., Orsal, S.E. and Nielsen, T.K. (2000) Emergence of resistance to fluoroquinolones among bacteria causing infections in food animals in Demmark. *Vet Rec* **146,** 76-78.

2. Avrain, L., Humbert, F., L´Hospitalier, R.L., Sanders, P., Vernozy-Rozand, C. and Kempf, I. (2003) Antimicrobial resistance in *Campylobacter* from broilers: association with production type and antimicrobial use. *Vet Microbiol* **96,** 267-276.

3. Bager, F. and Helmuth, R. (2001) Epidemiology of resistance to quinolones in *Salmonella*. *Vet Res* **32,** 285-290.

4. Barrow, P.A., Novell, M.A., Szmolleny, G. and Murphy, C.K. (1998) Effect of enrofloxacin administration on excretion of *Salmonella enteritidis* by experimentally infected chickens and on quinolone resistance of their *Escherichia coli* flora. *Avian Pathol* **27,** 586-590.

5. Berrang, M.E., Smith, D.P., Windham, R.W. and Feldner, P.W. (2004) Effect of intestinal content contamination on broiler carcass *Campylobacter* counts. *J Food Prot* **67,** 235-238.

6. EEC Council Directive 86/609/ of November 24 on the approximations of laws, regulations and administrative provisions of the member states regarding the protection of animals used for experimental and other scientific purposes. *OJL* **358,** December 18, 1986.

7. DANMAP (1999). 1998 Consumption of Antimicrobials Agents and Occurrence of Antimicrobial Resistance in Bacteria from Food Animals, Food and Humans in Denmark ed. Bager, F. Copenhagen: Danish Zoonosis Centre.

8. DANMAP (2003) 2002 Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark. Copenhagen Danish Zoonosis Center, Danish Veterinary Institute.

9. Davidson, D. (2004) In the matter of enrofloxacin for poultry: withdrawal of approval of Bayer Corporation's new animal drug application 1 (NADA) 140-828 (Baytril). *FDA Docket* No 00N-1571.

10. Desmonts, M.H., Dufour-Gesbert, F., Avrain, L. and Kempf, I. (2004) Antimicrobial resistance in *Campylobacter* strains isolated from french broilers before and after antimicrobial grown promoter bans. *J Antimicrob Chemother* **54,** 1025-1030.

11. Devriese, L.A., Hommez, J., Wijfels, R. and Haesebrouck, F. (1991) Composition of the enterococcal and Streptococcal intestinal flora of chickens. *J Appl Bacteriol* **71,** 46-50.

12. Gibreel, A., Sjögren, E., Kaijser, B., Wretlind, B., and Sköld, O. (1998) Rapid emergence of high-level resistance to quinolones in *Campylobacter jejuni* associated with mutational changes in *gyrA* and *par*C. *Antimicrob Agents Chemother* **42,** 3276-3278.

13. Griggs, D.J., Johnson, M.M., Frost, J.A., Humphrey, T., Jorgensen, F., and Piddock, L.J.V. (2005) Incidence and mechanism of ciprofloxacin resistance in *Campylobacter* spp isolated from commercial poultry flocks in the United Kingdon before, during and after fluoroquinolone treatment. *Antimicrob Agents Chemother* **49,** 699-707.

14. Gupta A, Nelson, J:M., Barrett, T.J., Tauxe, R.V., Rossiter, S.P., Friedman, C.R., Joyce, K.W., Smith, K.E., Jones, T.F., Hawkins, M.A., Shiferaw, B., Beebe, J.L., Vugia, D.J., Rabatsky-Ehr, T., Benson, J.A., Root, T.P. and Angulo, F.J. (2004) Antimicrobial resistance among *Campylobacter* strains, United States, 1997–2001. *Emerg Infect Dis* **10,** 1102–1109.

15. Knarreborg, A., Simon, M.A., Engberg, R.M., Jensen, B.B. and Tannock, G.W. (2002) Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. *Appl Environ Microbiol* **68,** 5918-5924

16. Klare, I., Konstabel, C., Badstübner, D., Werner, G. and Witte, W. (2003) Occurrence and spread of antibiotic resistances in *Enterococcus faecium. Int J Food Microbiol* **88,** 269-290.

17. Mannu, L., Paba, A., Daga, E., Comunian, R., Zanetti, S., Duprè, I. And Sechi, L.A. **(**2003) Comparison of the incidence of virulence determinants and antibiotic resistance between *Enterococcus faecium* strains of dairy, animal and clinical origin. *Int J Food Microbiol* **88,** 291-304.

18. McDermott, P.F., Bodeis, S.M., English, L.L., White, D.G., Walker, R.D., Zhao, S., Simjee, S. and Wagner, D. (2002) Ciprofloxacin resistance in *Campylobacter jejuni* envolves rapidly in chickens treated with fluoroquinolones. **185,** 837-840.

19. Netherwood, T., Gilbert, H.J., Parker, D.S. and O´Donnell, A.G. (1999) Probiotics shown to change bacterial community structure in the avian gastrointestinal tract. *Appl Environ Microbiol* **65,** 5134-5138.

20. NCCLS (2002) Performance standards for antimicrobial disk and dilution susceptibility test for bacteria isolated from animals: approved standard M31-A2, 2nd ed. Wayne, PA: National Committee for Clinical Laboratory Standards.

21. Pedersen, K. and Wedderkopp, A. (2003) Resistance to quinolones in *Campylobacter jejuni* and *Campylobacter coli* from Danish broilers at farm level. *J Appl Microbiol* **94,** 111-119.

22. Peters, J., Mac, K., Wichman-Schauer, H., Klein, G. and Ellerbroek, L. (2003) Species distribution and antibiotic resistance patters of enterococci isolated from food of animal origin in Germany. *Int J Food Microbiol* **88,** 311-314.

23. Piddock, L.J.V. (1995) Quinolone resistance and *Campylobacter* spp. *J Antimicrob Chemother* **35,** 891-898.

24. Poole, K. (2000a) Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria. *Antimicrob Agents Chemother* **44,** 2233-2241.

25. Poole, K. (2000b) Efflux-mediated resistance to fluoroquinolones in Gram-positive bacteria and the *Mycobacteria. Antimicrob Agents Chemother* **44,** 2595-2599.

26. Sáenz, Y., Zarazaga, M., Briñas, L., Lantero, M., Ruiz-Larrea, F. and Torres, C. (2001) Antibiotic resistance in *Escherichia coli* isolates obtained from animals, foods and humans in Spain. *Int J Antimicrob Agents* **18,** 353-358.

27. Stern, N.J. and Robach, M.C. (2003) Enumeration of *Campylobacter* spp in broiler feces and in corresponding processed carcasses. *J Food Protect* **66,** 1557-1563.

28. Tran, J.H., Jacoby, G.A., and Hooper, D. (2005) Interaction of the plasmid-encoded quinolone resistance protein Qnr with *Escherichia coli* DNA gyrase. *Antimicrob Agents Chemother* **49,** 118-125.

29. Van der Bogaard, A.E., London, N., Driessen, C. and Stobberingh, E.E. (2001) Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. *J Antimicrob Chemother* **47,** 763-771.

30. Van Boven, M., Veldman, K.T., De Jong, M.C.M. and Mevius, D.J. (2003) Rapid selection of quinolone resistance in *Campylobacter jejuni* but not in *Escherichia coli* in individually housed broilers. *J Antimicrob Chemother* **52,** 719-723.

31. Velazquez, J.B., Jimenez, A., Chomon, B. and Villa, T.G. (1995) Incidence and transmission of antibiotic resistance in *Campylobacter jejuni* and *Campylobacter coli*. *J Antimicrob Chemother* **35,** 173-178.

32. Von Baum, H. and Marre, R. (2005) Antimicrobial resistance of *Escherichia coli* and therapeutic implications. *Int J Med Microbiol* **295,** 503-511.

33. White, W. (1998) Medical consequences of antibiotic use in agriculture. *Science* **279,** 996-997.

34. Wiuff, C., Lykkesfeldt, J., Svendsen, O. and Aarestrup, F.M. (2003) The effects of oral and intramuscular administration and dose escalation of enrofloxacin on the selection of quinolone resistance among *Salmonella* and coliforms in pigs. *Res Vet Sci* **75,** 185-193.

RESUMEN

Los recuentos medios, así como las tasas de resistencia de las poblaciones intestinales de Enterobacteriaceae, *Enterococcus* spp., bacterias ácido-lácticas (BAL) y *Campylobacter* spp. en pollos fueron medidas durante un tratamiento terapéutico con enrofloxacina. Tanto el tratamiento como el correspondiente período de supresión fueron efectuados atendiendo a las instrucciones del fabricante de la presentación comercial utilizada.

Para este ensayo fueron utilizados un total de 36 pollos, de los cuales 18 fueron tratados con enrofloxacina durante 5 días y los otros 18 pollos fueron mantenidos sin tratamiento como controles.

A cada uno de ellos se les extrajo un mínimo de 0,5 g de heces mediante torundado de la cloaca antes de comenzar el tratamiento (día 0), así como en el 1er (dia 1), 3er (día 3) y 5º día de tratamiento (día 5), y en el 6º (día 11) y 12º día (día 17) del período de supresión. inmeditamente tras concluir el período de supresión. Estas heces fueron procesadas determinar el recuento de cada uno de los grupos de microorganismos estudiados. Asimismo, de cada uno de estos grupos fueron aisladas e identificadas un total de 432 cepas (18 por día de muestreo y grupo bacteriano estudiado en los días 0,5 y 17). Tras el correspondiente aislamiento e identificación bioquímica, estas cepas fueron utilizadas para determinar la CMI para enrofloxacina mediante el método de microdilución en caldo, excepto para el caso de *Campylobacter*, para el cual fue utilizada la técnica de dilución en agar.

Los resultados obtenidos mostraron en el caso de Enterobacteriaceae las poblaciones intestinales mostraron su nivel mínimo en el tercer día de tratamiento, mientras que en los casos de *Enterococcus* spp., BAL y *Campylobacter* alcanzaron su nivel mínimo en el quinto día de tratamiento. Asimismo, se encontraron diferencias significativas en la evolución de las poblaciones intestinales de los pollos tratados respecto a los controles para los casos de Enterobacteriaceae (P=0.003) y *Enterococcus* (P=0.0469).

Las tasas de resistencia de las poblaciones intestinales en los pollos tratados al finalizar el período de supresión fueron significativamente superiores a sus correspondientes en los pollos controles para los casos de Enterobacteriaceae (P=0.001) y *Campylobacter (P*=0.0207).

En consecuencia, la resistencia a antimicrobianos de las bacterias de origen animal es un factor muy a tener en cuenta en los tratamientos antimicrobianos a animales destinados al consumo humano, dado que el período de supresión en ocasiones no es suficiente para la recuperación de las tasas de resistencia pre-tratamiento.

3.3. Estudio de la evolución de la flora intestinal en pollos tratados con doxiciclina. Valoración de la aparición de resistencias durante el tratamiento terapéutico y el periodo de supresión.

Faecal bacterial dynamics and the selection of resistant strains during doxycycline therapeutic treatment and the withdrawal period in poultry

Miranda, J.M., Franco, C.M., Vázquez, B.I., Fente, C.A., Barros-Velázquez, J., Cepeda A.

Artículo enviado para su publicación en el Veterinary Microbiology.
Fecha de envío: Octubre 2006.

Faecal bacterial dynamics and the selection of resistant strains during doxycycline therapeutic treatment and the withdrawal period in poultry

[a]Miranda, J.M., [a]Franco, C.M., [a]Vázquez, B.I., [a]Fente, C.A., [b]Barros-Velázquez, J., [a]Cepeda A.

[a]Laboratorio de Higiene Inspección y Control de Alimentos. Dpto de Química Analítica, Nutrición y Bromatología. Facultad de Veterinaria. Universidad de Santiago de Compostela. 27002-Lugo. Spain.

[b]Area de Tecnología de los Alimentos. Dpto de Química Analítica, Nutrición y Bromatología. Facultad de Veterinaria. Universidad de Santiago de Compostela. 27002-Lugo. Spain.

Abstract

Antibiotics are used in poultry farming for therapy of bacterial infections. These practices can select antimicrobial-resistant bacteria in animals that can contaminate animal-origin food. The objective of the present work is to evaluate the effects of doxycycline on intestinal bacteria populations and to determine the doxycycline resistance level during a therapeutic treatment and withdrawal period. Doxycycline treatment was carried out on 18 birds over 5 days, while 18 birds fed with the same feed but without treatment were maintained as controls. Faecal samples (≥ 0.5 g) were obtained from chicken by cloacae swabbing before starting treatment (day 0), as well as on days 1, 3 and 5 of treatment and the second (day 7) and the last (day 12) of the withdrawal period. Populations of Enterobacteriaceae*, Enterococcus* spp.*,* Lactic Acid Bacteria (LAB), and *Campylobacter* spp. were estimated and a total of 432 strains were isolated before the start of treatment, at the end of treatment, and at the end of the withdrawal period. These strains were characterized and Minimal Inhibitory Concentrations (MICs) to doxycycline were determined. Significant differences in population evolution during the treatment and withdrawal period were found for *Enterococcus* (*P*=0.0261), LAB (*P*=0.0459), and *Campylobacter* (*P*=0.0259). For

MICs, significant differences were obtained between treated and control batches in the case of Enterobacteriaceae (*P*=0.0398) and *Campylobacter* (*P*=0.0283). In the case of *Campylobacter*, MICs of strains isolated from treated birds were significantly higher that those obtained from control poultry (*P*=0.0057) after the end of the withdrawal period. The results obtained show that animal origin bacteria could maintain acquired resistance after the withdrawal period.

Keywords: poultry, doxycycline, treatment, resistance, antimicrobial.

1. Introduction

Antibiotic use is considered the most important factor promoting the emergence, selection, and dissemination of antibiotic-resistant bacteria in both veterinary and human medicine (White, 1998). Antibiotics are used in animals and humans for therapy and the control of bacterial infections. However, in poultry farming, as well as in other intensively reared animals, antibiotics may be administrated to whole flocks rather than individual animals. In addition to therapeutic treatment, antimicrobial agents may be added directly to feed or water or can be administered in aerosols to animals in smaller dosage and over longer periods than those employed for therapeutic purposes in order to improve the rate of animal growth and feed conversion efficiency (Chopra and Roberts, 2001; Van den Boogard et al., 2001). In some countries these practices have been banned by the European Economic Community (EEC regulation 1831/2003). Epidemiological investigations suggest that several antimicrobial-resistant bacteria isolated from humans primarily originated from animals raised for human consumption and that they have been selected during the animal production stage rather than in the practice of human medicine (Franco Abuin et al., 1994; Aarestrup, 2000; Bager and Helmut, 2000). In the particular case of orally administered antimicrobials, the occurrence of resistant bacteria in the intestine of animals is of major concern, because meat products are readily contaminated with intestinal bacteria during slaughter and processing (Wiuff et al., 2003).

Among antimicrobial agents, the tetracyclines are one of the most commonly used antimicrobial families in veterinary therapy, especially in poultry and turkey (Antunes et

al., 2003; Avrain et al., 2003; Peters et al., 2003). This antimicrobial family, which was firstly described in the 1940s, inhibits protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome (Chopra et al., 1992). Some favourable properties of the tetracyclines, such as their broad spectrum, the possibility of oral administration, and the absence of major adverse side effects, have led to their extensive use in the therapy of human and animal infections. In addition, tetracyclines are one of the cheapest classes of antibiotic available, and their cost in real terms is further declining due to improved manufacturing technology (Chopra and Roberts, 2001). This pricing structure makes these agents particularly attractive for use in developing nations (Finch, 1997; Chopra and Roberts, 2001).

Among the tetracycline group, the possibility of using of doxycycline (6-Deoxy-5-hydroxytetracycline) in either oral or parenteral formulations that permit switching programs from intravenous to oral administration (Cunha, 1999), and its longer half-life than that of tetracycline (8 h *vs* 18-22 h) (Cunha, 2003), permitting once- or twice-daily dosing, make this antimicrobial very useful in veterinary medicine. This antimicrobial is therefore widely used in veterinary medicine (Chopra and Roberts, 2001) and is included in the WHO model list of essential drugs (WHO, 2005).

Although the tetracyclines retain important roles in both human and animal medicine, the emergence of microbial resistance has limited their effectiveness. In 1953 the first tetracycline-resistant bacteria (*Shigella dysenteriae*) were isolated (Falkow, 1975). The use of tetracyclines in human and veterinary clinical practice has undoubtedly been responsible for the selection of resistant organisms (Chopra and Roberts, 2001). When antimicrobials are added to the drinking water of animals such as poultry, a large number of both ill and healthy animals are exposed to the antimicrobial agent. In their intestines, the natural populations of bacteria probably contain small numbers of spontaneously resistant bacteria, which may then become the majority under the selective pressure of antimicrobial use (Gupta et al. 2004). This will initially occur only within that population, but as the numbers of resistant bacteria within that population (degree of resistance) increase, the chance of spilling over to other populations becomes greater. Thus, since the withdrawal period is determined as a function of the persistence of antimicrobial residues in animal tissues it is important to

know the resistance rates of the intestinal bacterial population when the withdrawal period has just finished, since it is legal to send animals to slaughter just after this time.

Doxycycline resistance has been widely documented in recent years in respiratory human pathogens such as *Haemophilus influenzae* and *Streptococcus pneumoniae* (Koeth et al. 2000a; Koeth et al. 2000b; Cunha, 2003; Jones at al. 2004) but there are few references in the literature concerning doxycycline resistance in bacteria of animal-origin and their capacity to develop resistance. The present study was undertaken in order to evaluate the effects of doxycycline on intestinal bacterial populations and to determine the degree of resistance of intestinal bacteria to doxycycline during the therapeutic treatment and withdrawal periods.

2. Materials and methods

2.1. Animals employed

A total of thirty-six healthy crossbred chickens aged 4-6 weeks were obtained from the same commercial hatchery. All birds were fed with the same antibiotic-free feed for 3 days before starting antimicrobial treatment. The birds were weighed and allocated in twelve groups of three birds each: treated chickens (groups 1-6) were treated with a therapeutic dose of doxycycline in water (1 g/l) of Doxidol® (Fatro Uriach Veterinaria, Barcelona, Spain) over 5 days, in accordance with the instructions on the package insert, and the other groups (groups 7-12) remained untreated, acting as controls. The chickens did not have direct physical contact with one another during the assays since they were housed in individual cages at the animal facility. Contamination between the treated and untreated batches was prevented by housing them in different rooms. The poultry were fed twice daily with antibiotic-free commercial poultry feed and had free access to the medicated water, in the case of the treated poultry, or to antimicrobial-free water in the case of the control batches. Observation of water intake was performed at 12 h intervals.

All assays were carried out according to EC Council Directive (86/609) concerning the approximations of laws, regulations and administrative provisions of the member states regarding the protection of animals used for experimental and other scientific purposes.

2.2. Experimental procedures

Each group of chickens was sampled immediately before starting treatment (day 0); on first day of treatment (day 1); on third day of treatment (day 3); on the last day of treatment (day 5); 3 days after the end of the treatment (day 8), and after the withdrawal time had ended (day 12). Faecal samples were taken by swabbing the cloacae of each bird with sterile swabs to obtain a minimum of 0.5 g of faecal contents and these were placed aseptically in sterile tubes. Samples were taken to the laboratory in an ice chest in less than half an hour for immediate processing. Samples of animals belonging to each group were placed in a sterile masticator bag with an appropriate volume (1/9) (w/v) of sterile buffered peptone water (Merck, Darmstadt, Germany) and subsequently homogenized with a masticator (Aes, Combourg, France) for 2 m. After homogenisation, samples were tested for quantification, isolation and identification of Enterobacteriaceae, *Enterococcus* spp., Lactic acid bacteria (LAB) and *Campylobacter* spp.

Enterobacteriaceae: Appropriate dilutions of homogenates were tested in poured plates of Crystal-violet neutral-red bile glucose agar (VRBD) agar prepared as specified by the manufacturer (Merck). After the agar had solidified, the plates were overlaid with 3-4 ml of melted VRBD and immediately incubated at 37 ºC for 24 h. After incubation, all pink to red colonies were counted.

Enterococcus spp.: Appropriate dilutions of homogenates were tested by surface plating in Chromocult® enterococci agar, prepared as specified by the manufacturer (Merck). Plates were incubated at 37 ºC for 48 h and all red colonies were counted.

LAB: Appropriate dilutions were tested by surface plating in De Man, Rogosa and Sharpe agar (MRS), prepared as specified by the manufacturer (Merck). Plates were incubated at 31 ºC for 72 h under a micro-aerobic atmosphere generated with the Anaerocult C® system in anaerobic jars (Oxoid, Basingstoke, UK). White circular colonies were counted.

Campylobacter spp.: Appropriate dilutions of homogenates were tested by surface plating in Campylossel® agar (BioMérieux, Marcy l`Etoile, France). Plates were incubated at 42 ºC for 48 h under a micro-aerobic atmosphere generated with the

Campygen® system (Oxoid) in anaerobic jars (Oxoid). White colonies without haemolytic character were counted.

Only plates containing 20 to 250 colonies with the typical morphology of target bacteria were enumerated. Target bacteria count numbers were converted to $\log_{10}$ values and expressed as $\log_{10}$ CFU/g faeces after calculating the bacterial population obtained from triplicate assays for each culture media.

The sampling and processing procedures of poultry faeces described in this work were always carried out by the same laboratory personnel. Agar media types were prepared by the same research assistant throughout the study.

2.3. Isolation and identification of bacteria

After incubation and counting, 3 typical colonies for each bacterial group were picked and transferred onto Columbia agar with 5% sheep blood (BioMérieux) and incubated under the same conditions of temperature, atmosphere and time as specified above in order to obtain pure cultures. This was done for each batch of chickens (three birds) on days 0, 5 and 12, obtaining a total of 432 strains (18 strains per bacterial group per treatment day).

These pure cultures were characterized as follows:

Enterobacteriaceae: Presumptive colonies were characterized by colony and cell morphology, Gram stain, oxidase, and catalase activity.

Enterococcus spp.: Presumptive colonies were characterized by colony and cell morphology, haemolytic character, Gram stain, oxidase, and catalase activity, and pyrrolidonyl arylamidase test (Oxoid).

LAB: Presumptive colonies were characterized by colony and cell morphology, Gram stain, and catalase activity.

Campylobacter spp.: Presumptive colonies were characterized by colony and cell morphology, haemolytic character, Gram strain, oxidase and catalase activity and Dyspot *Campylobacter* test (Oxoid).

All isolates were stored at -80 °C until further analysis using Maintenance Freeze Medium units (Oxoid).

2.4. Antibiotic susceptibility testing of bacteria

Antibiotic susceptibility testing for Enterobacteriaceae, *Enterococcus* spp. and LAB were performed using a broth microdilution susceptibility test on microtitre plates to determine the Minimum Inhibitory Concentration (MIC). MICs and levels of resistance were determined according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (2002).

The microtitre plates were incubated for 18 to 24 h at 37ºC in the atmosphere in which the microbial group had been grown and with a source of moisture to prevent dehydration of the outer wells. Doxycycline was tested in two-fold increases from 0.125 to 128 µg/ml on the microtitre plates. Target MIC ranges were verified with *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 reference strains as quality controls (CLSI, 2002; Ross et al. 2004).

For *Campylobacter* the susceptibility testing was done by the agar doubling dilution procedure, in accordance with the CLSI Campylobacter Working group (McDermott et al. 2004), using *C. jejuni* ATCC 33560 as a quality control. Test medium consisted on Mueller-Hinton agar containing 5% defibrinated sheep blood in a range of doxycycline from 0.125 to 128 µg/ml. Plates were incubated for 48 h at 36 ºC under a micro-aerobic atmosphere generated with the Campygen® system (Oxoid) .

The endpoint or MIC used was that recommended by the CLSI (2002) for veterinary pathogens: ≥ 16 µg/ml. The MICs that inhibited 50% (MIC50) and 90% (MIC90) of total strains were calculated from the MIC values obtained from each bacterial group. Doxycycline standard dilutions were obtained from Doxycycline hyclate (Flucka, St Gallen, Switzerland).

2.5. Statistical analysis

The amounts of water ingested by the treated and control birds were compared using an unpaired Student's t test. The mean of log CFU/g values in faeces were analysed by analysis of variance (ANOVA), with treatment and day as models. The distributions of resistant strains were compared by means of the X^2 test and Fisher's exact test. Differences were considered significant when probabilities were lower than 0.05. All statistical analyses were carried out using Statgraphics version 5.0.1. (SAS Institute, North Carolina, USA).

3. Results

The amount of water ingested by the treated animals was 429.67 mL (S.D. =113.28) and, in the case of the untreated birds was 524.17 ml (S.D. =173.30), *P*=0.290. This shows that the doxycycline treatment had no effect on the final amount of water ingested. Thus, the mean antimicrobial consumption per bird during the 5 days of treatment was 42.97 mg doxycycline (8.59 mg/bird and day) (S.D. = 13.28). Since the mean weight of the chickens used on day 0 was 638 g (SD= 109.26), the real dose ingested was 13.47 mg/kg of body weight/day.

Immediately after the start of treatment, all the microbial populations underwent a decrease in its average counts. These losses in viable counts were different, depending on the bacterial group considered. No significant differences were obtained with treatment day as a model for any bacterial group. Significant differences were obtained with treatment as a model for *Enterococcus* (*P*=0.0261), *Campylobacter* (*P*=0.0259) and LAB (*P*=0.0459). No significant differences were observed for the Enterobacteriaceae population. In the case of Enterobacteriaceae, after 3 days of treatment the bacterial counts reached their lowest level and then began to increase, exceeding the level in control poultry populations before the end of the withdrawal period. In the case of *Enterococcus*, LAB and *Campylobacter* the lowest counts were observed on the fifth day of treatment (end of treatment), and after this the populations began to increase, remaining under control bird counts during the entire assay in the

case of *Enterococcus* and LAB and exceeding them on day 12 in the case of *Campylobacter*.

These characterisations revealed the following specificities for the media employed: Enterobacteriaceae 87.04 %; *Enterococcus* 96.3 %; LAB 94.44 %, and *Campylobacter* 70.37 %.

The results obtained for the MIC_{50}, MIC_{90}, MIC ranges, and resistance rates are shown in Tables 1-4. There were no significant differences between the control and treated batches in regard the MIC profile for each bacterial group on day 0. Likewise, as expected, there were no significant differences in the control batches during the assay in any of the microbial groups tested.

During the course of the treatment, doxycycline efficiently selected resistant strains onto Enterobacteriaceae population (Table 1). Significant differences were obtained (P=0.0398) in regard resistance rates between treated and control chickens during the treatment period. This resistance reached its highest rate on day 5 (35.5%). In the case of the control batches, resistance rates reached their highest value at 11.1%.

In the case of *Enterococcus* (Table 2) and LAB (Table 3), the resistance rates observed at the end of treatment were 17.6% for enterococci and 11.1% for LAB. Nevertheless, at the end of the withdrawal period resistance rates were similar for both bacterial groups (16.7%). No significant differences were found in MICs for either enterococci or the LAB populations.

Campylobacter (Table 4) was the bacterial group showing the lowest initial resistance. The MICs obtained pointed to significant differences between the treated and control batches (P=0.0283). In the treated group significant differences for MICs were found between days 0 and 5 (P=0.0380) and between days 0 and 12 (P=0.0057).

All bacterial groups tested showed higher resistance rates after the withdrawal period had finished in the treated batches with respect to day 0, but only in the case of *Campylobacter* (P=0.0057) did this difference remain significant. However, significant differences were obtained between days 0 and 5 in the cases of Enterobacteriaceae (P=0.0153) and *Campylobacter* (P=0.0057). No significant differences were observed for the resistance rates in any case for *Enterococcus* and LAB.

4. Discussion

The faeces obtained from the poultry before the start of doxycycline treatment contained a variable population average for the bacterial groups studied, although always in concordance with the data reported by other authors (Devriese et al. 1991; Frei et al. 2001; Knarreborg et al. 2002; Stern and Robach, 2003).

The mean counts of Enterobacteriaceae (Figure 1) fell until the third day of treatment, when the resistance rates obtained for these populations peaked. The initial number of Enterobacteriaceae counts only returned to the initial values when treatment finished. In the case of *Enterococcus* (Figure 2), LAB (Figure 3) and *Campylobacter* (Figure 4), the decrease persisted up to the end of doxycycline treatment (day 5), when the populations stopped decreasing and began to increase. Only in the gram-negative bacterial groups did the mean counts of bacteria isolated from the treated poultry exceed levels in control poultry populations until the end of the withdrawal period. This suggests a faster recovery of populations of Gram-negative than Gram-positive bacteria.

In this work, the resistance rates obtained for Enterobacteriaceae before the start of treatment (12.5% resistant strains, MIC_{50}=0.5 mg/l and MIC_{90}=8 mg/l) were relatively lower than those established by other authors for tetracycline sensitivity in Enterobacteriaceae. In *Salmonella* spp. isolated from poultry products in Portugal, Antunes et al. (2003) obtained a 36% of resistant-strains to tetracycline; in *E. coli* collected from poultry faeces in Germany Frei et al. (2001) harbored 20% tetracycline-resistant strains, with a MIC_{50}=1.5 mg/l and MIC_{90}=192 mg/l. Nevertheless, this is understandable since some tetracycline-resistant bacteria may be sensitive to doxycycline (Ge et al. 2003).

The results obtained here show that Enterobacteriaceae population are able to acquire a high level of doxycycline resistance and maintain part of such acquired resistance until the end of the withdrawal period.

In the case of enterococci and LAB, less work has been devoted to the effect of antimicrobials against enterococci and LAB obtained from poultry in comparison with Enterobacteriaceae and *Campylobacter*. The resistance rates obtained in the control batches were lower than those reported by other authors such as Peters et al. (2003), who obtained 38% resistance in *E. faecalis* and 18% in *E. faecium* in animal-origin

feed, or Frei et al. (2001), who obtained 76% resistance in *E. faecalis* to tetracycline, with MIC_{50}= 0.75 µg/ml and MIC_{90}= 192 µg/ml and 57% resistance in LAB with MIC_{50}= 16 µg/ml and MIC_{90}>256 µg/ml.

The resistance rates, MIC_{50} and MIC_{90} obtained for *Campylobacter* in the control birds were compatible with the data reported by other authors such as Frei et al. (2001), who obtained 0% resistance to doxycycline in *Campylobacter* from poultry samples with MIC_{50}= 0.125 µg/ml and MIC_{90}= 0.125 µg/ml, or Ronner et al. (2004), who obtained 1% of resistant strains to doxycycline in *Campylobacter* isolated from chicken samples in Sweden. Nevertheless, the results reported by other authors such as Ge et al. (2003) show considerably higher resistance levels: 82% tetracycline resistance and 77% doxycycline resistance in strains obtained from retail raw poultry meat. The results obtained in this work point to a significant difference in the evolution of MICs during treatment (P=0.0283), and significant differences in comparison of the MICs obtained on days 0 and 5 (P=0.02809), and those obtained on days 5 and 12 (P=0.057). This may be due to the low resistance rates obtained in the *Campylobacter* isolated before the start of treatment.

One of the important goals of the present work was to determine whether important resistance rates might persist at the end of the withdrawal period. Studies aimed at approving antimicrobials for specific use in animal medicine include specific drug depletion assays in order to determine when the maximum residual limit has been attained and hence to establish the corresponding withdrawal period. However, although recovery of the bacterial population not resistant to the drug was expected to occur during the withdrawal period, in our hands the resistance rates, MIC_{50} and MIC_{90} obtained at the end of the withdrawal period were always higher than those obtained on day 0, and were always higher than those obtained on day 12 in control batches, except in the case of the MIC_{50} in Enterobacteriaceae and *Enterococcus*.

Taking into account the faecal contamination of future poultry carcasses as well as the fact that treated chickens are often sent to the slaughterhouse immediately after the withdrawal period, it is expected that the enteric pathogens responsible for food-borne diseases, such as Enterobacteriaceae and *Campylobacter*, could select a high rates of resistant strains to doxycycline during treatment and, in the case of *Campylobacter*,

could maintain this higher resistance after the withdrawal period has finished, resulting in a possible public safety risk after poultry slaughter and processing.

Acknowledgments

Special appreciation is extended to Carmen Carreira for technical assistance.

Table 1. MIC_{50} and MIC_{90}, range and resistance rates for Enterobacteriaceae strains obtained from treated and control birds during and after doxycycline treat ment in their drinking water.

	Sampling Day		
	Day 0	**Day 5**	**Day 12**
Treated Birds			
No. strains	16	17	18
MIC_{50}	0,5	8	1
MIC_{90}	8	16	16
Range	<0.125-16	1-16	<0.125-32
No. resistant strains (%)	2(12.5)	6(35.5)	3(16.7)
Control Birds			
No. strains	17	16	18
MIC_{50}	1	2	2
MIC_{90}	4	8	8
Range	<0.125-16	<0.125-16	<0.125-16
No. resistant strains (%)	1(5.9)	1(6.3)	2(11.1)

MIC_{50}, MIC_{90} and range expressed in µg/ml.

Day 0: Before start of treatment. Day 5: End of treatment. Day 12: End of withdrawal period.

Table 2. MIC_{50} and MIC_{90}, range and resistance rates for *Enterococcus* spp. strains obtained from treated and control birds during and after doxycycline application in their drinking water.

	Sampling Day		
	Day 0	**Day 5**	**Day 12**
Treated Birds			
No. strains	16	17	18
MIC_{50}	0.5	2	1
MIC_{90}	8	8	16
Range	<0.125-16	1-16	<0.125-32
No. resistant strains (%)	1 (6.3)	3 (17.6)	3 (16.7)
Control Birds			
No. strains	17	18	18
MIC_{50}	1	1	1
MIC_{90}	4	8	8
Range	<0.125-16	<0.125-16	<0.125-16
No. resistant strains (%)	1 (5.9)	1(5.6)	2(11.1)

MIC_{50}, MIC_{90} and range expressed in µg/ml.

Day 0: Before start of treatment. Day 5: End of treatment. Day 12: End of withdrawal period.

Table 3. MIC_{50} and MIC_{90}, range and resistance rates for LAB strains obtained from treated and control birds during and after doxycycline application in their drinking water.

	Sampling Day		
	Day 0	**Day 5**	**Day 12**
Treated Birds			
No. strains	17	18	18
MIC_{50}	2	2	4
MIC_{90}	4	8	16
Range	<0.125-8	0.5-16	<0.125-16
No. resistant strains (%)	0(0)	2(11.1)	3 (16.7)
Control Birds			
No. strains	18	16	18
MIC_{50}	2	1	2
MIC_{90}	4	4	4
Range	<0.125-16	<0.125-8	<0.125-4
No. resistant strains (%)	1 (5.6)	0 (0)	1 (6.7)

MIC_{50}, MIC_{90} and range expressed in µg/ml.

Day 0: Before start of treatment. Day 5: End of treatment. Day 12: End of withdrawal period.

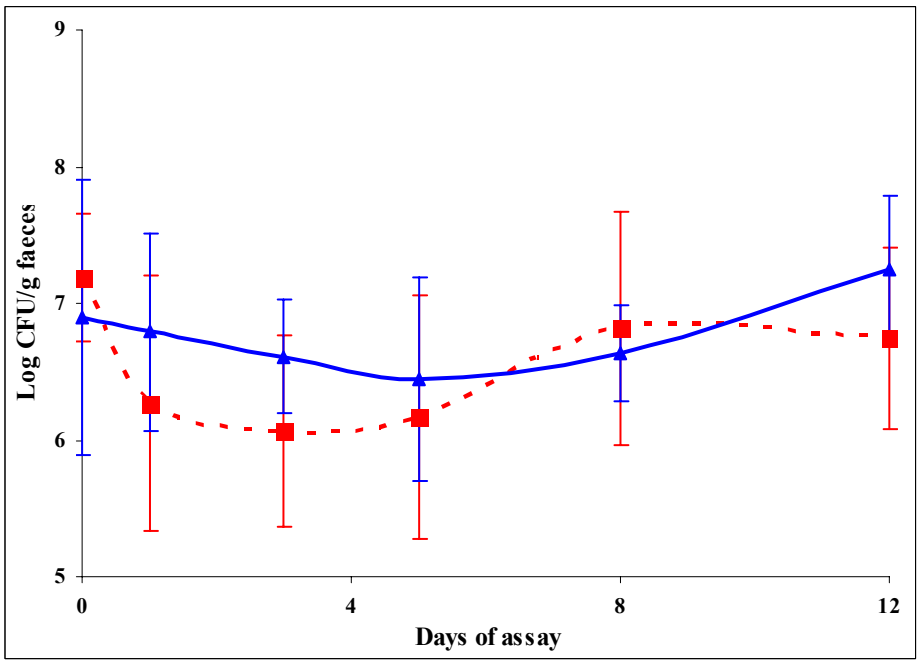
Table 4. MIC_{50} and MIC_{90}, range and resistance rates for *Campylobacter* spp. strains obtained from treated and control birds during and after doxycycline application in their drinking water.

	Sampling Day		
	Day 0	**Day 5**	**Day 12**
Treated Birds			
No. strains	18	18	18
MIC_{50}	<0.125	<0.125	1
MIC_{90}	4	2	8
Range	<0.125-4	<0.125-8	<0.125-16
No. resistant strains (%)	0 (0)	0 (0)	1 (5.88)
Control Birds			
No. strains	18	18	18
MIC_{50}	<0.125	<0.125	<0.125
MIC_{90}	2	1	1
Range	<0.125-4	<0.125-4	<0.125-4
No. resistant strains (%)	0(0)	0 (0)	0 (0)

MIC_{50}, MIC_{90} and range expressed in µg/ml.

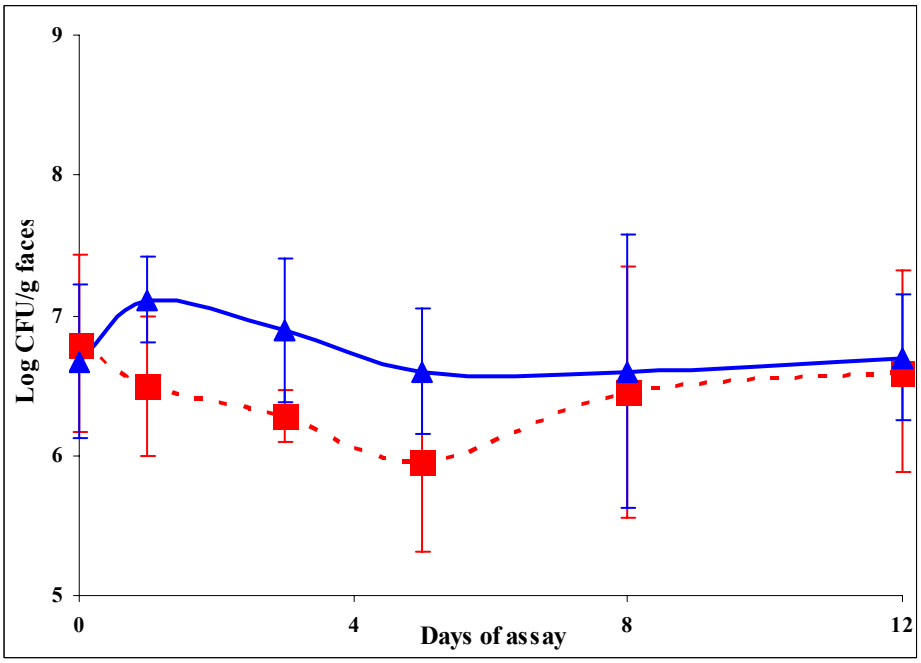
Day 0: Before start of treatment. Day 9: End of treatment. Day 12: End of withdrawal period.

Figure 1. Evolution of intestinal Enterobacteriaceae populations during doxycycline treatment and in the withdrawal period



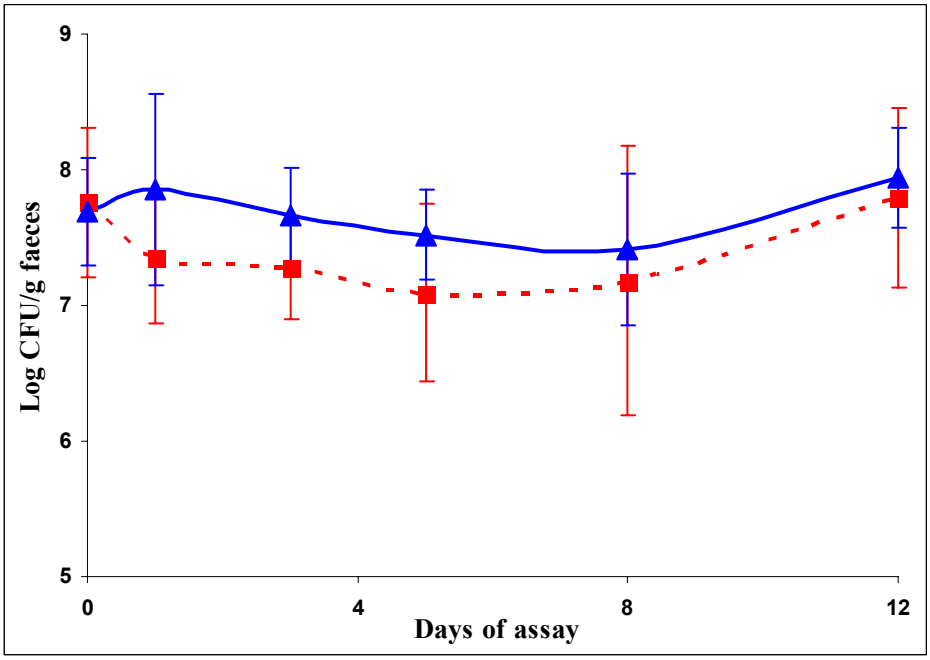
Square points denote treated chickens and triangular points denote control chickens.

Figure 2. Evolution of intestinal *Enterococcus* spp. during doxycycline treatment and in the withdrawal period



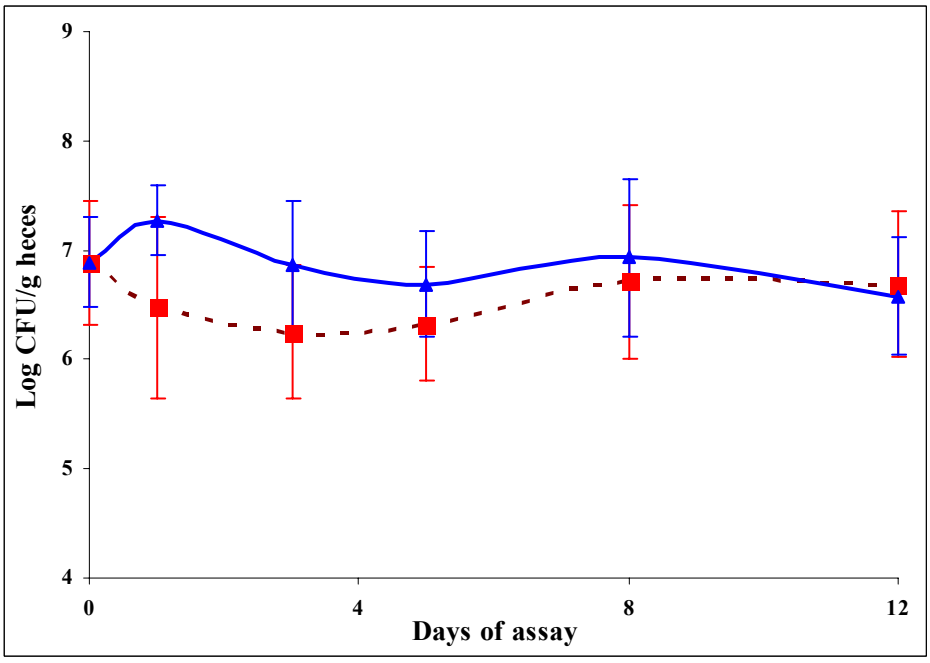
Square points denote treated chickens and triangular points denote control chickens.

Figure 3. Evolution of intestinal LAB during doxycycline treatment and in the withdrawal period



Square points denote treated chickens and triangular points denote control chickens.

Figure 4. Evolution of intestinal *Campylobacter* spp. during doxycycline treatment and in the withdrawal period



Square points denote treated chickens and triangular points denote control chickens.

References

Aarestrup, F.M., 2000. Occurrence, selection and spread of resistance to antimicrobial agents used for growth promotion for food animals in Denmark. APMIS 108 (Supplementum No. 101), 5-48.

Antunes, P., Réu, C., Sousa, J.C., Peixe, L., Pestana, N., 2003. Incidence of *Salmonella* from poultry products and their susceptibility to antimicrobial agents. Int. J. Food Microbiol. 82, 97-103.

Avrain, L., Humbert, F., L´Hospitalier, R.L., Sanders, P., Vernozy-Rozand, C., Kempf, I., 2003. Antimicrobial resistance in *Campylobacter* from broilers: association with production type and antimicrobial use. Vet. Microbiol. 96, 267-276.

Bager, F., Helmuth, R., 2000. Epidemiology of resistance to quinolones in *Salmonella*. Vet. Res. 32, 285-290.

Chopra, I., Hawkey, P.M., Hinton, M., 1992. Tetracyclines, molecular and clinical aspects. J. Antimicrob Chemother. 29, 245-277.

Chopra, I., Roberts, M., 2001. Tetracycline antibiotics: Mode of action, applications, molecular biology and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev. 65, 232-260.

Cunha, B.A., 1999. Doxycicline re-visited. Arch. Intern. Med. 159:1006-1007.

Cunha, B.A., 2003. Doxycycline for community-acquired pneumonia. Clin. Infect. Dis. 35, 632.

Devriese, L.A., Hommez, J., Wijfels, R., Haesebrouck, F., 1991. Composition of the enterococcal and Streptococcal intestinal flora of chickens. J. Appl. Bacteriol. 71, 46-50.

European Economic Community. 1986. Council Directive (EC) Nº 86/609/ of 24 November 1986 on the approximations of laws, regulations and administrative provisions of the member states regarding the protection of animals used for experimental and other scientific purposes. Off. J. Eur. Union L358. 18/12/1986, pp. 0001-0028.

Regulation (EC) No. 1831/2003 of the European Parliament and the Council of 22 September 2003 on additives for use in animal nutrition. Off. J. Eur. Union 2003, L268, 29-43.

Falkow, S., 1975. Infectious multiple drug resistance. London: Pion Ltd.

Franco Abuín, C.M., Quinto Fernández, E.J., Fente Sampayo, C., Rodríguez Otero, J.L., Domínguez Rodríguez, L., Cepeda Sáez, A., 1994. Susceptibilities of *Listeria* species isolated from food to nine antimicrobial agents. Antimicrob. Agents Chemother. 38, 1655-1657.

Finch, R.G., 1997. Tetracyclines, p. 469-484. In O`Grady HP, Lambert RG. Finch, Greenwood D. eds. Antibiotic and chemotherapy, 7th ed. Edingurgh: Churchill Livingstone Ltd., Edinburgh.

Frei, A., Goldenberge, D., Teuber, M., 2001. Antimicrobial susceptibility of intestinal bacteria from Swiss poultry flocks before the ban of antimicrobial grown promoters. Syst. Appl. Microbiol. 24, 116-121.

Ge, B., White, D.G., McDermott, P.F., Girard, W., Zhao, S., Hubert, S., Meng, J., 2003. Antimicrobial-resistant *Campylobacter* species from retail raw meats. Appl. Environ. Microbiol. 69, 3005-3007.

Gupta, A., Nelson, J.M., Barrett, T.J., Tauxe, R.V., Rossiter, S.P., Friedman, C.R., Joyce, K.W., Smith, K.E., Jones, T.F., Hawkins, M.A., Shiferaw, B., Beebe, J.L., Vugia, D.J., Rabatsky-Ehr, T., Benson, J.A., Root T.P., Angulo, F.J., 2004. Antimicrobial resistance among *Campylobacter* strains, United States, 1997–2001. Emerg. Infect. Dis. 10, 1102–1109.

Jones, R.N., Sader, H.S., Fritsche, T.R., 2004. Doxycycline use for community-acquired pneumonia: Comtemporary in vitro spectrum of activity against *Streptococcus pneumoniae* (1999-2002) Diagn. Microbiol. Infect. Dis. 49, 147-149.

Knarreborg, A., Simon, M.A., Engberg, R.M., Jensen, B.B., Tannock, G.W., 2002. Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. Appl. Environ. Microbiol. 68, 5918-5924.

Koeth, L.M., Felmingham, D., Jacobs, M.R., Rossi, F., 2004a. Antimicrobial resistance of *Streptococcus pneumoniae* and *Haemophilus influenzae* in Sao Paulo, Brazil from 1996 to 2000. Int. J. Antimicrob. Agents 23, 356-361.

Koeth, L.M., Jacobs, M.R., Good, C.E., Bajaksouzian, S., Windau, A., Jakielaszek, C., Saunders, K.A., 2004b. Comparative in vitro activity of a pharmacokinetically enhanced

oral formulation of amoxicillin/clavulanic acid (2000/125 mg twice daily) against 9172 respiratory isolates collected woldwide in 2000. Int. J. Infec. Dis. 8, 362-373.

McDermott, P.F., Bodeis, S.M., Aarestup, F.M., Brown, S., Traczewski, M., Fedorka-Cray, M., Wallace, M., Critchley, I.A., Thornsberry, C., Graff, S., Flamm, R., Beyer, J., Shortridge, D., Piddock, L.J., Ricci, V., Johnson, M.M., Jones, R.N., Reller, B., Mirrett, S., Aldrobi, J., Rennie, R., Brosnikoff, C., Turnbull, L., Stein, G., Schooley, S., Hanson, R.A., Walker, R.D., 2004. Development of a standardized susceptibility test for *Campylobacter* with quality-control ranges for ciprofloxacin, doxycycline, erythromycin, gentamicin, and meropenem. Microb. Drug Resist. 10, 124-131.

National Committee for Clinical Laboratory Standards. 2002. Performance standards for antimicrobial disk and dilution susceptibility test for bacteria isolated from animals: approved standard M31-A2, 2nd ed. Wayne, PA: NCCLS.

Peters, J., Mac, K., Wichman-Schaue, H., Klein, G., Ellerbroek, L., 2003. Species distribution and antibiotic resistance patterns of enterococci isolated from food of animal origin in Germany. Int. J. Food Microbiol. 88, 311-314.

Stern, N.J., Robach, M.C., 2003 Enumeration of *Campylobacter* spp. in broiler faeces and in corresponding processed carcasses. J. Food Prot. 66, 1557-1563.

Ronner, A.C., Olsson Engvall, E., Andersson, L., Kaijser, B., 2004. Species identification by genotyping and determination of antibiotic resistance in *Campylobacter jejuni* and *Campylobacter coli* from humans and chickens in Sweden. J. Clin. Microbiol. 96, 173-179.

Ross, J.E., Jones, R.N., 2004. The Quality Control Working Group. MIC quality control guidelines for doxycycline when testing Gram-positive control strains by the reference methods. Diagn. Microbiol. Infect. Dis. 50, 295-297.

Van den Bogaard, A.E., London, N., Driessen, C., Stobberingh, E.E., 2001. Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. J. Antimicrob. Chemother. 47, 763-771.

White, W., 1998. Medical consequences of antibiotic use in agriculture. Science 279, 996-997.

Wiuff, C., Lykkesfeldt, J., Svendsen, O., Aarestrup, F.M., 2003. The effects of oral and intramuscular administration and dose escalation of enrofloxacin on the selection of

quinolone resistance among *Salmonella* and coliforms in pigs. Res. Vet. Sci. 75, 185-193.

World Health Organization. 2005. WHO model list of essential medicines 14th Edition [Online.] http://www.who.int/medicines/publications/essentialmedicines/en/. Accessed 12 January 2006.

RESUMEN

Durante la producción aviar es cada vez más habitual el uso y/o abuso de antimicrobianos en tratamientos terapéuticos. Estas prácticas pueden conllevar un aumento en el número de cepas resistentes a dichos antimicrobianos, cepas que pueden diseminar su resistencia haciendo que en un futuro no muy lejano, algunos de estos tratamientos se vuelvan ineficaces.

El objetivo de este trabajo es evaluar los efectos de la doxiciclina sobre la flora intestinal y sobre la aparición de resistencias en pollos.Para ello, se utilizaron 36 pollos de un mismo lote que se dividieron en 2 grupos: grupo control (18 pollos) y grupo tratado (18 pollos), a este último y durante 5 días se les adicionó doxiciclina a través del agua de bebida (cuya ingesta se controlaba cada 12 horas), en las dosis terapéuticas establecidas por el fabricante, así como también se respetó el periodo de supresión indicado por el mismo.

Se tomaron muestras de heces (≥ 0.5 g) de la cloaca mediante torundaje antes de iniciar el tratamiento (día 0) y durante el primer, tercer y último día del tratamiento (días 1, 3 y 5). A continuación se tomaron muestras durante el tercer (día 8) y último día (día 12) del periodo de supresión.

Se estimaron las poblaciones de *Enterobacteriaceae*, *Enterococus* spp., Bacterias Ácido Lácticas (BAL) y *Campylobacter* spp. en los medios de cultivo apropiados. A continuación, de cada uno de estos grupos se aislaron e identificaron bioquímicamente un total de 432 cepas (18 por día de muestreo y grupo bacteriano) de las que se determinó su CMI, CMI_{50} y CMI_{90} para la doxiciclina mediante el método de microdilución en caldo, excepto para el caso del género *Campylobacter* para el cual fue utilizada la técnica de dilución en agar.

De los datos obtenidos hay que destacar que se encontraron diferencias estadísticamente significativas en la evolución de las poblaciones microbianas de los pollos tratados con doxiciclina (13.47 mg/kg peso/día) durante los periodos de tratamiento y de supresión estudiados, con recuentos claramente menores para el caso de *Enterococcus* (P = 0.0261), LAB (P = 0.0459) y *Campylobacter* (P = 0.0259) respecto al grupo control, lo que coincide con los datos encontrados en la literatura científica, y además se observa que efectivamente al final del periodo de supresión, las

poblaciones de bacterias gram-negativas se recuperan más rápidamente que las gram-positivas.

Por otra parte, los resultados mostraron también que las tasas de resistencia de *Enterobacteriaceae* en cualquier período siempre fueron mayores ($P = 0.0398$) que las de los grupos control incluido el último día del periodo de supresión. En el caso de *Campylobacter* hay que indicar que no se encontraron cepas resistentes en las muestras del grupo control en ningún momento y sí en cambio en los pollos tratados en los últimos días de muestreo ($P = 0.0057$). Se supone que el periodo de supresión establecido para los medicamentos permite el que las poblaciones bacterianas originales se recuperen y sin embargo este trabajo demuestra que en determinados casos esto no ocurre. El hecho de que persista una cierta tasa de cepas fecales resistentes después del periodo de supresión, puede suponer un riesgo para la salud pública si estas cepas llegan a diseminar su resistencia durante el sacrificio o posterior procesado de estos pollos.

3.4. Estudio de la evolución de bacterias fecales y de la selección de cepas resistentes durante el tratamiento con sulfonamidas y durante el periodo de supresión, en pollos

Faecal bacterial dynamics and selection of resistant strains during sulfonamides therapeutic treatment and the withdrawal period in poultry

Miranda, J.M., Franco, C.M., Vázquez, B.I., Fente, C.A., Barros-Velázquez, J., Cepeda A.

Artículo enviado para su publicación en el British Poultry Science.
Fecha de envío: Abril 2007.

Faecal bacterial dynamics and selection of resistant strains during sulfonamides therapeutic treatment and the withdrawal period in poultry

Miranda, J.M., Vázquez, B.I., Fente, C.A., Franco, C.M., and Cepeda A.

Laboratorio de Higiene Inspección y Control de Alimentos. Dpto de Química Analítica, Nutrición y Bromatología. Facultad de Veterinaria. Universidad de Santiago de Compostela. 27002-Lugo. Spain.

Running title: Effects of Sulfonamides treatment in poultry

ABSTRACT

Faecal bacterial populations were counted during a therapeutic treatment of poultry with sulfonamides. Treatment was carried out on 18 birds over 9 days, and 18 birds fed with the same feed but without treatment were maintained as controls. Faecal samples (≥ 0.5 g) were obtained from chicken by cloacae swabbing before starting treatment, as well as on days 1, 4 and 9 of treatment and days 16 and 24 of the withdrawal period. Populations of Enterobacteriaceae, Lactic Acid Bacteria (LAB) and *Campylobacter* spp. were estimated by plating onto appropriate selective media. A total of 324 strains were isolated before the start of treatment, at the end of treatment, and at the end of the withdrawal period. These strains were characterized and Minimal Inhibitory Concentrations to sulfisoxazole were determined using a broth microdilution method for Enterobacteriaceae and LAB and using an agar dilution for *Campylobacter*. No differences were obtained for the populations of Enterobacteriaceae and *Campylobacter*. Siginicative differences were obtained for LAB (P=0.0282), in which population were observed an increase in counts after began the treatment and a decrease in these counts immediately after stop this treatment. For the evolution of resistance rates, significant differences were obtained between treated and control batches along the treatment in the case of Enterobacteriaceae (P<0.0001), and *Campylobacter* (P=0.0025). These significative differences were maintained until the end of the withdrawal period in the

case of Enterobacteriaceae (*P*=0.0001) and *Campylobacter* (*P*=0.034). The results obtained in the present paper shows that sulfonamides treatment produces an increase in the poultry intestinal bacteria than can persist after the withdrawal period.

Keywords: poultry, sulfonamide, resistance, treatment, antimicrobial.

INTRODUCTION

Sulfonamides was the first chemical antimicrobial sintetized by the human. Since then, this antimicrobial family was used successfully in human as veterinary medicine until the emergence of bacterial resistance and the development of more potent drugs have limited their clinical massive use in human medicine to some bacterial protozoal or fungal infections (Patel et al. 2004; Meneau et al. 2004) and to coccidiostatic purposes and the treatment of some enteric and genital animal diseases in veterinary medicine, especially in reared-grown animals as poultry and turkey (Gibreel and Sköld 1999; Botana et al. 2003, Posyniak et al., 2005).

Sulfonamides are bacteriostatic and broad spectrum agents and their target is the enzyme dihydropterase synthase (DHPS), which catalyses the formation of dihydropteroic acid in bacteria and some eukaryotic cells. Antimicrobial mechanism is similar in all sulfonamides. This cause that bacteria resistant to one of then often can resist all them. However, in poultry farming as well as other intensively reared animals, antibiotic may be administrated to whole flocks rather than individual animals. In addition to therapeutic treatments, sulfonamides as other antimicrobial agents may be added directly to feed or water or can be administered in aerosols to animals in smaller dosage and in longer periods than those employed for therapeutic purposes to improve the animal rate of growth and feed conversion efficiency (Van den Boogard, Wiuff), although this practice is now banned in some countries as the EC. Epidemiological investigations suggest that several antimicrobial-resistant bacteria isolated from humans are primary originated from animals raised for human consumption and have been selected during the animal production stage rather than in the human medicine practice (Bager and Helmut). These resistant bacteria originated from animals can contaminate meat products easily during slaughter and processing (Franco Abuin; Wiuff).

The present study was undertaken in order to evaluate the sulfonamides effects in the intestinal bacteria populations and to determinate the intestinal bacteria degree of resistance against sulfonamides along the treatment and in the withdrawal period. This is because the latter is mainly determined as a function of the persistence of antimicrobial residues in animal tissues and it is crucial to know the resistance rates of intestinal bacterial populations when the withdrawal period has just finished because it is legal to send birds to slaughter after the withdrawal period has just ended.

MATERIALS AND METHODS

Animals employed

A total of thirty-six healthy label chickens aged 4-6 weeks were obtained from the same commercial hatchery. All birds were fed with the same antibiotic-free feed for 3 days before starting antimicrobial treatment. The birds were weighed and allocated in twelve groups of three birds each: treated chickens (groups 1-6) were treated with a therapeutic dose of sulfonamides in water (15 ml/l) of Cunisan Aviar® (Arimany, Barcelona, Spain) over 4 days, 2 days of repose and other 3 days of treatment, in accordance with the instructions on the package insert, and the other groups (groups 7-12) remained untreated, acting as controls. None of the chickens had direct physical contact with one another during the assays since they were housed in individual cages at the animal facility. Contamination between the treated and untreated batches was prevented by housing them in different rooms. The poultry were fed twice daily with antibiotic-free commercial feed (Biona, Palencia, Spain) and had free access to the medicated water, in the case of the treated poultry, or to antimicrobial-free water in the case of the control batches. Observation of water intake was performed at 12 h intervals.

All assays were carried out according to EC Council Directives (8) concerning the approximations of laws, regulations and administrative provisions of the member states regarding the protection of animals used for experimental and other scientific purposes.

Experimental procedures

Each group of chickens was sampled immediately before starting treatment (day 0), on first day on treatment (day 1) on fourth day of treatment (day 4), the last day of treatment (day 9), seven days after the end of the treatment (day 16), and after the withdrawal time had ended (day 24). Faecal samples were taken by swabbing the cloacae of each bird with sterile swabs to obtain a minimum of 0.5 g of faecal contents and these were placed aseptically in sterile tubes. Samples were taken to the laboratory in an ice chest in less than half an hour for immediate processing. Samples of animals belonging to each group were placed in a sterile masticator bag with an appropriate volume (1/9) (w/v) of sterile buffered peptone water (Merck, Darmstadt, Germany) and subsequently homogenized with a masticator (Aes, Combourg, France) for 2 m. After homogenisation, samples were tested for quantification, isolation and identification of Enterobacteriaceae, Lactic acid bacteria (LAB) and *Campylobacter* spp.

Enterobacteriaceae: Appropriate dilutions of homogenates were tested in poured plates of Crystal-violet neutral-red bile glucose agar (VRBG) agar prepared as specified by the manufacturer (Merck). After the agar had solidified, the plates were overlaid with 3-4 ml of melted VRBG and immediately incubated at 37 ºC for 24 h. After incubation, all pink to red colonies were counted.

LAB: Appropriate dilutions were tested by surface plating in De Man, Rogosa and Sharpe agar (MRS), prepared as specified by the manufacturer (Merck). Plates were incubated at 31 ºC for 72 h under a micro-aerobic atmosphere generated with the Anaerocult C® system in anaerobic jars (Oxoid, Basingstoke, UK). White circular colonies were counted.

Campylobacter spp.: Appropriate dilutions of homogenates were tested by surface plating in Campylossel® agar (BioMérieux, Marcy l`Etoile, France). Plates were incubated at 42 ªC for 48 h under a micro-aerobic atmosphere generated with the Campygen® system (Oxoid) in anaerobic jars (Oxoid). White colonies without haemolytic character were counted.

Only plates containing 20 to 250 colonies with the typical morphology of target bacteria were enumerated. Target bacteria count numbers were converted to $\log_{10}$ values

and expressed as $\log_{10}$ CFU/g faeces after calculating the bacterial population obtained from triplicate assays for each culture media.

The sampling and processing procedures of poultry faeces described in this work were always carried out by the same laboratory personnel. Agar media types were prepared by the same research assistant throughout the study.

Isolation and identification of bacteria

After incubation and counting, 3 typical colonies for each bacterial group were picked and transferred onto Columbia agar with 5% sheep blood (BioMérieux) and incubated under the same conditions of temperature, atmosphere and time as specified above in order to obtain pure cultures. This was done for each batch of chickens (three birds) on days 0, 9 and 24, obtaining a total of 324 strains (18 strains per bacterial group and treatment day).

These pure cultures were characterized as follows:

Enterobacteriaceae: Presumptive colonies were characterized by colony and cell morphology, Gram strain, oxidase, and catalase activity.

LAB: Presumptive colonies were characterized by colony and cell morphology, Gram stain, and catalase activity.

Campylobacter spp.: Presumptive *Campylobacter* colonies were characterized by colony and cell morphology, haemolytic character, Gram strain, oxidase and catalase activity and Dyspot Campylobacter test (Oxoid).

All isolates were stored at -80 $^{\circ}$C until further analysis using Maintenance Freeze Medium units (Oxoid).

Antibiotic susceptibility testing of bacteria

Antibiotic susceptibility testing for Enterobacteriaceae and LAB was performed using a broth microdilution susceptibility test on microtitre plates to determine the Minimum Inhibitory Concentration (MIC) to Sulfisoxazole. MICs and levels of

resistance were determined according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) [22].The microtitre plates were incubated for 18 to 24 h at 37ºC in the atmosphere in which the microbial group had been grown and with a source of moisture to prevent dehydration of the outer well. Sulfisoxazole was tested in two-fold increases from 4 to 2048 µ/ml on the microtitre plates. Target MIC ranges were verified with *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 reference strains as quality control.

For *Campylobacter* the susceptibility testing was done by the agar dilution procedure (Gibreel et al, 1999; Luber et al. 2003). Test medium consisted on Mueller-Hinton agar containing 5% defibrinated sheep blood in a range of sulfisoxazole from 4 to 2048 µ/ml. Plates were incubated for 48 h at 36 ºC under a micro-aerobic atmosphere generated with the Campygen® system (Oxoid) . Target MIC ranges were verified with *E. faecalis* ATCC 29212 reference strains as quality control since no reference values of sulfisoxazole susceptibility have been reported for *Campylobacter* reference strains.

The endpoint or MIC used was that recommended by the NCCLS [22] for Veterinary Pathogens: ≥ 512 µ/ml. The MICs that inhibited 50% (MIC50) and 90% (MIC90) of total strains were calculated from the MIC values obtained from each bacterial group. Sulfisoxazole standard dilutions were obtained from Sulfisoxazole (Sigma, St Louis, USA).

Statistical analysis

The amounts of water ingested by the treated and control birds were compared using an unpaired Student's t test. The mean of log CFU/g values in faeces were analysed by analysis of variance (ANOVA), with treatment and day as models. The distributions of resistant strains were compared by means of the X^2 test and Fisher's exact test. Differences were considered significant when probabilities were lower than 0.05. All statistical analyses were carried out using Statgraphics version 5.0.1. (SAS Institute, North Carolina, USA).

RESULTS

The amount of water ingested by the treated animals was 623 mL (SD =182.18) and for untreated birds it was 596 ml (SD= 109.86). This shows that sulfonamides treatment had no effect on the final amount of water ingested. Thus, since the medication used contained 9.31 g sulfamides/100 ml commercial drug, total consumption of the antimicrobials along the 9 days of treatment was 870.02 mg sulfonamides/ bird (SD = 254.42) (124.29 mg/bird and day). Since the mean weights of the chicken employed on day 0 was 678 g (SD= 175.26), the real dose ingested was 183.32 mg/kg of body weight/day.

The results for the evolution of the microbial population in faeces along treatment as well as in the withdrawal period for Enterobacteriaceae, LAB and *Campylobacter* are shown in figures 1 to 3 respectively.Sulfonamides treatment not caused a significative decrease in the microbial populations tested. No significant differences were obtained with treatment day as model for any bacterial group. Nevertheless, significant differences were obtained with treatment as model only for LAB (P=0.0282). No significant differences were obtained for the Gram negative populations tested. For all but *Campylobacter* populations increased during the course of treatment and reached the high level at the end of the treatment (day 9), and began to decrease after it. . For *Campylobacter* a little decrease was observed after starting the treatment, but it rapidly recovered the initial counts.

The results obtained for the MIC_{50}, MIC_{90}, MIC ranges and resistance rates for Enterobacteriaceae, LAB and *Campylobacter* are shown in Tables 1-3 respectively. There were no significant differences between the control and treated batches as regards the MIC profile for each bacterial group on day 0, except in the case of *Enterobacteriaceae*, in which MIC ranges were significantly higher in the case of treated batches (P=0.0299). Likewise, there were no significant differences in the control batches along the assay in any of the microbial groups tested except *Campylobacter (*P=0.026), in which an unexpected increase in resistance rates of controls were obtained in day 9. During the course of the treatment, sulfonamides efficiently increased resistance rates of Enterobacteriaceae (P=<0.001) and *Campylobacter* (P=0.0025). No significant differences was obtained for LAB

(*P*=0.802). After withdrawal period, resistance rates remained higher than controls in the case of *Enterobacteriaceae*, and *Campylobacter*.

DISCUSSION

The counts obtained from the poultry before the start of sulfonamides treatment contained a variable population average for the bacterial groups studied, although always in concordance with the data reported by other authors (Devriese, Frei, Knarrebourg, Stern).

The slight increase in viable counts of Enterobacteriaceae, and LAB observed after the start of treatment probably can be due the high level of resistance showed by these populations in day 0. For *Campylobacter*, the little decrease observed, as was expected due to the low level of resistance showed by *Campylobacter* in day 0 was rapidly stopped due to the selection of resistant strains, since in day 4, a 75% of intestinal *campylobacter* were already resistant to sulfisoxazole.

In this work, the resistance rates obtained for Enterobacteriaceae before the start of treatment (77.8% resistant strains, MIC_{50}=0512 mg/l and MIC_{90}=2048 mg/l) were compatible with those established by other authors for sulfonamides sensitivity in bacteria belonging to Enterobacteriaceae order. Thus, for *E. coli* isolated from turkeys, Cornican et al. (2001) found a 47.6% of resistance and for *E. coli* isolated from hens, and a 68% of resistant strains to sulfamides were obtained from turkeys. In Spain, other authors as Saenz et al. (2001) found in *E. coli* isolated from broilers faeces 65% of sulfa-resistant strains. Blanco et al. (1997) found frequencies in the range of 59 to 92% for trimethoprim-sulfamethoxazole resistance in *E. coli* isolated from healthy and sick chickens. Nevertheless, for other Enterobacteriaceae as Salmonella, significative lower resistance rates had been reported. So, Antunes et al (2003), who reported in *Salmonella* isolated from poultry products a 3% of resistance to trimethropim-sulphametoxazole.

The results obtained here shows that Enterobacteriaceae have a good ability to increase sulfa-resistance rates and to maintain part of this higher resistance rates until the end of withdrawal period. This is a very important task, since in addition of the loss of usefulness of sulfonamides for therapeutic purposes, in recent years, a number of multi-drug resistance phenotypes have been associated with large transferable plasmids,

on which may be other mobile DNA elements. For example, class I integrons, which have been frequently found located on plasmids in *E. coli*, cointain as a hallmark feature the Stull gene, and thus, are typically resistant to sulfonamides. Because class I intergrons may also cointain several antimicrobial resistance genes "cassettes", selection of sulpha resistance can co-select for resistance to other antimicrobials. (Schroeder et al. 2004).

For the case of LAB, it is known that they are often resistant to sulfonamides, since Enterococcus, a genus included in LAB are naturally resistant to sulfonamides and it was reported that Gram + bacteria are more frequently resistant than Gram – bacteria. In this sense, Bothe and Arnold obtained a higher resistnce rate to sulfonamides in Gam + bacteria (71%) than Gram – (56%). These data are in order with the obtained in the present work. Thus, this suggests that other LAB not included in the genus *Enterococcus* posses a lower sulphonamide resistance than *Enterococcus* genus.

The resistance rates obtained for *Campylobacter* in the control birds were compatible with the data reported by other authors such as Luber et al. (2003), who in German retail market chicken poultry obtained a 54.6% of trimetropim-sulfamethoxazole in *Campylobacter Jejuni* and a 47.1% in *Campylobacter coli*.

One of the important goals of the present work was to determine whether important resistance rates might persist at the end of the withdrawal period. Studies aimed at approving antimicrobials for specific use in animal medicine include specific drug depletion assays in order to determine when the maximum residual limit has been attained and hence to establish the corresponding withdrawal period. However, although recovery of the bacterial population not resistant to the drug was expected to occur during the withdrawal period, in our hands the resistance rates, MIC_{50} and MIC_{90} obtained at the end of the withdrawal period were always higher than those obtained on day 0, and were always higher than those obtained on day 12 in control batches, except in LAB and the case of the MIC_{50} in Enterobacteriaceae.

Taking into account the faecal contamination of future poultry carcasses as well as the fact that treated chickens are often sent to the slaughterhouse immediately after the withdrawal period, it is expected that enteric pathogens responsible for food-borne diseases such as Enterobacteriaceae and *Campylobacter* could increase its sulpha resistance rates during the treatment and, in the case of *Campylobacter*, could maintain

this higher resistance after the withdrawal period has finished and could be a risk for public safety after poultry slaughter and processing, since it is known that this sulpha resistance can co-select for resistance to other antimicrobials.

Table 1. MIC_{50} and MIC_{90}, range and resistance rates for Enterobacteriaceae strains obtained from treated and control birds during and after sulfonamides treatment in their drinking water.

	Sampling Day		
	Day 0	**Day 9**	**Day 24**
Treated Birds			
No. strains	18	18	18
MIC_{50}	512	2048	2048
MIC_{90}	2048	<2048	>2048
Range	<4-2048	<4->2048	1024->2048
No. resistant strains (%)	14(77.8)	16(88.9)	18(100)
Control Birds			
No. strains	18	18	18
MIC_{50}	512	<4	512
MIC_{90}	1024	2048	1024
Range	<4-1024	<4-2048	<4-2048
No. resistant strains (%)	10(55.6)	6(33.3)	11(61.1)

MIC_{50}, MIC_{90} and range expressed in μg/ml.

Day 0: Before start of treatment. Day 9: End of treatment. Day 24: End of withdrawal period.

Table 2. MIC_{50} and MIC_{90}, range and resistance rates for LAB strains obtained from treated and control birds during and after sulfonamides application in their drinking water.

	Sampling Day		
	Day 0	**Day 9**	**Day 24**
Treated Birds			
No. strains	18	16	18
MIC_{50}	1024	64	256
MIC_{90}	1024	1024	2048
Range	<4->2048	<4-1024	<4->2048
No. resistant strains (%)	12(66.7)	6(37.5)	8 (44.4)
Control Birds			
No. strains	18	18	18
MIC_{50}	256	256	256
MIC_{90}	1024	1024	2048
Range	<4-2048	<4-2048	<4-2048
No. resistant strains (%)	7 (38.9)	7 (38.9)	9 (50)

MIC_{50}, MIC_{90} and range expressed in µg/ml.

Day 0: Before start of treatment. Day 9: End of treatment. Day 24: End of withdrawal period.

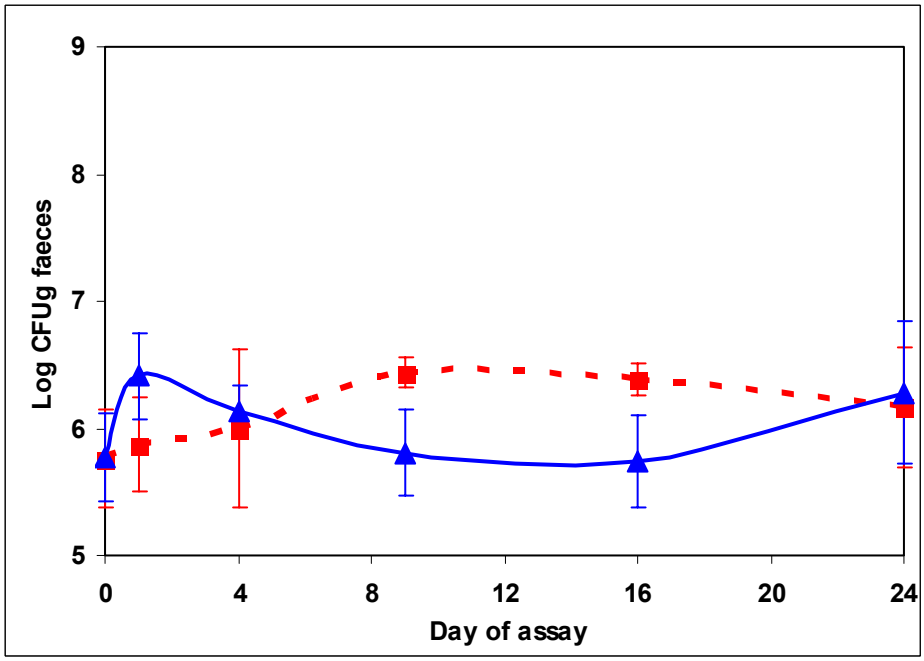
Table 3. MIC_{50} and MIC_{90}, range and resistance rates for *Campylobacter* spp. strains obtained from treated and control birds along and after sulfonamides application in their drinking water.

	Sampling Day		
	Day 0	**Day 9**	**Day 24**
Treated Birds			
No. strains	17	16	18
MIC_{50}	128	1024	512
MIC_{90}	512	2048	>2048
Range	<4-1024	<4-2048	<4-<2048
No. resistant strains (%)	4 (23.5)	12 (75)	14 (77.8)
Control Birds			
No. strains	16	18	18
MIC_{50}	16	512	64
MIC_{90}	1024	2048	2048
Range	<4-2048	<4->2048	<4-2048
No. resistant strains (%)	2(12.5)	11 (61.8)	4 (28.6)

MIC_{50}, MIC_{90} and range expressed in µg/ml.

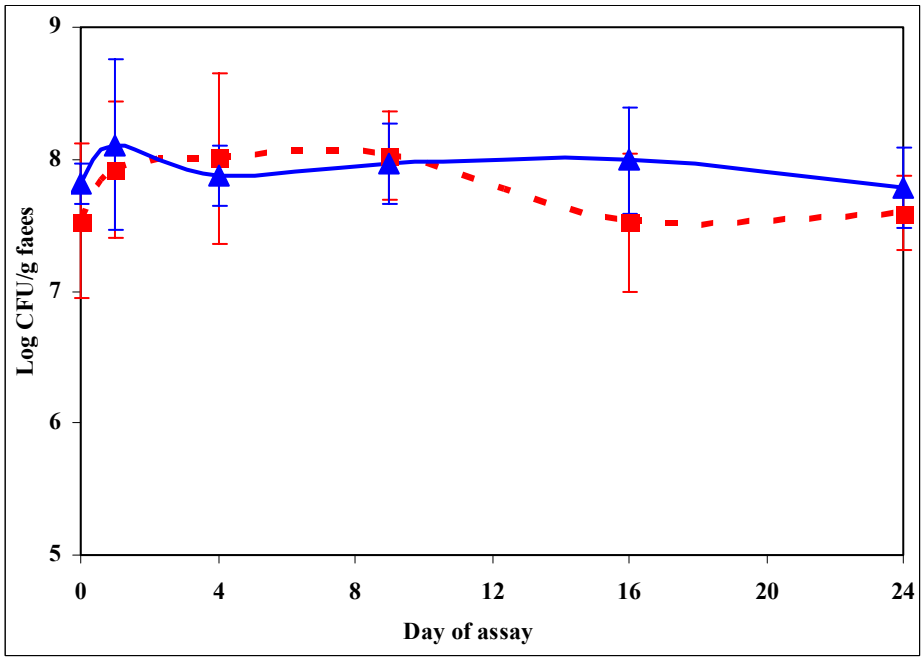
Day 0: Before start of treatment. Day 9: End of treatment. Day 24: End of withdrawal period.

Figure 1. Evolution of intestinal Enterobacteriaceae. during sulfonamides treatment and in the withdrawal period.



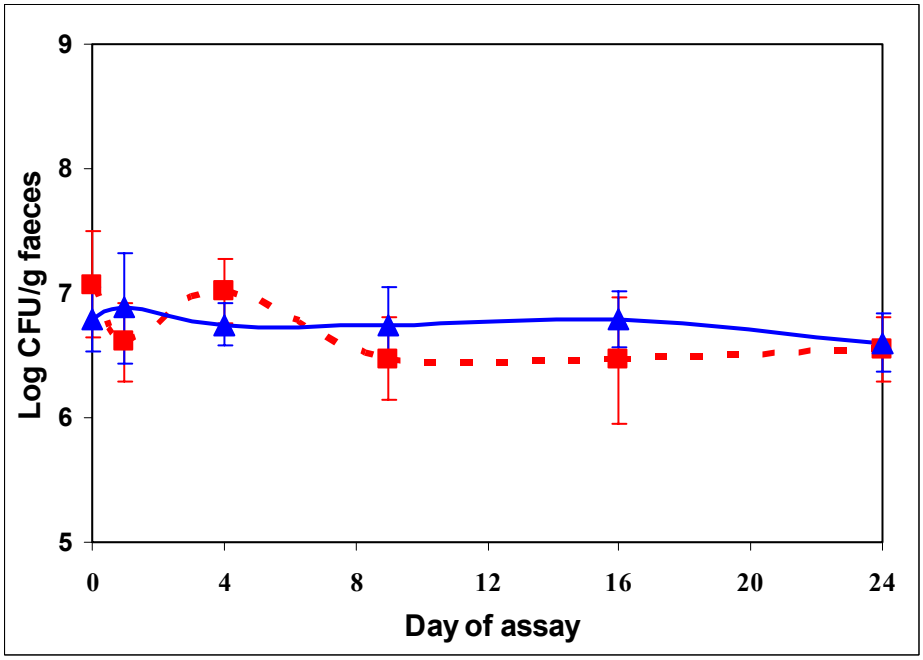
Squares denote treated chickens and triangles denote control chickens.

Figure 2. Evolution of intestinal LAB during sulfonamides treatment and in the withdrawal period.



Squares denote treated chickens and triangles denote control chickens.

Figure 3. Evolution of intestinal *Campylobacter* during sulfonamides treatment and in the withdrawal period.



Squares denote treated chickens and triangles denote control chickens.

References

Bager, F., and R. Helmuth. 2001. Epidemiology of resistance to quinolones in *Salmonella*. Vet. Res. 32: 285-290.

Blanco, J. E., M. Blanco, A. Mora, and J. Blanco. 1997. Prevalence of bacterial resistance to quinolones and other antimicrobials among avian *Escherichia coli* strains isolated from septicemia and healthy chickens in Spain. J. Clin. Microbiol. 35: 2184-2185.

Boothe, D.H., and J. W. Arnold. 2003. Resistance of bacterial isolates from poultry products to therapeutic veterinary antibiotics. J. Food Prot. 66: 94-102.

Chopra I, P. M. Hawkey, and M. Hinton. 1992. Tetracyclines, molecular and clinical aspects. J. Antimicrob. Chemother. 29: 245-277.

Cornican, M., V. Buckley, G. Corbett-Feeney, and F. Sheridan. 2001. Antimicrobial resistance is *Escherichia coli* isolates from turkeys and hens in Ireland. J. Antimicrob. Chemother. 48: 587-595.

Devriese, L. A., J. Hommez, R. Wijfels, and F. Haesebrouck. 1991. Composition of the enterococcal and Streptococcal intestinal flora of chickens. J. Appl. Bacteriol. 71: 46-50.

EEC Council Directive 86/609/ of November 24 on the approximations of laws, regulations and administrative provisions of the member states regarding the protection of animals used for experimental and other scientific purposes. OJL 358, December 18, 1986.

Frei, A., D. Goldenberger, and M. Teuber. 2001. Antimicrobial susceptibility of intestinal bacteria from Swiss poultry flocks before the ban of antimicrobial grown promoters. Syst. Appl. Microbiol. 24: 116-121.

Franco Abuín, C.M., E. J. Quinto Fernández, C. Fente Sampayo, J. L. Rodríguez Otero, L. Domínguez Rodríguez, and A. Cepeda Sáez. 1994. Susceptibilities of *Listeria* species isolated from food to nine antimicrobial agents. Antimicrob. Agents Chemother. 38: 1655-1657.

Gibreel, A. and O. Sköld. 1999. Sulfonamide resistance in clinical isolates of *Campylobacter jejuni*: mutational changes in the chromosomal diydropteroate synthase. Antimicrob. Agents Chemother. 43:2156-2160.

Knarreborg, A., M. A. Simon, R. M. Engberg, B. B. Jensen, and G. W. Tannock. 2002. Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. Appl. Environ. Microbiol. 68: 5918-5924.

Luber, P., E. Bartelt, E. Genschow, J. Wagner, and H. Hahn. 2003. Comparison of broth microdilution, E test, and agar dilution methods and antibiotic susceptibility testing of *Campylobacter jejuni* and *Campylobacter coli*. J. Clin. Microbiol. 41: 1062-1068.

Luber, P., J. Wagner, H. Hahn, and E. Bartelt. 2003. Antimicrobial resistance in *Campylobacter coli* strains isolated in 1991 and 2001-2002 from poultry and humans in Berlin, Germany. Antimicrob. Agents chemother. 47: 3825-3830.

Meneau, I., D. Sanglard, J. Bille, and P. M. Hauser. 2004. *Pneumocystis jiroveci* Dihidropterase synthase polymorphisms confer resistance to sulfadoxine and sulfanilamide in *Saccharomyces cerevisiae*. Antimicrob. Agents. Chemother. 48: 2610-2616.

National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk and dilution susceptibility test for bacteria isolated from animals: approved standard M31-A2, 2nd ed. Wayne, PA: NCCLS; 2002.

Patel, O. G., E. K. Mberu, A. M. Nzila, and I. G. Macreadie. 2004. Sulfa drugs strike more than once. Trends Parasitol. 20: 1-3.

Posyniak, A., J. Zmudzki, and K. Mitrowska. 2005. Dispersive solid-phase extraction for the determination of sulfonamides in chicken muscle by liquid chromatography. J. Chomatogr. A. 1087: 259-264.

Sáez, Y., M. Zarazaga, L. Briñas, M. Lantero, F. Ruiz-Larrea, and C. Torres. 2001. Antibiotic resistance in *Escherichia coli* isolates isolated from animals, foods and humans in Spain. Int. J. Antimicrob. Agents 18: 353-358.

Schroeder, C.M., D.G. White, and J. Meng. 2004. Retail meat and poultry as an important reservoir of antimicrobial-resistant *Escherichia coli*. Food Microbiol. 21: 249-255.

Stern, N.J., and M. C. Robach. 2003 Enumeration of *Campylobacter* spp. in broiler faeces and in corresponding processed carcasses. J. Food Prot. 66: 1557-1563.

Van der Bogaard, A.E., N. London, C. Driessen, C. and E. E. Stobberingh. 2001. Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. J. Antimicrob. Chemother**. 47:** 763-771.

Wiuff, C., J. Lykkesfeldt, O. Svendsen, O. and F. M. Aarestrup. 2003. The effects of oral and intramuscular administration and dose escalation of enrofloxacin on the selection of quinolone resistance among *Salmonella* and coliforms in pigs. Res. Vet. Sci. 75: 185-193.

RESUMEN

Las sulfamidas han sido los primeros antimicrobianos de gran efectividad sintetizados por el hombre y desde entonces se han utilizado ampliamente tanto en la medicina humana como en la animal. Posteriores investigaciones demostraron que, quizás debido a su masivo uso, estaban apareciendo elevadas tasas de bacterias resistentes a estos medicamentos, y que estas resistencias que presumiblemente se habían originado en los tratamientos en animales, de allí se habían expandido a los humanos. Es por esto que su uso actualmente se encuentra bastante limitado.

En el estudio que aquí se presenta, se pretende evaluar el efecto de las sulfamidas en las poblaciones bacterianas intestinales en pollos y el grado de cepas resistentes que se encuentran durante el periodo de tratamiento, pero sobretodo determinar las que permanecen al final del periodo supresión puesto que presumiblemente estos pollos pueden ser enviados al matadero justo al final dicho periodo.

Para ello y durante 7 días, a 18 pollos se les suministró agua adicionada con sulfamidas (15 ml l^{-1}) y se dejaron otros 18 pollos con la misma agua pero sin el medicamento (controles); todos ellos de 4-6 semanas de edad y procedentes del mismo criadero. Se tomaron muestras fecales (≥ 0,5 g) de la cloaca de cada pollo mediante torundaje: antes de comenzar el tratamiento y a los 1,4 y 9 días del tratamiento; y a los 16 y 29 días que se corresponden con el periodo de supresión.

Tanto el protocolo de tratamiento como el tiempo correspondiente al periodo de supresión, fueron efectuados atendiendo a las instrucciones del fabricante de la presentación comercial utilizada.

En las muestras de heces, se realizaron los recuentos de la microbiota fecal, en concreto de poblaciones bacterianas de la Fam. *Enterobacteriaceae*, Bacterias Ácido Lácticas (LAB) y *Campylobacter* spp., que se hicieron crecer en los medios cultivo adecuados.

Se aislaron un total de 324 cepas (18 por día de muestreo y grupo bacteriano) antes de comenzar el tratamiento, durante el tratamiento y durante el periodo de supresión. Todas ellas se identificaron bioquímicamente y se estableció la Concentración Mínima Inhibitoria (CMI) para el sulfisoxazol usando el método de microdilución en caldo para

Enterobacteriaceae y LAB y usando el método de dilución en agar para las cepas pertenecientes al género *Campylobacter*.

De los resultados obtenidos, hay que destacar que en general las sulfamidas no causaron disminución en las poblaciones bacterianas fecales estudiadas, de hecho, no se pudieron apreciar diferencias estadísticamente significativas entre las poblaciones de *Enterobacteriaceae* y *Campylobacter* spp. procedentes de los pollos tratados y control durante el periodo de estudio. Sólo se encontraron diferencias estadísticamente significativas para LAB (P = 0.0282) en el sentido de que su población crece justo después de iniciar el tratamiento, aunque decrece inmediatamente después de suspenderlo.

Respecto a la evolución de las tasas de aparición de resistentes, se encontraron diferencias estadísticamente significativas entre los pollos tratados con sulfonamidas y los pollos control, siendo mayores para el caso de enterobacterias (P <0.0001) y *Campylobacter* spp. (P = 0.0025). Pero es que cabe resaltar que el considerable grado de resistencia encontrado para *Enterobacteriaceae* ya antes del inicio del tratamiento (77.8 %, CMI_{50}=0512 mg l^{-1} y CMI_{90}=2048 mg l^{-1}) es compatible con los establecidos por otros autores; así que los datos que se hallaron en este estudio corroboran la habilidad de este grupo bacteriano para aumentar e incluso mantener posteriormente estas resistencias. En el caso del género Campylobacter igualmente se observa un aumento claramente progresivo en las tasas de cepas resistentes desde el inicio hasta el final del periodo de supresión (desde 23.5 a 77.8 %).

En cuanto a las bacterias ácidolácticas, se encuentra que a menudo son resistentes a las sulfamidas sobretodo porque se ha demostrado que los enterococos (género incluido en este grupo) presenta una resistencia natural a estos fármacos. En este trabajo sin embargo no se pudieron establecer diferencias con el grupo control puesto que es posible que en las muestras tomadas abunden otros géneros no tan resistentes como *Enterococcus* spp.

Por último, y teniendo en cuenta que estos pollos pueden ser enviados al matadero inmediatamente después de finalizar el periodo de supresión establecido por las autoridades sanitarias, hay que destacar que el mayor grado de cepas resistentes encontradas para los grupos fecales *Enterobacteriaceae* y *Campylobacter* se mantenía perfectamente en las muestras analizadas justo el último día correspondiente al periodo

de supresión (P = 0.0001, para ambos) lo que puede conllevar un riesgo potencialmente considerable si esas cepas son capaces de transmitir su capacidad a otras bacterias durante el procesado y/o consumo de las carnes que contaminen.

3.5. Resistencia a antimicrobanos en *Enterococcus* spp. aislados de carne de pollo ecológico, pollo convencional y pavo.

Antimicrobial resistance in *Enterococcus* spp. strains isolated from organic chicken, conventional chicken and turkey meat: A comparative survey.

Miranda, J.M., Guarddon, M., Mondragón, A., Vázquez, B.I., Fente, C.A., Cepeda, A., Franco, C.M.

Journal of Food Protection, 70 (4): 1021-1024.

Journal of Food Protection, Vol. 70, No. 4, 2007, Pages 1021–1024

Research Note

Antimicrobial Resistance in *Enterococcus* spp. Strains Isolated from Organic Chicken, Conventional Chicken, and Turkey Meat: A Comparative Survey

J. M. MIRANDA,[1] M. GUARDDON,[1] A. MONDRAGÓN,[2] B. I. VÁZQUEZ,[1] C. A. FENTE,[1] A. CEPEDA,[1] AND C. M. FRANCO[1]*

[1]*Laboratorio de Higiene Inspección y Control de Alimentos, Dpto de Química Analítica, Nutrición y Bromatología, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002-Lugo, Spain; and* [2]*Centro de Investigaciones Químicas, Universidad Autónoma del Estado de Hidalgo, Carretera Pachuca-Tulancingo, Kilometre 4.5, Ciudad Universitaria, 42074, Pachuca de Soto, Hidalgo, México*

MS 06–301: Received 31 May 2006/Accepted 4 October 2006

ABSTRACT

The mean counts of *Enterococcus* spp. were determined for 30 samples each of organic chicken meat, conventional chicken meat, and turkey meat, and differences for *Enterococcus* contamination in meat were determined. Two enterococci strains from each sample were isolated to obtain a total of 180 strains, and resistance to ampicillin, chloramphenicol, doxycycline, ciprofloxacin, erythromycin, gentamicin, nitrofurantoin, and vancomycin was determined by a disk diffusion method. Average counts obtained showed that *Enterococcus* mean counts from organic chicken meat (3.18 log CFU/g) were significantly higher than those obtained from conventional chicken meat (2.06 log CFU/g) or conventional turkey meat (1.23 log CFU/g). However, the resistance data obtained showed that isolates from organic chicken meat were less resistant than enterococci isolates from conventional chicken meat to ampicillin ($P = 0.0067$), chloramphenicol ($P = 0.0154$), doxycycline ($P = 0.0277$), ciprofloxacin ($P = 0.0024$), erythromycin ($P = 0.0028$), and vancomycin ($P = 0.0241$). In addition, isolates from organic chicken were less resistant than conventional turkey meat isolates to ciprofloxacin ($P = 0.001$) and erythromycin ($P = 0.0137$). Multidrug-resistant isolates were found in every group tested, but rates of multidrug-resistant strains were significantly higher in conventional chicken and turkey than those obtained from organic chicken meat. *Enterococcus faecalis* was the most common species isolated from organic chicken (36.67%), whereas *Enterococcus durans* was the most common species isolated from conventional chicken (58.33%) and turkey (56.67%). The rates obtained for antimicrobial resistance suggest that although organic chicken meat may have higher numbers of *Enterococcus,* these bacteria present a lower level of antimicrobial resistance.

Organic and other nonconventional meat products are now readily available at retail in developed countries to satisfy consumers' demand for high-quality products that meet the following requirements: (i) guarantee animal welfare during production; (ii) absence of chemical agents during animal feeding; (iii) environmental friendliness; and (iv) better taste than conventional products *(8).* In addition, many consumers believe that because growth conditions are more natural for organic rather than conventional products, the former will have less pathogenic bacteria *(4).* However, little is known about the microbiological status of organic animal products and the potential microbiological risks linked to organic meat production. Thus, raising of animals outdoors, use of slow-growing breeds, strict restrictions in therapeutic use of antimicrobial agents, or use of very small slaughtering facilities may not guarantee strict microbiological control of animals destined for human consumption *(8, 19).*

Currently, it is well known that several antimicrobial-resistant bacteria isolated from humans primarily originate from animals raised for human consumption *(1)* and that such resistant bacteria may contaminate meat derived from those animals *(16).* Although this contamination declines in the absence of antimicrobial agents *(15),* antimicrobial-resistant bacteria may persist in meat even after the withdrawal period *(24, 26).* Thus, the development of antibiotic resistance among bacterial isolates from animals can also represent a potential hazard to consumers via foodborne infections caused by antibiotic-resistant bacteria.

Enterococcus spp. are found in a wide variety of environments, including dairy and food products *(5, 9),* humans, and animals *(25). Enterococcus* can be used as an indicator of both fecal contamination of foods *(5, 20)* and the dissemination of antimicrobial resistance related to the use of antimicrobials in poultry farming *(3, 10, 13, 21, 25, 27).* Although enterococci are generally considered to be only mildly pathogenic, today they are one of the leading causes of major nosocomial infections *(14, 23).*

Recently, other authors have reported on the development of antimicrobial resistance by bacteria isolated from organic dairy products *(17, 18, 22)* as well as *Salmonella* and *Campylobacter* in poultry products *(4, 7, 19).* However, little information relative to commensal bacteria isolated from organic poultry meat is currently available. Conse-

* Author for correspondence. Tel: +34982252231, Ext 22407; Fax: +34982254592; E-mail: cmfranco@lugo.usc.es.

quently, the main goal of this study was to investigate the prevalence of antimicrobial susceptibility of enterococci strains isolated from organic chicken meat as compared with conventional chicken and turkey meats.

MATERIALS AND METHODS

Collection of poultry meat samples. A total of 90 fresh prepackaged skin-on drumstick samples were taken during 2005 from supermarkets and butcher shops: 30 organically reared chicken samples, 30 conventionally reared chicken samples, and 30 conventionally reared turkey samples. All conventional poultry samples were taken on different days and from different supermarkets and butcher shops. The organic chicken samples were obtained from five supermarkets (six samples per supermarket) with the supermarkets visited on different days. All supermarkets and butcher shops were located in Galicia (northwestern Spain). All samples were processed 3 to 4 days before the expiration date indicated on the label.

Microbiological analyses. Portions of 25 g were obtained from each meat sample, placed in a sterile masticator bag, diluted 1:9 (wt/vol) in sterile 0.1% peptone water (Merck, Darmstadt, Germany), and homogenized in a masticator (Aes, Combourg, France) for 1 min. After homogenization, samples were analyzed for the presence of *Enterococcus* spp. by pour plating 1 ml of the 10^{-1} to 10^{4} dilutions using Chromocult enterococci agar prepared according to the manufacturer's instructions (Merck). After solidification, the plates were overlaid with 3 to 4 ml of Chromocult and incubated at 37°C for 48 h. Following incubation, pink to red colonies were counted as *Enterococcus* spp. All samples were processed in triplicate.

Two typical *Enterococcus* spp. colonies isolated from each meat sample were picked, transferred onto Columbia agar supplemented with 5% sheep blood (bioMérieux, Marcy l'Etoile, France), and incubated at 37°C for 48 h in order to obtain a total of 180 pure cultures. These isolates were identified by colony and cell morphology, Gram stain, oxidase, and catalase activity. Presumptive *Enterococcus* spp. were confirmed with API ID 32 Strep (bioMérieux) test kits and, when necessary, API 50 CH (bioMérieux) test kits to complete the identification.

All 180 *Enterococcus* spp. isolates were stored at −80°C in maintenance freeze medium units (Oxoid, Basingstoke, UK) until antimicrobial susceptibility testing.

Antimicrobial susceptibility testing. All 180 *Enterococcus* spp. isolates (60 from organic chicken meat, 60 from conventional chicken meat, and 60 from conventional turkey meat) were subjected to antimicrobial susceptibility testing by an agar disk diffusion method *(11)* on Mueller-Hinton agar plates (bioMérieux). The antimicrobial disks used included ampicillin (10 μg), chloramphenicol (30 μg), doxycycline (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), nitrofurantoin (300 μg), and vancomycin (200 μg) (Oxoid). The antibiotic resistance breakpoints used were the interpretative criteria for *Enterococcus* spp. as recommended by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) *(12). Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were used as controls.

Antimicrobials were chosen on the basis of their ability to provide diversity for representation of different antimicrobial agent classes. *Enterococcus* spp. isolates were classified as sensitive, intermediate, or resistant. Isolates resistant to two or more antimicrobial agents were considered to be multiresistant strains.

Statistical analysis. The mean log CFU per gram values in meats were compared using a Student's *t* test. The distributions of resistant strains, multiresistance patterns, enterococci species, and the antimicrobial resistance levels between the different species isolated were compared by using the χ^2 test and Fisher's exact test. For comparing resistance between the different species isolated, *Enterococcus* was split into *E. durans, E. faecalis,* and other species. Differences were considered significant when probabilities were <0.05. All statistical analyses were carried out with Statgraphics version 5.0.1. (SAS Institute, Cary, N.C.).

RESULTS

Mean counts obtained from the meat of organic chicken (3.18 log CFU/g) (standard deviation [SD] = 1.11), conventional chicken (2.06 log CFU/g) (SD = 1.04), and turkey (1.23 log CFU/g) (SD = 0.609) were significantly different between organic and conventional chicken ($P = 0.0002$), organic chicken and conventional turkey ($P < 0.0001$), and conventional chicken and turkey ($P = 0.0005$).

The antimicrobial resistance rates obtained for enterococci isolated from conventional chicken meat were significantly higher than those obtained for enterococci isolated from conventional turkey meat for chloramphenicol ($P = 0.0014$) and significantly lower for ciprofloxacin ($P = 0.0015$).

The incidence of antimicrobial resistance to ampicillin ($P = 0.0067$), chloramphenicol ($P = 0.0154$), doxycycline ($P = 0.0277$), ciprofloxacin ($P = 0.0024$), erythromycin ($P = 0.0028$), and vancomycin ($P = 0.0241$) was significantly lower in enterococci isolates from organic chicken samples compared with isolates from conventional chicken samples (Table 1). In addition, enterococci isolated from organic chicken meat showed lower antimicrobial resistance levels for ciprofloxacin ($P = 0.001$) and erythromycin ($P = 0.0137$) than enterococci isolated from turkey meat. Similarly, organic chicken contained fewer multiresistant isolates. (Table 2). Multiresistance rates for enterococci isolated from organic chicken (11.67%) were lower than those obtained from conventional chicken (33.33%) as well as those found for turkey (31.67%). Statistical evidence was obtained at a lower prevalence for multiresistant enterococci in organic chicken meat than conventional chicken meat ($P = 0.0021$) or conventional turkey meat ($P = 0.0266$).

The *Enterococcus* strains isolated from organic chicken meat showed a different species distribution compared with isolates from conventional chicken ($P = 0.0105$) or turkey meat ($P = 0.0052$). Organic chicken yielded the following species distribution pattern: *E. faecalis* (36.67%), *E. durans* (31.67%), *E. casseliflavus* (6.67%), *E. gallinarum* (6.67%), and *E. faecium* (1.67%). Other genera identified were *Aerococcus* spp. (5%), *Lactococcus* spp. (3.33%), and not identified (NI) (8.33%). Enterococci isolated from conventional chicken included the following: *E. durans* (58.33%), *E. faecalis* (21.67%), *E. avium* (1.67%), *E. faecium* (1.67%), and *E. hirae* (1.67%). Other genera identified were *Lactococcus* spp. (5%), *Aerococcus* spp. (5%), *Leuconostoc* spp. (1.67%), and NI (3.33%). In the case of enterococci isolated from conventional turkey, the presumptive species distribution was as follows: *E. durans* (56.67%), *E. faecalis*

TABLE 1. *Percentages of* Enterococcus *spp. isolated from organic chicken, conventional chicken, and conventional turkey susceptible (S), intermediate (I), and resistant (R) to microbial agents by disk diffusion method*[a]

Antimicrobial agent (μg)	Organic chicken			Conventional chicken			Conventional turkey		
	S	I	R	S	I	R	S	I	R
Ampicillin (10)	81.67	—	18.33	66.67	—	33.33	65	—	35
Chloranphenicol (30)	96.67	1.67	1.67	80	15	5	98.33	1.67	0
Doxycycline (30)	85	15	0	68.33	23.33	8.33	86.67	13.33	0
Ciprofloxacin (5)	55	40	5	58.33	18.33	23.33	26.67	40	33.33
Erythromycin (15)	31.67	66.67	1.67	45	40	15	56.67	40	3.33
Gentamicin (10)	75	20	5	70	18.33	11.67	73.33	18.33	8.33
Nitrofurantoin (300)	95	1.67	3.33	83.33	10	6.67	90	5	5
Vancomycin (30)	96.67	3.33	0	85	3.33	11.67	90	5	5

[a] $n = 60$ for each type of meat.

(36.67%), and *E. gallinarum* (1.67%), with *Aerococcus* spp. (5%) also identified.

Thus, the most common presumptive enterococci species isolated from organic chicken was *E. faecalis* (36.67%), whereas *E. durans* was the most common species isolated from conventional chicken (58.33%) and turkey (56.67%).

Significant differences in antimicrobial susceptibility of enterococci species were observed for ciprofloxacin, in which *E. durans* exhibited greater antimicrobial resistance than the other species ($P = 0.0245$), and for erythromycin, in which *E. faecalis* was significantly less resistant compared with the other isolates ($P = 0.0380$).

DISCUSSION

The mean counts obtained in the present work suggest a relation between enterococci contamination of meat and the farming procedures employed in animal rearing. Thus, as it is expected, in organic chicken, in which antimicrobial use was assumed to be less based on claims by the industry, the mean counts of enterococci were significantly higher than those obtained from conventional chicken and turkey meat, where higher quantities of antimicrobial agents are used *(6, 27)*.

During recent years, widely variable rates of antimicrobial resistance were reported for enterococci isolated from conventionally reared poultry and poultry meat *(10, 13, 21, 25, 27)*. The antimicrobial resistance levels determined in our study are in global terms the same or lower compared with those reported by these other authors. Nevertheless, the lower levels of *E. faecium* and higher levels of *E. durans* in our work compared with these other studies *(10, 13, 21, 25, 27)* can be a distorting factor when comparing resistance levels. Indeed, *E. faecium* is the enterococci species that has the broadest spectrum of natural and acquired antibiotic resistance *(11)*.

TABLE 2. *Multiresistance patterns in* Enterococcus *spp. strains isolated from organic chicken, conventional chicken, and conventional turkey*[a]

No. of antimicrobials	Organic chicken, No. (%)	Conventional chicken, No. (%)	Conventional turkey, No. (%)
0	44 (73.33)	22 (36.67)	27 (45)
1	9 (15)	18 (30)	14 (23.33)
2	5 (8.33)	8 (13.33)	13 (21.67)
3	2 (3.33)	7 (11.67)	4 (6.67)
4	0 (0)	2 (3.33)	2 (3.33)
≥5	0 (0)	3 (5)	0 (0)
Multiresistant strains	7 (11.67)	20 (33.33)	19 (31.67)

[a] $n = 60$ for each type of meat.

Our results also indicate that antimicrobial resistance in enterococci isolates can change based on the type of animal production system. Enterococci isolated from organic chicken are more susceptible to certain antimicrobials than counterpart enterococci strains isolated from conventionally farmed animals. Thus, the high differences observed for ciprofloxacin, probably due to quinolones, likely result from their widespread use in poultry medicine *(2, 6)*. Despite economic disincentives, ciprofloxacin is particularly popular in treating turkeys *(6)*. In recent years, quinolone use on turkey farms has also increased, especially for treating bacterial infections that are secondary viral rhinotracheitis *(6)*. Nevertheless, as was true for erythromycin, the low level of resistance found in enterococci isolates from organic chicken meat could be in part explained by the different rates of enterococci species isolated from organically and conventionally raised birds. In this sense, *E. durans* exhibited greater resistance to ciprofloxacin ($P = 0.0014$) than the other species isolated in this work ($P = 0.0014$), the latter being significantly less frequent in organic as compared with conventional poultry. As for erythromycin, significantly lower resistance levels were seen for *E. faecalis* than the other enterococci groups ($P = 0.047$), with this species also more frequently isolated from organic than conventional chicken. These results, in addition to the finding that *E. durans* was more frequently isolated from conventional than organic samples and that enterococci species other than *durans* and *faecalis* were more common in organic than in conventional samples, could be due to selection by the antimicrobials used in conventional poultry farming.

Because of more widespread use of antimicrobials in turkey farming *(6, 25)*, higher antimicrobial resistance rates were expected for the turkey meat isolates than those enterococci isolated from conventional or organic chicken meat. However, higher antimicrobial resistance was only seen for ciprofloxacin with respect to enterococci isolates

obtained from both conventional and organic chicken meat. Likewise, for the case of erythromycin, the resistance rates obtained for enterococci isolated from turkey meat were higher than those obtained for enterococci isolated from organic chicken meat but not higher than those obtained for enterococci isolated from conventional chicken meat. Furthermore, other surprising results included the higher chloramphenicol resistance of isolates from conventional chicken compared with those obtained from organic chicken or turkey. Because the use of chloramphenicol is banned in poultry medicine, it is difficult to attribute this higher resistance rate to antimicrobial exposure.

In summary, the lower rates of antimicrobial resistance in enterococci isolated from organically farmed chicken, as compared with conventionally farmed chicken and turkey, indicate that organic farming may limit the development and spread of antimicrobial resistance among foodborne bacteria. Considering that organic meats are more costly to purchase than their conventional counterparts, organizations regulating organic food must be able to assure the origin of organically farmed animals. In this sense, continuous antimicrobial-resistance monitoring could provide one means of preventing fraudulent activity because resistant bacteria developed during treatment survive longer than the antimicrobial residues in meat. However, more research would need to be carried out to confirm this strategy as a monitoring tool.

ACKNOWLEDGMENT

Special appreciation is extended to Carmen Carreira for technical assistance.

REFERENCES

1. Aarestrup, F. M. 2000. Occurrence, selection and spread of resistance to antimicrobial agents used for growth promotion for food animals in Denmark. *APMIS* 108(Suppl. 101):5–48.
2. Avrain, L., F. Humbert, R. L. L'Hospitalier, P. Sanders, C. Vernozy-Rozand, and I. Kempf. 2003. Antimicrobial resistance in *Campylobacter* from broilers: association with production type and antimicrobial use. *Vet. Microbiol.* 96:267–276.
3. Bager, F., M. Madsen, J. Christensen, and F. M. Aarestrup. 1997. Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant *Enterococcus faecium* on Danish poultry and pig farms. *Prev. Vet. Med.* 31:95–112.
4. Bailey, J. S., and D. E. Cosby. 2005. *Salmonella* prevalence in free-range and certified organic chickens. *J. Food Prot.* 68:2451–2453.
5. Chingwaru, W., S. F. Mpuchane, and B. A. Gashe. 2003. *Enterococcus faecalis* and *Enterococcus faecium* isolates from milk, beef, and chicken and their antibiotic resistance. *J. Food Prot.* 66:931–936.
6. Cornican, M., V. Buckley, G. Corbett-Feeney, and F. Sheridan. 2001. Antimicrobial resistance in *Escherichia coli* isolates from turkey and hens in Ireland. *J. Antimicrob. Chemother.* 48:587–588.
7. Cui, S., B. Ge, J. Zheng, and M. Jianghong. 2005. Prevalence and antimicrobial resistance of *Campylobacter* spp. and *Salmonella* serovars in organic chickens in Maryland retail stores. *Appl. Environ. Microbiol.* 71:4108–4111.
8. Dransfield, E., T. M. Ngapo, N. A. Nielsen, L. Bredahl, P. O. Sjödén, M. Magnusson, M. M. Campo, and G. R. Nute. 2005. Consumer choice and suggested price for pork as influenced by its appearance, taste and information concerning country of origin and organic pig production. *Meat Sci.* 69:61–70.
9. Giraffa, G., and F. Sisto. 1997. Susceptibility to vancomycin of enterococci isolated from dairy products. *Lett. Appl. Microbiol.* 25: 335–338.
10. Joseph, S. W., J. R. Hayes, L. L. English, L. E. Carr, and D. D. Wagner. 2001. Implication of multiple antimicrobial-resistant enterococci associated with the poultry environment. *Food Addit. Contam.* 18:1118–1123.
11. Klare, I., C. Konstabel, D. Badstübner, G. Werner, and W. Witte. 2003. Occurrence and spread of antibiotic resistances in *Enterococcus faecium*. *Int. J. Food Microbiol.* 88:269–290.
12. NCCLS. 2002. Performance standards for antimicrobial susceptibility testing, 12th informational supplement. M100-S12. NCCLS, Wayne, Pa.
13. Novais, C., T. M. Coque, M. J. Costa, J. C. Sousa, F. Baquero, and L. V. Peixe. 2005. High occurrence and persistence of antimicrobial-resistant enterococci in poultry food samples in Portugal. *J. Antimicrob. Chemother.* 56:1139–1143.
14. Peters, J., K. Mac, H. Wichmann-Shauer, G. Klein, and L. Ellerbroek. 2003. Species distribution and antibiotic resistance patterns of enterococci isolated from food of animal origin in Germany. *Int. J. Food Microbiol.* 88:305–309.
15. Phillips, I., M. Casewell, T. Cox, B. De Groot, C. Friis, R. Jones, C. Nightingale, R. Preston, and J. Waddell. 2004. Does the use of antimicrobials in food animals pose a risk to human health? A critical review of published data. *J. Antimicrob. Chemother.* 53:28–52.
16. Sáenz, Y., M. Zarazaga, L. Briñas, M. Lantero, F. Ruiz-Larrea, and C. Torres. 2001. Antibiotic resistance in *Escherichia coli* isolates obtained from animals, foods and humans in Spain. *Int. J. Antimicrob. Agents* 18:353–358.
17. Sato, K., P. C. Bartlett, J. B. Kaneene, and F. P. Downes. 2004. Prevalence and antimicrobial susceptibilities of *Campylobacter* spp. isolates from organic and conventional dairy hens in Wisconsin. *Appl. Environ. Microbiol.* 70:1442–1447.
18. Sato, K., T. W. Bennedsgaard, P. C. Barltett, R. J. Erskine, and J. B. Kaneene. 2004. Comparison of antimicrobial susceptibility of *Staphylococcus aureus* isolated from bulk tank milk in organic and conventional dairy herds in the Midwestern United States and Denmark. *J. Food Prot.* 67:1104–1110.
19. Soonthornchaikul, N., H. Garelick, H. Jones, J. Jacobs, D. Ball, and M. Choudhury. 2006. Resistance to three antimicrobial agents of *Campylobacter* isolated from organically- and intensively-reared chickens purchased from retail outlets. *Int. J. Antimicrob. Agents* 27: 125–130.
20. Stiles M. E., and W. H. Holzapfel. 1997. Lactic acid bacteria of foods and their current taxonomy. *Int. J. Food Microbiol.* 36:1–29.
21. Tejedor-Junco, M. T., J. L. Afonso-Rodriguez, J. L. Martín-Barrasa, and M. González-Martín. 2005. Antimicrobial susceptibility of *Enterococcus* strains isolated from poultry faeces. *Res. Vet Sci.* 78:33–38.
22. Tikofsky, L. L., J. W. Barlow, C. Santiesteban, and Y. H. Schukken. 2003. A comparison of antimicrobial susceptibility patterns for *Staphylococcus aureus* in organic and conventional dairy herds. *Microb. Drug Resist.* 9:39–45.
23. Tsai, J. C., P. R. Hsueh, H. M. Lin, H. J. Chang, S. W. Ho, and L. J. Teng. 2005. Identification of clinically relevant *Enterococcus* species by direct sequencing of *gro*Es and spacer region. *J. Clin. Microbiol.* 43:235–241.
24. Van den Bogaard, A. E., N. London, C. Driessen, and E. E. Stobberingh. 2001. Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. *J. Antimicrob. Chemother.* 47:763–771.
25. Van den Bogaard, A. E., R. Willens, N. London, J. Top, and E. E. Stobberingh. 2002. Antibiotic resistance of faecal enterococci in poultry, poultry farmers and poultry slaughterers. *J. Antimicrob. Chemother.* 49:497–505
26. Wiuff, C., J. Lykkesfeldt, O. Svendsen, and F. M. Aarestrup. 2003. The effects of oral and intramuscular administration and dose escalation of ciprofloxacin on the selection of quinolone resistance among *Salmonella* and coliforms in pigs. *Res. Vet. Sci.* 75:185–193.
27. Yoshimura, H., M. Ishimaru, Y. S. Endoh, and A. Kojima. 2000. Antimicrobial susceptibilities of enterococci isolated from faeces of broiler and layer chickens. *Lett. Appl. Microbiol.* 31:427–432.

RESUMEN

Enterococcus ha sido un género bacteriano ampliamente utilizado en numerosos estudios científicos como indicador de la contaminación fecal en alimentos, pero también ha servido para estudiar la posible diseminación de la resistencia microbiana relacionada con el uso de fármacos en la producción aviar.

Recientemente se ha realizado algún trabajo sobre la resistencia antimicrobiana de géneros como *Salmonella* o *Campylobacter* aislados de productos lácteos y productos a base de carne de pollo ecológicos. Sin embargo, puesto que hay poca información respecto a otros grupos bacterianos, es objeto del estudio que aquí se presenta, investigar la susceptibilidad de las bacterias pertenecientes al género *Enterococcus* a varios agentes antimicrobianos, en muestras de carne de pollo procedentes de producciones de carácter ecológico y compararlas con muestras procedentes de la cría convencional en intensivo.

Así, se determinaron los recuentos de *Enterococcus* spp. en 90 muestras de carne, compradas en supermercados y carnicerías, procedentes de pollos criados de forma ecológica y de pollos y pavos criados de forma convencional (30 muestras de cada). A continuación se aislaron 2 cepas de cada muestra, obteniendo finalmente un total de 180 cepas de enterococos.

Se determinó la resistencia a la ampicilina, cloranfenicol, doxyciclina, ciprofloxacina, eritromicina, gentamicina, nitrofurantoina y vancomicina por el método de difusión en disco.

La media de los recuentos del género *Enterococcus* (3.18 log UFC/g) en las muestras de pollo ecológico resultaron ser significativamente mayores que las obtenidas de la carne procedente de pollo (2.06 log UFC/g) y pavo (1.23 log UFC/g) de cría convencional ($P = 0.0002$ y $P < 0.0001$, respectivamente). Estos datos corroboran que existe una relación entre el grado de contaminación y los procedimientos de producción de estas carnes, siendo éste menor cuando se han usado medicamentos. Teniendo en cuenta que en la producción de carácter ecológico el uso de fármacos está muy restringido y aunque se intenta evitar la presencia de flora contaminante por otros medios (selección de razas, ambiente higiénico, cuidados especiales, etc.) está claro que la susceptibilidad de la carne de pollo a la contaminación bacteriana sigue siendo altamente notable.

Sin embargo, los datos sobre las tasas de resistencias de los enterococos aislados a partir de la carne de pollo ecológica fueron significativamente menores que las obtenidas para los enterococos aislados a partir de carne de pollo de cría convencional para el caso de 6 agentes antimicrobianos (ampilicina (P = 0.0067), cloranfenicol (P = 0.0154), ciprofloxacina (P = 0.0024), doxiciclina (P = 0.0277), eritromicina (P = 0.0028) y vancomicina (P = 0.0241)). Asimismo, también fueron significativamente menores que las tasas obtenidas para los enterococos aislados a partir de carne de pavo de cría convencional en el caso de 2 antimicrobianos (ciprofloxacina (P = 0.001) y eritromicina (P = 0.0137).

Se encontraron cepas multiresistentes (resistentes a dos antimicrobianos o más) en todos los grupos de aves muestreados. La proporción de cepas multiresistentes resultó ser siempre significativamente menor en los enterococos aislados a partir de carne ecológica (11.67 %) que en los aislados a partir de los otros dos tipos de carne de aves procedentes de la cría convencional (33.33 % y 31.67 % para pollos y pavos respectivamente).

Otro aspecto a destacar es que *Enterococcus faecalis* ha sido la especie mas frecuentemente aislada en pollo ecológico (36.67 %), mientras que *Enterococcus durans* lo fue en los pollos y pavos de cría convencional (58.33 % y 56.67 % respectivamente).
El grado de resistencias determinado en este trabajo en términos globales coincide o es algo menor que los establecidos por otros autores. Sin embargo hay que tener en cuenta que los menores aislamientos de *E. faecium* en contraposición con los mayores de *E. durans* hallados en este trabajo, pudieran constituir un cierto factor de distorsión cuando se comparan estas tasas de resistencia con la literatura científica, puesto que esta primera especie presenta generalmente un mayor espectro resistente frente a antimicrobianos.

Las mayores tasas de resistencia se encontraron para ampicilina y para ciprofloxacina en los 3 grupos de carnes. Esto puede ser debido, por ejemplo en el caso de la ciprofloxacina a que, a pesar de su mayor precio, está siendo cada vez más utilizada en toda la UE en terapéutica aviar, máxime en el caso de los pavos, donde efectivamente se corrobora en este trabajo que se encuentra un mayor grado de resistencia (33.33 %) que en pollos (23.33 %) (ambos de producción convencional).

Sorprendentemente, hay que señalar el hallazgo de cepas resistentes a cloranfenicol, mayor en pollos de producción intensiva (5%) (que en pollos de producción ecológica (1.67%), ninguna en pavos). Puesto que el uso de esta sustancia está prohibida desde

hace años, es difícil atribuir estas resistencias a la exposición actual de los animales al fármaco.

En conclusión, por una parte se puede indicar que la producción de carácter ecológico parece ser una estrategia eficaz para evitar la dispersión de la resistencia a los agentes antimicrobianos en los alimentos. Por otra parte y considerando que los alimentos ecológicos son más costosos que los obtenidos de forma convencional, las organizaciones que regulan este tipo de productos deberían ser capaces de asegurar la verdadera naturaleza de los mismos; en este sentido, se propone que una monitorización continua de la tasa de resistencias podría ser una herramienta eficaz para prevenir las posibles prácticas fraudulentas en el sector.

3.6. Resistencia de cepas pertenecientes a la Fam. *Enterobacteriaceae* aisladas de pollo y pavo de producción convencional y pollo de producción ecológica.

Antimicrobial resistance in Enterobacteriaceae strains isolated from organic chicken, conventional chicken and conventional turkey meat: A comparative survey

Miranda, J.M., Guarddon, M., Vázquez, B.I., Fente, C.A., Barros-Velázquez, J., Cepeda, A., Franco, C.M.

Food Control, Acepted 7-5-07, In Press.

Antimicrobial resistance in Enterobacteriaceae strains isolated from organic chicken, conventional chicken and conventional turkey meat: A comparative survey

[a]Miranda, J.M., [a]Guarddon, M., [a]Vázquez, B.I., [a]Fente, C.A., [b]Barros-Velázquez, J., [a]Cepeda, A. and [a]Franco, C.M.*

[a]Laboratorio de Higiene Inspección y Control de Alimentos. Dpto de Química Analítica, Nutrición y Bromatología. Facultad de Veterinaria. Universidad de Santiago de Compostela. 27002-Lugo. Spain;
[b]Area de Tecnología de los Alimentos. Dpto de Química Analítica, Nutrición y Bromatología. Facultad de Veterinaria. Universidad de Santiago de Compostela. 27002-Lugo. Spain.

Abstract

Mean counts of Enterobacteriaceae were determined for 30 samples each of organic chicken meat, conventional chicken meat and conventional turkey meat to assess differences in contamination. Two strains from each sample were isolated to obtain a total of 180 strains, which were examined for resistance to ampicillin, chloramphenicol, cephalothin, doxycycline, ciprofloxacin, gentamicin, nitrofurantoin, and sulfisoxazole. The average counts obtained showed that mean counts of Enterobacteriaceae from organic chicken meat were significantly higher than those obtained from conventional chicken ($P<0.0001$) or conventional turkey ($P<0.0001$) meat. However, the resistance data obtained showed that isolates from organic chicken meat were less resistant than isolates from conventional chicken meat to ampicillin ($P=0.0001$), chloramphenicol ($P=0.0004$), doxycycline ($P=0.0013$), ciprofloxacin ($P=0.0034$), gentamicin ($P=0.0295$) and sulfisoxazole ($P=0.0442$), and were less resistant than isolates from turkey meat to doxycycline ($P=0.0014$) and sulfisoxazole ($P=0.0442$). Multidrug resistant isolates were found in every group tested, but rates of multidrug resistant strains were higher in conventional chicken (63.3%) and turkey (56.7%) than organic chicken (41.7%) meat. The rates obtained for antimicrobial resistance support the theory that although organic

chicken meat contains more Enterobacteriaceae contamination, organic farming practices contribute to decreased dissemination of antibiotic resistance.

Keywords: poultry, organic, Enterobacteriaceae, antimicrobial, resistance.

1. Introduction

Organic and other non-conventional meat products are now readily available for retail in developed countries, to satisfy consumers' demand for high-quality products that meet the following requirements: (i) guaranteed animal welfare during production; (ii) absence of chemical agents during animal feeding; (iii) environmental-friendliness, and (iv) better taste than conventional products (Dransfield et al., 2005). However, little is known about the microbiological status of organic animal products and the potential microbiological risks linked to organic meat production. Thus, raising of animals outdoors, use of slow-growing breeds, strict restrictions in the therapeutic use of antimicrobial agents and use of small slaughtering facilities may not guarantee strict microbiological control of animals destined for human consumption (Dransfield et al., 2005; Soonthornchaikul et al., 2006).

Currently, it is well known that several antimicrobial-resistant bacteria isolated from humans originate primarily from animals raised for human consumption (Aarestrup, 2000) and that such resistant bacteria may contaminate the meat derived from those animals (Sáenz et al. 2001). Although this contamination declines in the absence of antimicrobial agents (Philips et al., 2004), the presence of antimicrobial-resistant bacteria may persist in meat even after the withdrawal period (Van den Boogard et al., 2001; Wiuff et al., 2003). Thus, the development of antibiotic resistance among bacterial isolates from animal sources can also represent a potential hazard to consumers *via* food-borne infections caused by antibiotic-resistant bacteria.

The Enterobacteriaceae family is commonly used as an indicator of faecal contamination during food microbiological analyses, and includes important zoonotic bacteria such as *Salmonella* spp., *Yersinia* spp. and *Escherichia coli.* Enterobacteriaceae are significant causes of serious infection, and many of the most important members of this family are becoming increasingly resistant to currently available antimicrobials

(Paterson, 2006). This is an important phenomenon that requires vigilance and find measures to control the further spread of resistance by pathogens included in this family.

Recently, antimicrobial resistance has been reported in bacteria isolated from organic dairy products (Sato et al., 2004a; Sato et al., 2004b; Tikofsky et al., 2003), and in poultry products related to *Salmonella* and *Campylobacter* (Cui et al., 2005; Soonthornchaikul et al., 2006). However, little information relative to commensal bacteria isolated from organic poultry meat products is currently available. Consequently, the main goal of the present study was to investigate the prevalence of antimicrobial susceptibility found in Enterobacteriaceae isolates derived from organic chicken meat as compared to conventional chicken and turkey meats. The potential implications of these results in terms of microbiological safety, especially concerning the development and spread of antimicrobial resistance to the food chain, are also discussed.

2. Methods

2.1. Collection of poultry meat samples

A total of 90 fresh pre-packaged skin-on drumstick samples were taken during 2005 from supermarkets and butcher shops: 30 organic-reared chicken samples, 30 conventionally-reared chicken samples, and 30 conventionally-reared turkey samples. All samples were taken on different days and in different supermarkets and butcher shops for the case of conventional poultry. For the case of organic chickens, certified products only were found in 5 supermarkets, so 6 organic samples were taken in each supermarket, but all of them on different days. All supermarkets and butcher shops were located in Galicia (North-Western Spain). All samples were processed between three and four days before the expiration date indicated on the label.

2.2. Microbiological analyses

All samples were processed following standard performance ISO 7402:1993 for plate count of Enterobacteriaceae: Portions of 25 g were obtained from each meat sample, placed in a sterile masticator bag with an appropriate volume (1/9) (w/v) of sterile 0.1% peptone water (Merck, Darmstadt, Germany), and subsequently homogenized for 1 min. with a masticator (Aes, Combourg, France). After homogenization, samples were investigated for the presence of Enterobacteriaceae. Thus, 10^{-1} to 10^{-4} dilutions of meat extracts were tested on poured plates of Crystal-violet neutral-red bile glucose agar (VRBG), which were prepared as specified by the manufacturer (Merck). Once the agar had solidified, plates were overlaid with 3-4 ml of melted VRBG and incubated at 35-37 ºC for 24 h. After incubation, red colonies were identified as Enterobacteriaceae and counted.

Once the bacterial counts were determined, two typical Enterobacteriaceae colonies isolated from each meat sample were picked, transferred onto Columbia agar supplemented with 5% sheep blood (BioMérieux, Marcy l`Etoile, France), and incubated at 35-37ºC for 24 h in order to obtain a total of 180 pure cultures. Such pure cultures were identified by colony and cell morphology, Gram stain, oxidase and catalase activity. Positive strains initially identified as Enterobacteriaceae were identified by API 20 E (BioMérieux) identification tests.

All 180 Enterobacteriaceae isolates were stored at -80°C in Maintenance Freeze Medium units (Oxoid, Basingstoke, UK) until antimicrobial susceptibility testing.

2.3. Antimicrobial susceptibility testing of bacteria

Antimicrobial susceptibility testing was performed for a total of 180 isolates of Enterobacteriaceae (60 from organic chicken meat, 60 from conventional chicken meat and 60 from conventional turkey meat). Antimicrobial susceptibility testing was carried out on Mueller-Hinton agar plates (Biomerieux) by the agar disk diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI, Formerly

NCCLS, 2002). Antimicrobial disks considered were: ampicillin (10 μg), cephalothin (30 μg), chloramphenicol (30 μg), doxycycline (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), nitrofurantoin (300 μg) and sulfisoxazole (300 μg) (Oxoid). The antibiotic resistance breakpoints considered were the interpretative criteria for Enterobacteriaceae recommended by the CLSI (2002). *E. coli* ATCC 25922 was used as quality control.

Antimicrobials were chosen on the basis of their ability to provide a diverse representation of different antimicrobial agent classes. Enterobacteriaceae isolates were classified as sensitive, intermediate or resistant. Isolates exhibiting resistance to at least two of the antimicrobial agents tested were considered to be multi-resistant strains.

2.4. Statistical analysis

The mean log CFU/g values in each meat sample analyzed were compared using a Student's t test. The distributions of resistant strains, multi-resistance patterns and Enterobacteriaceae genera distribution were compared by X^2 test and Fisher's exact test. Differences were considered significant when probabilities were less than 0.05. All statistical analyses were carried out using Statgraphics version 5.0.1. (SAS Institute, North Carolina, USA).

3. Results and Discussion

Our results indicate that the special characteristics of organic production give rise to higher Enterobacteriaceae counts in meats than conventional farming. Thus, as expected for organic chicken, in which antimicrobial use is more restricted, mean counts obtained (3.81 log CFU/g) (*SD*=1.10) were significantly higher *(P*<0.0001) than those obtained for conventional chicken (2.66 log CFU/g) (*SD*=1.3) and conventional turkey (1.44 log CFU/g) (*SD*=0.661) (*P*<0.0001) meat. In addition, mean counts obtained for conventional chicken were significantly higher than those obtained for conventional turkey meat, in which farming procedures require greater antimicrobial usage than chicken farming (Cornican et al., 2001; Van den Boogard et al., 2001).

During recent years, several studies have reported the antimicrobial resistance of some Enterobacteriaceae genera isolated from poultry, such as *Escherichia* and *Salmonella* (Cornican et al., 2001; Sáenz et al., 2001; Van den Boogard et al., 2001; Antunes et al., 2003; Guerra et al.,2003; Kijima-Tanaka et al., 2003). The antimicrobial resistance rates obtained in our study are in agreement with those described by these authors, and also provide evidence supporting the fact that antimicrobial resistance of Enterobacteriaceae isolates can change depending on the type of animal production system considered. Different patterns were observed in strains isolated from organic chicken meat compared to their counterparts isolated from conventional chicken and turkey meat (Table 1). Antimicrobial resistance was significantly lower in Enterobacteriaceae isolates from organic chicken samples for ampicillin (P=0.0001), chloramphenicol (P=0.0004), doxycycline (P=0.0013), ciprofloxacin (P=0.0034), gentamicin (P=0.0295), and sulfisoxazole (P=0.0442), as compared to Enterobacteriaceae isolated from conventional chicken samples. In addition, isolates from organic chicken meat demonstrated a lower level of antimicrobial resistance than their Enterobacteriaceae counterparts isolated from turkey meat for doxycycline (P<0.0001) and sulfisoxazole (P=0.0442). The resistance rates obtained for Enterobacteriaceae isolated from conventional chicken meat were significantly higher than those obtained from turkey meat for chloramphenicol (P=0.0044), ciprofloxacin (P=0.0199) and gentamicin (P=0.0067), and significantly lower for cephalothin (P=0.0063).

It should be noted that resistance rates obtained for isolates from organic chicken were significantly lower than those obtained from conventional chicken and turkey for the antimicrobial agents more commonly used in poultry medicine, such as quinolones (ciprofloxacin), tetracyclines (doxycycline) and sulphonamides (sulfisozaxole) (Cornican et al., 2001; Van den Boogard et al., 2001; Antunes et al., 2003; Avrain et al., 2003), with the exception of ciprofloxacin resistance in conventional turkey isolates. Conversely, due to the more common use of antimicrobial agents in turkey farming than chicken farming (Cornican et al., 2001; Van den Boogard et al., 2001), higher antimicrobial resistance rates were expected for Enterobacteriaceae isolates obtained from turkey meat than for those isolated from conventional or organic chicken meats. Nevertheless, the antimicrobial resistance rates obtained in our study were higher for the

strains isolated from conventional chicken meat in global terms. Remarkably, higher chloramphenicol resistance was observed in isolates from conventional chicken compared to organic chicken or conventional turkey. Since the use of this antimicrobial is banned in poultry medicine, it is difficult to attribute this difference in resistance rates to antimicrobial exposure.

In addition to individual resistance to tested antimicrobial agents, organic chicken isolates showed a lower level of multi-resistant bacteria (Table 2). Multi-resistance rates of Enterobacteriaceae isolated from organic chicken (41.7%) were lower than those obtained from conventional chicken (63.3%), as well as turkey (56.7%). Statistical analysis indicated a lower prevalence of multi-resistant Enterobacteriaceae in organic chicken meat versus conventional chicken meat (P=0.0197).

Identification of Enterobacteriaceae isolates from organic chicken, conventional chicken and turkey meat to the genus level is shown in Table 3. The most common Enterobacteriaceae genus isolated from every tested group was *Serratia* (45% of isolates from organic chicken, 28.3% of isolates from conventional chicken and 33.3% of isolates from conventional turkey). Other commonly isolated genera were *Enterobacter* (21.7% of isolates from organic chicken, 16.7% of isolates from conventional chicken and 23.3% of isolates from conventional turkey) and *Klebsiella* (6.7% of isolates from organic chicken, 6.7% of isolates from conventional chicken and 25% of isolates from conventional turkey). Enterobacteriaceae strains isolated from conventional turkey meat demonstrated different distribution profiles compared to their countherparts isolated from organic (P=0.0010) and conventional chicken meat (P=0.0055). These differences in classification could distort analyses used to compare resistance rates obtained from turkey isolates.

In summary, lower rates of antimicrobial resistance were observed in Enterobacteriaceae isolated from organically farmed chicken compared to counterpart strains derived from conventionally farmed chicken or turkey. This supports the idea that organic farming may limit the development and spread of antimicrobial resistance among food-borne bacteria. Taking into account that organic meats reach higher prices in market than conventional meat, organic food regulation agencies must assure that organic meat sold in markets is truly derived from organically farmed animals. In this sense, although more microbiological studies are necessary, continuous antimicrobial

resistance monitoring could be a useful tool to preclude fraudulent activities. Since resistant bacteria that develop during antimicrobial treatment can survive after the time than the persistence of antimicrobial residues in animal tissues, monitoring of this resistance could help to detect antimicrobial treatments not allowed in organic animal farming.

Acknowledgements

The authors wish to thank the Xunta de Galicia for garanting research project (PGIDIT05TAL003E). The authors also thank Carmen Carreira for her expert technical assistance.

References

Aarestrup, F. M. (2000) Occurrence, selection and spread of resistance to antimicrobial agents used for growth promotion for food animals in Denmark. APMIS 108 (Supplementum No. 101), 5-48.

Antunes, P., Réu, C., Sousa, J.C., Peixe, L. & Pestana, N. (2003) Incidence of *Salmonella* from poultry products and their susceptibility to antimicrobial agents. *Int. J. Food Microbiol*. 82, 97-103.

Cornican, M., Buckley, V., Corbett-Feeney, G. & Sheridan, F. (2001) Antimicrobial resistance in *Escherichia coli* isolates from turkey and hens in Ireland. *J. Antimicrob. Chemother.* 48, 587-588.

Cui, S., Ge, B., Zheng, J. & Jianghong, M. (2005) Prevalence and antimicrobial resistance of *Campylobacter* spp. and *Salmonella* serovars in organic chickens in Maryland retail stores. *Appl. Environ. Microbiol.* 71, 4108-4111.

Dransfield, E., Ngapo, T. M., Nielsen, N. A., Bredahl, L., Sjödén, P. O., Magnusson, M., Campo, M. M. & Nute, G. R. (2005) Consumer choice and suggested price for pork as influenced by its appearance, taste and information concerning country of origin and organic pig production. *Meat Sci.* 69, 61-70.

Guerra, B., Junker, E., Schoeter, A., Malorny, B., Lehmann, S. & Helmuth, R. **(**2003) Phenotypic and genotypic characterization of antimicrobial resistance in German *Escherichia coli* isolates from cattle, swine and poultry. *J. Antimicrob. Chemother.* 52, 489-492.

ISO Standard 7402. (1991) General Guidance for the Enumeration of Enterobacteriaceae Whithout Resuscitation-MPN Technique and Colony Count Technique. International Organization for Standardization, Geneva, Switzerland

Kijima-Tanaka, M., Ishihara, K., Morioka, A., Kojima, A., Ozono, T., Ogikubo, K., Takahashi, T. & Tamura, Y. (2003) A national surveillance of antimicrobial resistance in *Escherichia coli* isolated from food-producing animals in Japan. *J. Antimicrob. Chemother.* 51, 447-451.

National Committee for Clinical Laboratory Standards (2002) *Performance standards for antimicrobial susceptibility testing;* 12th informational supplement. M100-S12. NCCLS, Wayne, PA.

Paterson, D. L. (2006) Resistance in Gram-negative bacteria: Enterobacteriaceae. *Am. J. Med.* 119, 520-528.

Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preston, R. & Waddell, J. (2004) Does the use of antimicrobials in food animals pose a risk to human health? A critical review of published data. *J. Antimicrob. Chemother*. 53, 28-52.

Sáenz, Y., Zarazaga, M., Briñas, L., Lantero, M., Ruiz-Larrea, F. & Torres, C. (2001) Antibiotic resistance in *Escherichia coli* isolates obtained from animals, foods and humans in Spain. *Int. J. Antimicrob. Agents.* 18, 353-358.

Sato, K., Barlett, P. C., Kaneene, J. B. & Downes, F. P. (2004a) Prevalence and antimicrobial susceptibilities of *Campylobacter* spp. Isolates from organic and conventional dairy hens in Wisconsin. *Appl. Environ. Microbiol.* 70: 1442-1447.

Sato, K., Bennedsgaard, T. W., Barlett, P. C., Erskine, R. J. & Kaneene, J. B. (2004b) Comparison of antimicrobial susceptibility of *Sthapylococcus aureus* isolated from bulk tank milk in organic and conventional dairy herds in the Midwestern United States and Denmark. *J. Food Prot.* 67, 1104-1110.

Soonthornchaikul, N., Garelick, H., Jones, H., Jacobs, J., Ball, D. & Choudhury, M. (2006) Resistance to three antimicrobial agents of *Campylobacter* isolated from organically- and intensively-reared chickens purchased from retail outlets. *Int. J. Antimicrob. Agents*. 27: 125-130.

Tikofsky, L. L., Barlow, J. W., Santiesteban, C. & Schukken, Y. H. (2003) A comparison of antimicrobial susceptibility patterns for *Staphylococcus aureus* in organic and conventional dairy herds. *Microb. Drug Resist.* 9, 39-45.

Van den Bogaard, A.E., London, N., Driessen, C. & Stobberingh, E. E. (2001) Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. *J. Antimicrob. Chemother.* 47, 763-771.

Wiuff, C., Lykkesfeldt, J., Svendsen, O. & Aarestrup, F. M. (2003) The effects of oral and intramuscular administration and dose escalation of enrofloxacin on the selection of

quinolone resistance among *Salmonella* and coliforms in pigs. *Res. Vet. Sci.* 75, 185-193.

Table 1. Percentages of Enterobacteriaceae isolated from conventional chicken, organic chicken and poultry susceptible (S), intermediate (I) and resistant (R) to antimicrobial agents by disk diffusion methods

	Organic chicken (n=60)			Conventional chicken (n=60)			Turkey (n=60)		
Antimicrobial agent (μg)	S	I	R	S	I	R	S	I	R
Ampicillin (10)	40	38.3	21.7	35	16.7	48.3	33.3	30	36.7
Cephalothin (30)	30	25	45	40	13.3	46.7	20	21.7	58.3
Chloramphenicol (30)	98.3	1.7	0	81.7	11.7	6.7	95	5	0
Doxycycline (30)	95	5	0	78.3	16.7	5	70	26.7	3.3
Ciprofloxacin (5)	61.7	35	3.3	41.7	45	13.3	61.7	31.7	6.7
Gentamicin (10)	98.3	1.7	0	95	0	5	95	5	0
Nitrofurantoin (300)	60	20	20	60	13.3	26.7	55	25	20
Sulfisoxazole (200)	40	1.7	58.3	26.7	0	73.3	26.7	0	73.3

Table 2. Resistance patterns in Enterobacteriaceae isolated from organic chicken, conventional chicken and turkey.

Number of antimicrobials resistant	Organic chicken (n=60)	Conventional chicken (n=60)	Conventional turkey (n=60)
0	7 (11.7)	5 (8.3)	7 (11.7)
1	28 (46.7)	17 (28.3)	19 (31.7)
2	12 (20)	13 (21.7)	14 (23.3)
3	12 (20)	12 (20)	12 (20)
4	1 (1.7)	10 (16.7)	5 (8.3)
≥5	0 (0)	3 (5)	3 (5)
Multi-resistant strains (%)	25 (41.7)	38 (63.3)	34 (56.7)

Table 3. Genera distribution of Enterobacteriaceae isolated from organic chicken, conventional chicken and conventional turkey.

	Organic chicken (n=60)	Conventional chicken (n=60)	Conventional turkey (n=60)
Serratia spp.(%)	27 (45)	19 (31.7)	20 (33.3)
Enterobacter spp.(%)	12 (20)	11 (18.3)	14 (23.3)
Klebsiella spp. (%)	4 (6.7)	4 (6.7)	15 (25)
Yersinia spp. (%)	4 (6.7)	2 (3.3)	0 (0)
Hafnia spp. (%)	4 (6.7)	5 (8.3)	0 (0)
Escherichia spp. (%)	4 (6.7)	5 (8.3)	0 (0)
Other genera (%)	3 (5)	5 (8.3)	2 (3.3)
NI (%)	2 (3.3)	9 (15)	9 (15)

Other genera includes (Nº): *Erwinia* spp. (3); *Citrobacter* spp. (3); *Providencia* spp. (2); *Ewingella* spp. (1) and *Rahnella* spp (1). NI: Not identified.

RESUMEN

En los análisis microbiológicos sobre alimentos, se recurre habitualmente a la familia *Enterobacteriaceae* como indicador de la presencia de contaminación fecal, siendo un grupo bacteriano en el que se encuentran bacterias zoonóticas tales como *Salmonella* spp., *Yersinia* spp o *Escherichia coli*. Precisamente muchas de ellas han sido relacionadas con la aparición de resistencias a agentes antimicrobianos, tanto los usados en clínica veterinaria como los usados como promotores de crecimiento en producción animal. Cada vez más, desde los estamentos de control de medicamentos se está prestando más atención a este fenómeno por las implicaciones tanto directas como indirectas que supone sobre la salud pública.

Consecuentemente, el objetivo de este trabajo es investigar la prevalencia de la susceptibilidad antimicrobiana en cepas de enterobacterias aisladas en aves de producción ecológica y compararlas con las encontradas en aves procedentes de la producción convencional.

Para ello, se ha determinado la contaminación por bacterias de la Fam. *Enterobacteriaceae* en 30 muestras de carne de pollo ecológica, 30 de carne de pollo procedente de la cría convencional y otras 30 de pavo también procedentes de la cría convencional, recogidas en distintos días en varias carnicerías y supermercados de Galicia. De cada una de estas muestras se aislaron 2 cepas de enterobacterias hasta obtener un total de 180 cepas. Para todas se determinó su susceptibilidad a 8 tipos diferentes de agentes antimicrobianos, utilizados con asiduidad en terapéutica aviar (ampilicina, cloranfenicol, cefalotina, doxiciclina, ciprofloxacina, gentamicina, nitrofurantoina y sulfisoxazol), mediante el método de difusión en disco.

Los recuentos medios obtenidos revelaron que las muestras de carne de pollo ecológico estaban significativamente ($P < 0.0001$) más contaminadas por enterobacterias (3.81 log UFC/g) que las muestras procedentes de aves de cría convencional (2.66 UFC/g en pollos, 1.44 log UFC/g en pavos). Estos resultados parecen indicar que el mayor uso de antimicrobianos en durante la producción de pavos, y que es algo menor en pollos, tiene un efecto evidente en comparación con la no utilización de estos fármacos en durante la producción ecológica.

Sin embargo, las tasas de resistencia obtenidas entre las enterobacterias aisladas a partir de pollo ecológico fueron significativamente menores que las obtenidas entre las

enterobacterias aisladas a partir de pollo de producción convencional en el caso de 6 agentes antimicrobianos (ampilicina (P = 0.0001), cloranfenicol (P = 0.0004), ciprofloxacina (P = 0.0034), doxiciclina (P = 0.0013), gentamicina (P = 0.0295) y sulfisoxazol (P = 0.0442)). Asi mismo, también fueron significativamente menores que las obtenidas para las enterobacterias aisladas a partir de pavo para el caso de la doxiciclina (P = 0.0141) y del sulfisoxazol (P = 0.0442). El mayor grado de resistencias resultaron encontrarse para el sulfisoxazol y a continuación para la cefalotina y ampicilina, en los tres grupos de muestras. También se puede señalar que aunque debía de esperarse una mayor aparición de resistencias en las muestras de pavo debido a la mayor exposición a estos fármacos, en cambio, de forma general se encontraron más cepas resistentes en el caso de muestras procedentes de carne de pollo de cría convencional, siendo únicamente en éstas donde aparecieron también casos (6.7 %) de resistencia al cloranfenicol (prohibido desde hace años).

Aparte hay que destacar que se encontraron cepas multirresistentes sobretodo en las muestras de carne de pollo (63.3 %) y de pavo (56.7 %) de cría convencional que resultaron ser más abundantes que las encontradas en las muestras de carne de pollos de cría ecológica (41.7 %).

El género perteneciente a la orden *Enterobacteriaceae* más frecuentemente aislado a partir de los tres tipos de muestras fue *Serratia* spp. (45 % en carnes ecológicas, 28.3 % y 33.3 % en pollo y pavo no ecológicos). Otros géneros aislados con bastante frecuencia fueron *Enterobacter* y *Klebsiella*. Cabe destacar que se encontraron diferencias estadísticamente significativas en la distribución de estos géneros en los distintos grupos, de muestras sobretodo para el caso de las carnes de pavo (P = 0.0010 y P = 0.0055 respecto a las carnes de pollo de producción ecológica y no ecológica, respectivamente) lo que podría tener alguna influencia en el aislamiento de cepas resistentes en los distintos grupos de muestras.

En resumen, los resultados del presente trabajo establecen que aunque las carnes procedentes de pollos de producción ecológica se hallan más contaminadas por enterobactaerias, las cepas pertenecientes a este grupo presentan menos resistencia a los antimicrobianos más comúnmente utilizados en la clínica aviar, de acuerdo a los resultados obtenidos también por otros autores para casos similares. Así, se podría

indicar que la cría de aves de forma ecológica es un mecanismo que contribuye a disminuir la selección y/o diseminación de bacterias resistentes hacia el consumidor.

3.7. Estudio sobre la resistencia a antimicrobianos de *Escherichia coli*, *Staphylococcus aureus* y *Listeria monocytogenes* aislados de muestras de carne procedentes de pollos de ecológicos y de pollos de producción convencional

Comparison of antimicrobial resistance in *Escherichia coli, Staphylococcus aureus and Listeria monocytogenes* strains isolated from organic and conventional poultry meat

Miranda, J.M., Vázquez, B.I., Fente, C.A., Barros-Velázquez, J., Cepeda, A., Franco, C.M.

Enviada para su publicación al Food Research Internacional.

Fecha de envío: Enero 2007.

Comparison of antimicrobial resistance in *Escherichia coli, Staphylococcus aureus and Listeria monocytogenes* strains isolated from organic and conventional poultry meat

[a]Miranda, J.M., [a]Vázquez, B.I., [a]Fente, C.A., [b]Barros-Velázquez, J., [a]Cepeda, A., [a]Franco, C.M.

[a]Laboratorio de Higiene Inspección y Control de Alimentos. Dpto de Química Analítica, Nutrición y Bromatología. Facultad de Veterinaria. Universidad de Santiago de Compostela. 27002-Lugo. Spain;
[b]Area de Tecnología de los Alimentos. Dpto de Química Analítica, Nutrición y Bromatología. Facultad de Veterinaria. Universidad de Santiago de Compostela. 27002-Lugo. Spain.

Abbreviated running head: Antimicrobial resistance in organic poultry

Abstract

The presence of *Escherichia coli, Staphylococcus aureus and Listeria monocytogenes* was determined in 55 samples of organic poultry meat and in 61 samples of conventional poultry meat. A total of 220 *E. coli,* 192 *S. aureus and* 71 *L. monocytogenes* strains were analyzed by an agar disk diffusion assay for their resistance to ampicillin, chloramphenicol, cephalotin, ciprofloxacin, doxycycline, fosfomycin, gentamicin, nitrofurantoin, sulfisoxazole and streptomycin (*E. coli*), chloramphenicol, clindamycin, ciprofloxacin, doxycycline, erythromycin, gentamicin, oxacillin, nitrofurantoin and sulfisoxazole (*S. aureus*) and ampicillin, chloramphenicol, ciprofloxacin, doxycycline, erythromycin, gentamicin, streptomycin, sulfisoxazole and vancomycin (*L. monocytogenes*). The results indicated a higher presence of *E. coli* ($P<0.0001$) but not of *S. aureus* and *L. monocytogenes* in organic poultry meat as compared to conventional poultry meat. *E. coli* isolated from organic poultry meat exhibited lower levels of antimicrobial resistance against 7 of the antimicrobials tested as compared to isolates recovered from conventional meat. In the case of *S. aureus* and

L. monocytogenes, isolated from conventional poultry, antimicrobial resistance was significantly higher only for doxycycline as compared to strains isolated from organic poultry. Moreover, the presence of multi-resistant strains was significantly higher in conventional poultry meat as compared to organic poultry meat ($P<0.0001$) only in the case of *E. coli*. Organically-farmed poultry samples showed significantly lower development of antimicrobial resistance in intestinal bacteria as *E. coli*, thus contributing to a reduction in the development and spread of antimicrobial resistance among these food-borne bacteria.

Keywords: E. coli, organic, antimicrobial, resistance, poultry, meat.

1. Introduction

In recent years the sales of organic foods have increased significantly. Thus, organic and other non-conventional meat products are now available in the retail market of developed countries with a view to satisfy the consumers' demand for products that meet specific requirements. Among them, the following can be highlighted: guaranteed animal welfare during production, absence of chemical agents during animal feeding, environmental friendliness, and better taste as compared to conventional products (Dransfield et al., 2005). In addition, there is a perception on a part of consumers that, due to the fact that conditions for growth are more natural for organic farming, such products would harbour less pathogenic bacteria than conventional food products (Bailey & Cosby, 2005). However, little is known about the microbiological status of organic animal products and the potential microbiological risks linked to organic meat production. Thus, it has been reported that the raising of animals outdoors and the use of slow-growing breeds as well as the strict restrictions in therapeutic use of antimicrobial agents, may not guarantee a strict microbiological control of animals destined for human consumption (Dransfield et al., 2005; Soonthornchaikul, Garelick, Jones, Jacobs, Ball & Choudhury, 2006).

Meat and meat products are the major source of food-borne infections and the most important link between food-producing animals and humans (Mayrhofer, Paulsen, Smulders & Hilbert, 2004). In this sense, it has been previously reported that several

antimicrobial-resistant bacteria isolated from humans primarily originated from animals raised for human consumption (Aarestrup, 2000; Philips et al., 2004) and that such resistant bacteria may contaminate meat derived from those animals (Sáenz, Zarazaga, Briñas, Lantero, Ruiz-Larrea & Torres, 2001). Thus, antibiotic resistance of bacterial isolates from animal origins can also represent a potential hazard to consumers *via* food-borne infections caused by antibiotic-resistant bacteria.

Monitoring of the use of antimicrobial agents in veterinary medicine in animals destined for human consumption is considered to be a risk-management option to prevent the development and spread of antimicrobial resistance in microorganisms present in food-producing animals (Philips et al., 2004). Recently, other authors have reported antimicrobial resistance by bacteria isolated from organic animal products. Data reported in such studies are specially referred to *Campylobacter* or *Salmonella* genera in dairy and poultry products and to *Staphylococcus aureus* isolated from dairy products (Bailey et al., 2005; Cui, Ge, Zheng & Jianghong, 2005, Sato, Barlett, Kaneene & Downes, 2004; Sato, Bennedsgaard, Barlett, Erskine & Kaneene, 2004; Soonthornchaikul et al., 2006; Tikofsky, Barlow, Santiesteban & Schukken, 2003). However, little information relative to *Escherichia coli*, *Staphylococcus aureus* or *Listeria monocytogenes* isolated from organic food products is currently available. As a consequence of this, the main goal of this study was to investigate the prevalence of the antimicrobial susceptibility of *E. coli, S. aureus* and *L. monocytogenes* strains isolated from organic poultry as compared to strains isolated from conventional poultry. The implications of these results in terms of microbiological safety, especially concerning the development and spread of antimicrobial resistance to the food chain, are also discussed.

2. Materials and Methods

2.1. Collection of poultry meat samples

A total of 116 fresh pre-packaged skin-on drumstick samples were taken during 2006 from supermarkets and butcher shops. Fifty-five samples corresponded to organic-reared chicken samples and 61 samples corresponded to conventionally-reared chicken

samples. All samples were taken on different days and from different lots in 12 different supermarkets and butcheries. In the case of organic chickens, commercial products certified by an official agency were found in only 5 supermarkets, so 11 organic samples were taken in each supermarket, but all of them on different days. All supermarkets and butcheries were located in Galicia (North-Western Spain). All samples were processed between three and four days before the expiration date indicated on the label.

2.2. Microbiological analyses and isolation procedure

Twenty-five g portions were obtained from each poultry meat sample, placed in a sterile masticator bag together with an appropriate volume (1/9) (w/v) of sterile 0.1% peptone water (Merck, Darmstadt, Germany), and homogenized in a masticator (AES, Combourg, France) for 1 min. After homogenization, samples were investigated for the presence of *E. coli*, *S. aureus* and *L. monocytogenes*.

2.2.1. E. coli

One ml of 10^{-1} to 10^{-4} dilutions of meat extracts were processed on plates of Fluorocult® Agar prepared following the manufacturers' instructions (Merck). Once the agar had solidified, the plates were overlaid with 3-4 ml of melted Fluorocult® and incubated at 44ºC for 24 h. After incubation, pink to red colonies exhibiting blue florescence after exposure to a 365 nm ultraviolet lamp (Vilbert Lourmat, Marne, France) were identified as *E. coli* and counted. Once bacterial counts were determined, one-to-three typical *E. coli* colonies isolated from each poultry sample were picked, transferred onto Columbia agar supplemented with 5% sheep blood (BioMérieux, Marcy l`Etoile, France), and incubated at 44ºC for 24 h in order to obtain pure cultures. Such pure cultures were preliminarily investigated by colony and cell morphology, Gram stain, oxidase and catalase activity, methyl red stain and indole production. Positive strains preliminarily identified as *E. coli* were confirmed by the API 20E identification system (BioMérieux).

2.2.2. S. aureus

One ml of 10^{-1} to 10^{-2} dilutions of meat extracts were processed on plates of Baird Parker Agar prepared following the manufacturers' instructions (BioMérieux), and incubated at 37ºC for 48 h. Grey to black colonies with a white halo showing coagulase activity were isolated. Once bacterial counts were determined, one-to-three typical *S. aureus* colonies isolated from each poultry sample were picked, transferred onto Columbia agar supplemented with 5% sheep blood (BioMérieux), and incubated at 37ºC for 48 h in order to obtain pure cultures. Such pure cultures were preliminarily investigated for their colony and cell morphology, Gram stain, oxidase and catalase activity. Positive strains preliminarily identified as *S. aureus* were confirmed by the API ID 32 STAPH identification system (BioMérieux).

2.2.3. L. monocytogenes

Twenty-five g portions obtained from each poultry sample were analyzed according to ISO-11290-1 and ISO-11290-2 procedures and differentiated by streaks onto ALOA agar plates (AES) and incubated for 24 h at 37 ºC. Blue/green colonies with an opaque halo were considered as *L. monocytogenes*. Once bacterial counts were determined, one-to-three typical *L. monocytogenes* colonies isolated from each poultry sample were picked, transferred onto Columbia agar supplemented with 5% sheep blood (BioMérieux), and incubated for 24 h at 37 ºC in order to obtain pure cultures. Such pure cultures were preliminarily investigated for their colony and cell morphology, Gram stain, oxidase and catalase activity, tumbling motility at 25 ºC and hemolysis on sheep blood agar. Positive strains preliminarily identified as *L. monocytogenes* were confirmed by the API listeria identification system (BioMérieux).

All isolates were stored at -80°C in Maintenance Freeze Medium units (Oxoid, Basingstoke, UK) until antimicrobial susceptibility was tested. All meat samples were processed in triplicate. Sampling and processing of poultry meat samples were always

carried out by the same laboratory personnel. Agar media plates were prepared by the same research assistant throughout the study.

2.3. Antimicrobial susceptibility testing of bacteria

Antimicrobial susceptibility testing was performed for a total of 220 isolates of *E. coli*, 192 isolates of *S. aureus* and 71 isolates of *L. monocytogenes*. Antimicrobial susceptibility testing was carried out by agar disk diffusion on Müeller-Hinton agar plates (Oxoid) according to the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) guidelines (NCCLS, 2002). Antimicrobial disks considered for *E. coli* testing were: ampicillin (10 µg), cephalotin (30 µg), chloramphenicol (30 µg), doxycycline (30 µg), ciprofloxacin (5 µg), fosfomycin (200 µg), gentamicin (10 µg), nitrofurantoin (300 µg), streptomycin (10 µg), and sulfisoxazole (300 µg) (Oxoid). Antimicrobial disks considered for *S. aureus* testing were: chloramphenicol (30 µg), clindamycin (2 µg), ciprofloxacin (5 µg), doxycycline (30 µg), erythromycin (15 µg), gentamicin (10µg), oxacillin (1 µg), nitrofurantoin (300 µg) and sulfisoxazole (300 µg). Antimicrobial disks considered for *L. monocytogenes* testing were: ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), erythromycin (15 µg), gentamicin (10µg), streptomycin (10 µg), sulfisoxazole (300 µg) and vancomycin (30 µg).

Antimicrobial agents were selected in terms of their different structure and mechanism of action. Antibiotic resistance breakpoints considered were those recommended by the CLSI (NCCLS, 2002; NCCLS, 1993). *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 were used as reference strains for this study. Isolates were classified as sensitive, intermediate (moderately sensitive) or resistant. Isolates exhibiting resistance to at least two of the antimicrobial agents tested were considered to be multi-resistant strains.

2.4. Statistical analysis

E. coli, S. aureus and *L. monocytogenes* counts were grouped into different categories (<1 log CFU g^{-1}; 1-2 log CFU g^{-1} and >2 log CFU g^{-1} for *E. coli*; <2 log CFU g^{-1} and 2-3 log CFU g^{-1} for *S. aureus* and absence; <2 log CFU g^{-1} and 2-3 log CFU g^{-1} for *L. monocytogenes*) prior to the performance of statistical analyses. The distributions of bacterial counts (log CFU g^{-1}) as well as the distributions of resistant strains and multi-resistance patterns were compared by means of the X^2 test. The differences were considered to be significant when *P* was lower than 0.05. All statistical analyses were performed by means of the Statgraphics version 5.0.1 software (SAS Institute, North Carolina, USA).

3. Results

The results indicated that the special characteristics of organic production provided a higher prevalence of *E. coli* but not of *S. aureus* and *L. monocytogenes* as compared to conventionally farmed poultry. In organic poultry meat, *E. coli* was detected (>1 log CFU g^{-1}) in 45 of the 55 samples tested (81.8%). The counts determined were >2 log CFU g^{-1} for 18 of the 55 samples tested (32.7%), while 27 samples (49.1%) harboured *E. coli* in the 1-2 log CFU g^{-1} range. In the case of poultry reared conventionally, *E. coli* was detected in only 38 of the 61 samples investigated (62.3% of the total), in the 1-2 log CFU g^{-1} range in all cases. According to these results, the *E. coli* counts obtained for organic poultry samples were significantly higher ($P<0.0001$) than those obtained for conventional poultry.

In the case of *S. aureus* in organic poultry meat, this food-borne pathogen was detected at concentrations higher than 2 log CFU g^{-1} in 37 of the 55 samples tested (67.3%). The bacterial numbers indicated that in 32 of the samples *S. aureus* was present at concentrations below 2 log CFU g^{-1} (58.2% of the total), while 5 samples reached counts above 2 log CFU g^{-1} (9.1%). In the case of poultry reared conventionally, *S. aureus* was detected only in 35 of the 61 samples investigated (57.4 % of the total). 34 of the samples in which *S. aureus* was detected reached counts below 2 log CFU g^{-1} (55.7%), while only one sample (1.6%) reached counts above 2 log CFU

g^{-1} range. According to these results, the recovery of *S. aureus* isolates from organic poultry samples was significantly higher (P=0.1436) than in conventional poultry.

In the case of *L. monocytogenes*, this pathogen was recovered (presence in 25 g) from organic poultry meat in 27 of the 55 samples tested (49.1%). The bacterial counts indicated that 25 of the samples in which *L. monocytogenes* was detected exhibited counts below 2 log CFU g^{-1} (45.5 % of the total), while only 2 samples reached counts above 2 log CFU g^{-1} range (3.6%). In the case of poultry reared conventionally, *L. monocytogenes* was detected in 25 of the 61 samples investigated (41% of the total). 24 of the samples in which *L. monocytogenes* were detected exhibited concentrations below 2 log CFU g^{-1} (39.3%), while only one sample reached counts higher than 2 log CFU g^{-1} (1.6%). According to these results, no statistically-significant differences were obtained between the *L. monocytogenes* counts obtained for organic poultry samples and those obtained for conventional poultry (P=0.4045).

With respect to the antimicrobial resistance of isolates, different patterns were observed in the *E. coli* strains isolated from organic poultry samples as compared to their counterparts isolated from conventional poultry (Table 1). Antimicrobial resistance was significantly higher in *E. coli* isolates derived from conventional poultry samples for the antibiotics ampicillin (P<0.0001), cephalotin (P<0.0001), doxycycline (P<0.0001), ciprofloxacin (P=0.0026), gentamicin (P<0.0001), streptomycin (P<0.0001) and sulfisoxazole (P<0.0001) as compared to their *E. coli* counterparts isolated from organic poultry (Table 1). In the case of *S. aureus* (Table 2), antimicrobial resistance was also significantly higher in isolates derived from conventional poultry samples for doxycycline (P=0.0001) as compared to *S. aureus* isolated from organic poultry. Nevertheless, in the case of clindamycin (P=0.0239), the resistance rates observed in *S. aureus* isolates derived from conventional poultry were significantly lower than those observed in the *S. aureus* counterparts isolated from organic poultry (Table 2). In the case of *L. monocytogenes* (Table 3), antimicrobial resistance was significantly higher in isolates derived from conventional poultry samples as compared to *L. monocytogenes* isolates recovered from organic poultry samples only in the case of doxycycline (P=0.0446).

In addition, it should be highlighted that organic poultry meat showed lower isolation rates of multi-resistant *E. coli* strains than conventional poultry samples (Table

4). The percentage of *E. coli* isolates that exhibited resistance to at least two antimicrobial agents was significantly lower ($P<0.0001$) in organic poultry samples (34.3%) than in the conventional poultry counterparts (76.5%). Nevertheless, although the percentages of recovery of multiresistant strains were always higher in isolates from conventional poultry, no statistically-significant differences were obtained between the percentage of multi-resistant *S. aureus* (Table 5) or *L. monocytogenes* (Table 6) strains isolated from organic and conventional poultry meat, respectively.

4. Discussion

The results obtained in our study support the hypothesis that the prevalence of *E. coli* isolates differs depending on the type of poultry production system (conventionally raised versus organically raised) considered. Thus, as expected for conventional poultry, in which rearing procedures require more intensive antimicrobial consumption than organic poultry farming, the average counts of *E. coli* and *S. aureus* were significantly lower than in the organic poultry samples, in which the use of antimicrobial agents is seriously restricted. Nevertheless, for the case of *S. aureus* and *L. monocytogenes*, no significant differences were obtained in their recovery rates between both types of poultry production systems. These results suggest that contamination derived from handling in processing plants is more important for these bacteria than for the case of *E. coli*. In this sense, other authors have reported that food handlers may be important sources of *S. aureus* or *L. monocytogenes* contamination (Gundogan, Citak, Yucel & Devren, 2005; Loura, Almeida & Almeida, 2004).

Recently, widely variable rates of antimicrobial resistance were reported for *E. coli, S. aureus* and *L. monocytogenes* strains isolated from conventionally-farmed food products (Aureli, Ferrini, Mannoni, Hodzic, Wedell-Weergaard & Oliva, 2003; Bywater et al., 2004; Cornican, Buckley, Corbett-Feeney & Sheridan, 2001; Franco et al., 1994; Gundogan et al., 2005; Mayrhofer et al., 2004; Sáenz et al., 2001; Van den Bogaard, London, Driessen & Stobberingh, 2001). The antimicrobial resistance rates determined in our study for conventionally-farmed poultry are, in global terms, compatible with the results reported by these authors.

Other authors have described a correlation between the percentages of antimicrobial resistant isolates and the use of antimicrobial agents in animal farming (Aarestrup, 1999; Asai, Kojima, Harada, Ishihara, Takahashi & Tamura, 2005). The results obtained in our study indicated a relationship between the levels of antimicrobial resistance in *E. coli* and the tolerance in the use of antimicrobial agents employed in the poultry production systems considered in this work. Thus, while organic poultry meat proved to be more contaminated with *E. coli*, these isolates were more sensitive to certain antimicrobial agents than their counterpart *E. coli* strains isolated from conventionally-farmed animals. In this sense, the differences for antimicrobial agents commonly used in poultry medicine, such as tetracyclines, quinolones, aminoglycosides, β-lactams or sulphonamides (Asai et al., 2005; Emborg & Heuer, 2003) should be noted. Moreover, the fact that no significant differences were observed for the antimicrobial agents banned from poultry medicine in the European Union, such as chloranphenicol and nitrofurantoin, provides additional support for the hypothesis that the development of antimicrobial resistance may be a direct consequence of the use of certain antimicrobial agents in veterinary medicine.

In addition, the statistically-significant higher rates of multi-resistant *E. coli* isolates in conventional poultry underlines the possibility that stable resistance elements may exist and that different resistance mechanisms may be linked. Thus, drug application may not only select for resistance against the applied drug, but for multiresistance phenotypes having a selection advantage. Nowadays, a variety of genetic elements involved in cross-resistance events have been described. In the case of *E. coli* it is frequent that strains that can produce Extended Spectrum Beta-Lactamase enzymes (ESLBs) also exhibit cross-resistance to other antimicrobial families such as tetracyclines or sulphonamides (Meunier, Jouy, Lazizzera, Kobisch & Madec, 2006, Von Baum & Marre, 2005]

The relationship between the levels of antimicrobial resistance in *S. aureus* or *L. monocytogenes* and the tolerance of antimicrobial agents used in the poultry production systems considered in this study is less evident than for the case of *E. coli*. Thus, *S. aureus* and *L. monocytogenes* isolates derived from organic poultry were more sensitive to doxycycline than their counterpart strains isolated from conventionally-farmed animals. Remarkably, *S. aureus* strains isolated from organic poultry meat were more

resistant to clindamycin than their counterpart *S. aureus* isolates from conventional poultry meat. These differences might be caused by differences in the primary habitat of the bacteria. *E. coli* are intestinal while *S. aureus* live on the skin and mucous membranes and *L. monocytogenes* is found in the environment. Thus, antimicrobials that are usually administrated orally to whole flocks in poultry medicine seem to exert selection pressure of resistant phenotypes in poultry faecal flora (Van den Bogaard et al., 2001). The high frequency of mutation exhibited by *E. coli* and leading to the development of antimicrobial resistance should also be considered, as compared to other microorganisms frequently found in animals and foods (Vom Baum & Marre, 2005).

Although organic meat sells for higher prices in the market than conventional meat, the sale of organic foods has increased in recent years (Walshe, Sheeman, Delahunty, Morrisey & Kerry, 2005). For this reason, the regulations concerning organic food production should ensure that meat derived from organic farming present in the markets truly originated from organically-reared animals. In this sense, the investigation of antimicrobial resistance could be a useful tool to detect fraudulent practices, since the detection of bacterial resistance to the antimicrobial agents most commonly used in poultry farming might point to the fraudulent substitution of organic poultry meat with meat derived from conventional farming. The results obtained in the present work clearly indicate that the study of intestinal bacteria such as *E. coli*, a bacterial species exhibiting an unique ability to develop resistance after antimicrobial treatments, is more accurate than other bacteria such as *S. aureus* or *L. monocytogenes*. The statistically-significant lower percentages of antimicrobial resistant *E. coli* isolates found in organically-raised poultry support that the organic rearing of poultry may limit the presence of antibiotic-resistant intestinal bacteria in such animal foods.

Acknowledgements

The authors wish to thank the financial support of Xunta de Galicia (Project PGIDIT05TAL003E). The authors also thank Carmen Carreira for her expert technical assistance.

Table 1. Percentages of *Escherichia coli* strains isolated from conventional and organic poultry meat that exhibited a sensitive (S), intermediate (I) or resistant (R) phenotype with respect to antimicrobial agents, as determined by the agar disk diffusion method.

	Conventional poultry meat (n=115)			Organic poultry meat (n=105)			
Antimicrobial agent (μg)	S	I	R	S	I	R	*P*
Ampicillin (10)	33.9	12.2	53.9	74.3	3.8	21.9	<0.0001
Cephalotin (30)	47	18.3	34.8	92.4	2.9	4.8	<0.0001
Chloranphenicol (30)	90.4	4.4	5.2	95.2	1.9	2.9	0.3841
Doxycycline (30)	4.3	47.8	47.8	57.1	17.1	25.7	<0.0001
Ciprofloxacin (5)	60	12.2	27.8	75.2	15.2	9.5	0.0026
Fosfomycin (200)	98.3	1.7	0	96.2	1	2.9	0.1687
Gentamicin (10)	77.4	13	9.6	98.1	1	1	<0.0001
Nitrofurantoin (300)	84.3	12.2	3.5	93.3	4.8	1.9	0.1059
Sulfisoxazole (300)	56.6	7	36.5	77.1	1	21.9	0.0021
Streptomycin (10)	7	47	46.1	44.8	31.4	23.8	<0.0001

Table 2. Percentages of *Staphylococcus aureus* strains isolated from conventional and organic poultry meat that exhibited a sensitive (S), intermediate (I) or resistant (R) phenotype with respect to antimicrobial agents, as determined by the agar disk diffusion method.

	Conventional poultry meat (n=101)			Organic poultry meat (n=91)			
Antimicrobial agent (μg)	S	I	R	S	I	R	*P*
Chloranphenicol (30)	76.2	21.8	2	89	9.9	1.1	0.0679
Clindamycin (2)	27.7	5	67.3	12.1	4.4	83.5	0.0239
Doxycycline (30)	23.8	17.8	58.4	53.8	12.1	34.1	0.0001
Ciprofloxacin (5)	56.4	25.7	17.8	64.8	23.1	12.1	0.4401
Erythromycin (15)	70.3	8.9	20.8	72.5	11	16.5	0.699
Gentamicin (10)	100	0	0	100	0	0	ND*
Oxacillin (1)	29.7	0	70.3	38.5	0	61.5	0.2003
Nitrofurantoin (300)	51.5	19.8	28.7	62.6	22	15.4	0.0840
Sulfisoxazole (300)	68.3	6.9	24.8	70.3	4.4	25.3	0.7519

*Not Determined

Table 3. Percentages of *Listeria monocytogenes* strains isolated from conventional and organic poultry that exhibited a sensitive (S), intermediate (I) or resistant (R) phenotype with respect to antimicrobial agents, as determined by the agar disk diffusion method.

	Conventional poultry meat (n=32)			Organic poultry meat (n=39)			
Antimicrobial agent (μg)	S	I	R	S	I	R	*P*
Ampicillin (10)	100	0	0	100	0	0	ND*
Chloranphenicol (30)	84.4	12.5	3.1	79.5	20.5	0	0.3795
Ciprofloxacin (5)	100	0	0	97.4	2.6	0	0.3616
Doxycycline (30)	62.5	18.8	18.8	84.6	12.8	2.6	0.0446
Erythromycin (15)	84.4	9.4	6.3	97.4	2.6	0	0.1217
Gentamicin (10)	100	0	0	100	0	0	ND*
Streptomycin (10)	93.8	6.3	0	100	0	0	0.3882
Sulfisoxazole (300)	87.5	0	15.6	79.5	0	20.5	0.7781
Vancomycin (30)	100	0	0	100	0	0	ND*

*Not Determined

Table 4. Resistance patterns in *Escherichia coli* strains isolated from conventional and organic poultry meat.

Number of antimicrobials*	Conventional poultry (n=115) No.(%) of isolates	Organic poultry (n=105) No.(%) of isolates
0	12 (10.4)	35 (33.3)
1	15 (13)	34 (32.4)
2	32 (27.8)	22 (21)
3	22 (19.1)	9 (8.6)
4	16 (13.9)	3 (2.9)
≥ 5	18 (15.7)	2 (1.9)
Multi-resistant strains (%)	88 (76.5)	36 (34.3)

*The percentage of multi-resistant strains is expressed between brackets. Differences between conventional and organic poultry were estimated by means of the X^2 test ($P<0.0001$).

Table 5. Resistance patterns in *Staphylococcus aureus* strains isolated from conventional and organic poultry.

Number of antimicrobials*	Conventional poultry (n=101) No.(%) of isolates	Organic poultry (n=91) No.(%) of isolates
0	5 (5)	9 (9.9)
1	10 (9.9)	14 (15.4)
2	16 (15.8)	17 (18.7)
3	33 (32.7)	21 (23.1)
4	23 (22.8)	26 (28.6)
≥ 5	14 (13.9)	4 (4.4)
Multi-resistant strains (%)	86 (85.1)	68 (74.4)

*The percentage of multi-resistant strains is expressed between brackets. Differences between conventional and organic poultry were estimated by means of the X^2 test (P=0.0826).

Table 6. Resistance patterns in *Listeria monocytogenes* strains isolated from conventional and organic poultry.

Number of antimicrobials*	Conventional poultry (n=32) No.(%) of isolates	Organic poultry (n=39) No.(%) of isolates
0	23 (71.9)	31 (79.5)
1	5 (15.6)	7 (17.9)
2	3 (9.4)	1 (2.6)
3	1 (3.1)	0 (0)
4	0 (0)	0 (0)
≥ 5	0 (0)	0 (0)
Multi-resistant strains (%)	4 (12.5)	1 (2.6)

*The percentage of multi-resistant strains is expressed between brackets. Differences between conventional and organic poultry were estimated by means of the X^2 test (P=0.4143).

References

Aarestrup, F. M. (1999). Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. *International Journal of Antimicrobial Agents,* 12, 279-285.

Aarestrup, F. M. (2000). Occurrence, selection and spread of resistance to antimicrobial agents used for growth promotion for food animals in Denmark. *APMIS* 108 (Supplementum No. 101), 5-48.

Asai, T., Kojima, A., Harada, K., Ishihara, K., Takahashi, T. & Tamura, Y. (2005). Correlation between the usage volume of veterinary therapeutic antimicrobials and resistance in *Escherichia coli* isolated from the faeces of food-producing animals in Japan. *Japanese Journal of Infectious Disesases,* 58, 369-372.

Aureli, P., Ferrini, A. M., Mannoni, V., Hodzic, S., Wedell-Weergaard, C. & Oliva, B. (2003). Susceptibility of *Listeria monocytogenes* isolated from food in Italy to antibiotics. *International Journal of Food Microbiology*, 83, 325-330.

Bailey, J. S. & Cosby, D. E. (2005). *Salmonella* prevalence in free-range and certified organic chikens. *Journal of Food Protection,* 68, 2451-2453.

Bywater, R., Deluyker, H., Deroover, E., Jong, A., Marion, H., McConville, M., Rowan, T., Shryock, T., Shuster, D., Thomas, V., Vallé, M. & Walters, J. (2004). A European survey of antimicrobial susceptibility among zoonotic and commensal bacteria isolated from food-producing animals. *Journal of Antimicrobial Chemotherapy,* 54, 744-754.

Cornican, M., Buckley, V., Corbett-Feeney, G. & Sheridan, F. (2001). Antimicrobial resistance in *Escherichia coli* isolates from turkeys and hens in Ireland. *Journal of Antimicrobial Chemotherapy,* 48, 587-95.

Cui, S., Ge, B., Zheng, J. & Jianghong, M. (2005). Prevalence and antimicrobial resistance of *Campylobacter* spp. and *Salmonella* serovars in organic chickens in Maryland retail stores. *Applied and Environmental Microbiology,* 71, 4108-4111.

Dransfield, E., Ngapo, T. M., Nielsen, N. A., Bredahl, L., Sjödén, P. O., Magnusson, M., Campo, M. M. & Nut, G.R., (2005). Consumer choice and suggested price for pork as influenced by its appearance, taste and information concerning country of origin and organic pig production. *Meat Science,* 69, 61-70.

Emborg, H. D. & Heuer, O. E. (2003). DANMAP 2002- *Use of antimicrobial agents and occurence of antimicrobial resistance in bacteria from food producing animals, foods and humans in Demmark*. Statens Serum Institut, Danish Veterinary and Food Administration, Danish Medicines Agency, Danish Veterinary Institute, Copenhagen, Demmark.

Franco Abuín, C. M., Quinto Fernández, E. J., Fente Sampayo, C., Rodríguez Otero, J. L., Domínguez Domínguez, L. & Cepeda Sáez, A. (1994). Susceptibilities of *Listeria* species isolated from food to nine antimicrobial agents. *Antimicrobial Agents and Chemotherapy*, 38, 1655-1657.

Gundogan, N., Citak, S., Yucel, N. & Devre, A. (2005). A note on the incidence and antibiotic resistance of *Staphylococcus aureus* isolated from meat and chicken samples. *Meat Science,* 69, 807-810.

Loura, C. A. C., Almeida, R. C. C. & Almeida, P. (2005). The incidence and level of *Listeria* spp. and *Listeria monocytogenes* contamination in processed poultry at a poultry processing plant. *Journal of Food Safety*, 25, 19-29.

Mayrhofer, S., Paulsen, P., Smulders, F. J. M. & Hilbert, F. (2004). Antimicrobial resistance profile of five major food-borne pathogens isolated from beef, poultry and poultry. *International Journal of Food Microbiology*, 97, 23-29.

Meunier, D., Jouy, E., Lazizzera, C., Kobisch, M. & Madec, J. Y. (2006). CTX-M-1- and CTX-M-15-type β-lactamases in clinical *Escherichia coli* isolates recovered from food-producing animals in France. *International Journal of Antimicrobial Agents,* 28, 402-407.

National Committee for Clinical Laboratory Standards (NCCLS). (2002). *Performance standards for antimicrobial susceptibility testing;* 12th informational supplement. M100-S12. NCCLS, Wayne, PA.

National Committee for Clinical Laboratory Standards (NCCLS). (1993). Performance Standards for antimicrobial disk susceptibility. Approved Standard M2-A5, vol. 13, n. 24, NCCLS, Villanova, PA.

Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preston, R. & Waddell, J. (2004). Does the use of antimicrobials in food animals pose a risk to human health? A critical review of published data. *Journal of Antimicrobial Chemotherapy,* 53, 28-52.

Sáenz, Y., Zarazaga, M., Briñas, L., Lantero, M., Ruiz-Larrea, F. & Torres, C. (2001). Antibiotic resistance in *Escherichia coli* isolates obtained from animals, foods and humans in Spain. *International Journal of Antimicrobial Agents,* 18, 353-358.

Sato, K., Barlett, P. C., Kaneene, J. B. & Downes, F. P. (2004). Prevalence and antimicrobial susceptibilities of *Campylobacter* spp. Isolates from organic and conventional dairy hens in Wisconsin. *Applied and Environmental Microbiology,* 70, 1442-1447.

Sato, K., Bennedsgaard, T. W., Barlett, P. C., Erskine, R. J. & Kaneene, J. B. (2004) Comparison of antimicrobial susceptibility of *Sthapylococcus aureus* isolated from bulk tank milk in organic and conventional dairy herds in the Midwestern United States and Denmark. *Journal of Food Protection,* 67, 1104-1110.

Soonthornchaikul, N., Garelick, H., Jones, H., Jacobs. J., Ball, D. & Choudhury, M. (2006). Resistance to three antimicrobial agents of *Campylobacter* isolated from organically- and intensively-reared chickens purchased from retail outlets. *International Journal of Antimicrobial Agents,* 27, 125-130.

Tikofsky, L. L., Barlow, J. W., Santiesteban, C. & Schukken, Y. H. (2003). A comparison of antimicrobial susceptibility patterns for *Staphylococcus aureus* in organic and conventional dairy herds. *Microbial Drug Resistance,* 9, 39-45.

Van den Bogaard, A. E., London, N., Driessen, C. & Stobberingh, E. E. (2001). Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. *Journal of Antimicrobial Chemotherapy,* 47, 763-771.

Von Baum, H. & Marre, R. (2005). Antimicrobial resistance of *Escherichia coli* and therapeutic implications. *International Journal of Medical Microbiology,* 295, 503-511.

Walshe, B. E., Sheeman, E. M., Delahunty, C. M., Morrisey, P. A. & Kerry, J. P. (2005). Composition, sensory and shelf life stability analyses of *Longissimus dorsi* muscle from steers reared under organic and conventional production systems. *Meat Science,* 73, 319-325.

RESUMEN

El consumo de productos ecológicos ha experimentado un verdadero auge estos últimos años en los países desarrollados, debido a que los consumidores los perciben como algo de mayor calidad, concepto en el que supuestamente se incluye una mayor seguridad sanitaria. Sin embargo, actualmente no hay demasiados estudios científicos sobre el estatus microbiano real de estos productos y aunque entre las condiciones de producción se contempla una higiene lo más cuidada posible, no garantiza en absoluto que éstos se hallen exentos de patógenos.

Por otra parte, aunque hay muy pocos trabajos al respecto, algunos autores ya han demostrado recientemente la presencia de cepas resistentes a antimicrobianos en alimentos ecológicos, en concreto en productos lácteos, y para el caso de los géneros *Campylobacter*, *Salmonella* o *Staphylococcus*.

Por eso, el motivo de este trabajo es investigar la prevalencia de la susceptibilidad a determinados agentes microbianos en cepas pertenecientes a los géneros *E. coli*, *S. aureus* y *L. monocytogenes*, aisladas en carnes de pollo procedentes de la producción ecológica y compararlos con carnes igualmente de pollo pero procedentes de la producción industrial, actualmente de carácter intensivo.

Para ello, se determinó la presencia de *Escherichia coli*, *Staphylococcus aureus* y *Listeria monocytogenes* en 55 muestras de carne de pollo de cría ecológica y de 61 muestras de carne de pollo de cría convencional, todas ellas adquiridas en 12 carnicerías y supermercados diferentes localizados en Galicia.

Se determinó, por el método de difusión en disco, la resistencia de 220 cepas de *E.coli*, 192 de *S. aureus* y 71 de *L. monocytogenes*, a varios agentes antimicrobianos: cloranfenicol, doxiciclina, ciprofloxacina, gentamicina, sulfisoxazol (*E.coli, S. aureus, L. monocytogenes*), ampilicina y estreptomicina (*E.coli, L. monocytogenes*), nitrofurantoína (*E.coli, S. aureus*), eritromicina (*S. aureus, L. monocytogenes*), cefalotina y fosfomicina (*E.coli*), clindamicina y oxacillina (*S. aureus),* vancomicina (*L. monocytogenes*).

Los resultados obtenidos pusieron de manifiesto que la carne de pollo ecológico está significativamente más contaminada por *E. coli* ($P < 0.0001$) que la carne de pollo convencional, pero no se encontraron diferencias en lo referente a la contaminación por

S. aureus o *L. monocytogenes*. Presumiblemente estas diferencias sean debidas a las especiales características de crecimiento de los animales en las producciones ecológicas, en las que no se pueden utilizar determinados fármacos que pueden afectar de forma diferente a las distintas poblaciones bacterianas. Incluso la contaminación por *S. aureus* y/o *L. monocytogenes* en carnes ecológicas podría ser debida a unas deficientes prácticas de manipulación durante el procesado de las carnes ecológicas y por tanto tratarse de una contaminación posterior a la producción, tal y como apuntan algunos autores.

Respecto a la resistencia a antimicrobianos, en el caso de *E.coli*, las cepas aisladas a partir de carne de pollo ecológica mostraron tasas de resistencia significativamente menores a las aisladas en carne de pollo convencional para 7 de los 10 antimicrobianos ensayados. En el caso de *S. aureus* y *L. monocytogenes* sólo se observó este hecho para el caso de la doxiciclina. Las diferencias en los porcentajes de cepas resistentes parecen están influenciadas por el tipo de agente antibacteriano y el principal origen de los microorganismos, y así como ejemplo, *E.coli* se encuentra principalmente en intestino de los animales, mientras que *S. aureus* predomina en la piel y en las mucosas y *L. monocytogenes* en el ambiente, por lo que el efecto de los fármacos sería capaz de ejercer una selección distinta en estos grupos bacterianos.

Los datos obtenidos en este trabajo para carnes de cría convencional coinciden con los encontrados recientemente en la literatura científica para otros productos animales también procedentes de la cría convencional. Además algunos autores han demostrado una relación entre los aislamientos de cepas resistentes y el uso de antimicrobianos durante este tipo de cría. En este trabajo, probablemente la mayor prevalencia de *E. coli* en los pollos ecológicos esté también relacionada con el no uso de estos fármacos, tal y como hemos indicado, pero es que además este hecho sería la causa del menor aislamiento de cepas resistentes.

A mayores, es posible que el uso de agentes antimicrobianos sea también la causa de la presencia de una mayor proporción de cepas multiresistentes (resistentes a 2 antimicrobianos o más) en las carnes de cría convencional, sobretodo para el caso de cepas de *E. coli* procedentes de carne ecológica (menores aislamientos 34.3 %, estadísticamente significativo con $P < 0.0001$). Aunque este efecto también se observó para el caso de *S. aureus* o *L. monocytogenes* , no se pudo demostrar estadísticamente.

En función de los resultados obtenidos en esta parte del trabajo, se deduce que aunque la carne de pollo que procede de una producción de tipo ecológico presenta una mayor contaminación por *E. coli*, presenta al mismo tiempo un menor nivel de resistencia a los antimicrobianos ensayados, que son los más habitualmente utilizados en la producción aviar general. La ausencia de diferencias significativas tanto en lo referente al grado de contaminación debido a *S. aureus* y *L. monocytogenes* entre los dos tipos de carne de diferente origen, así como en lo referente a la resistencia a antimicrobianos de estas cepas, sugiere que la contaminación por estos dos géneros bacterianos no depende especialmente de la forma de producción de dichas carnes. Por tanto, otros factores tales como la higiene durante los procesos de sacrificio, despiece o envasado, así como del personal encargado de realizarlos, posiblemente jueguen un papel más importante en la presencia de estos microorganismos en el producto final.

3.8. Estudio sobre cepas de *Escherichia coli* resistentes a antimicrobianos, aisladas de carne de cerdo procedente de producción ecológica y producción convencional

Antimicrobial resistance in *Escherichia coli* strains isolated from organic and conventional pork meat: A comparative survey

Miranda, J.M., Vázquez, B.I., Fente, C.A., Barros-Velázquez, J., Cepeda, A., Franco, C.M.

European Food Research and Tecnology, DOI 10.1007/s00217-006-0547-y

Eur Food Res Technol
DOI 10.1007/s00217-006-0547-y

ORIGINAL PAPER

Antimicrobial resistance in *Escherichia coli* strains isolated from organic and conventional pork meat: a comparative survey

J. M. Miranda · B. I. Vázquez · C. A. Fente · J. Barros-Velázquez · A. Cepeda · C. M. Franco Abuín

Received: 26 September 2006 / Revised: 24 November 2006 / Accepted: 6 December 2006

Abstract Mean counts of *Escherichia coli* were determined for 54 samples of organic pork meat, and in 67 samples of conventional pork meat. Up to three *E. coli* strains from each sample were analysed by an agar disk diffusion assay for their resistance to ampicillin, chloramphenicol, cephalotin, doxycycline, enrofloxacin, gentamicin, nitrofurantoin, sulfisoxazole and streptomycin by the agar disk diffusion method. Results indicated that the presence of *E. coli* was significantly ($P < 0.05$) higher in organic pork meat as compared to conventional pork meat. Isolates from organic pork meat exhibited lower levels of antimicrobial resistance against ampicillin ($P < 0.0001$), doxycycline ($P < 0.0001$) and sulfisoxazole ($P < 0.0001$), as compared to isolates from conventional meat. Moreover, presence of multi-resistant *E. coli* strains was significantly ($P < 0.0001$) higher in conventional pork meat as compared to organic pork meat, the largest differences being observed for isolates resistant to combinations of ampicillin, sulfisoxazol and/or doxycycline. Organically-farmed pork samples showed significantly lower development of antimicrobial resistance in *E. coli*, thus contributing to reduce the development and spread of antimicrobial resistance among these foodborne bacteria.

Keywords *E. coli* · Organic · Antimicrobial · Resistance · Pork · Meat

J. M. Miranda · B. I. Vázquez · C. A. Fente · A. Cepeda · C. M. Franco Abuín (✉)
Laboratorio de Higiene Inspección y Control de Alimentos, Dpto de Química Analítica, Nutrición y Bromatología, Facultad de Veterinaria pabellón, Universidad de Santiago de Compostela, 4 p.b. Campus Universitario, 27002 Lugo, Spain
e-mail: cmfranco@lugo.usc.es

J. Barros-Velázquez
Area de Tecnología de los Alimentos, Dpto de Química Analítica, Nutrición y Bromatología, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002 Lugo, Spain

Introduction

Organic and other non-conventional meat products are now readily available for retail in developed countries, to satisfy consumers' demand of high-quality products that meet the following requirements: (1) guaranteeing animal welfare during production; (2) absence of chemical agents during animal feeding; (3) environmental consideration and (4) better taste than conventional products [7]. In addition, there is a perception on a part of consumers that due to the fact that conditions for growth are more natural for organic farming than for the other one meat produts will have less pathogenic bacteria [5]. However, little is known about microbiological status of organic animal products and the potential microbiological risks linked to organic meat production. Thus, raising of animals outdoors, use of slow-growing breeds, the strict restrictions in therapeutic use of antimicrobial agents and use of very small slaughtering facilities may not guarantee a strict microbiological control of animals destined to human consumption [7, 19].

It has been previously reported that several antimicrobial-resistant bacteria isolated from humans primarily originated from animals raised for human consumption [1, 4] and that such resistant bacteria may contaminate meat derived from those animals [15].

Although this populations of resistant bacteria declines in the absence of the antimicrobial agent [14], antimicrobial-resistant bacteria may persist in meat even after the withdrawal period [24, 28]. Thus, antibiotic resistance of bacterial isolates from animal origin can also represent a potential hazard to consumers via food-borne infections caused by antibiotic-resistant bacteria.

Monitoring of the use of antimicrobial agents in veterinary medicine in animals destined for human consumption is considered to be a risk-management option to prevent development and spread of antimicrobial resistance in microorganisms present in food-producing animals [26]. In this sense, *Escherichia coli* has been described to be a very useful biomarker to evaluate the development of antimicrobial resistance [25]. This is due to the high frequency of mutation exhibited by *E. coli* leading to the development of antimicrobial resistance, as compared to other microorganisms frequently found in animals and foods [10, 15, 25].

Recently, other authors have reported of antimicrobial resistance by bacteria isolated from organic animal products. The data reported in such studies are referred to poultry and dairy products [5, 6, 16, 17, 19, 21]. However, little information relative to organic pork meat products is currently available. As a consequence of this, the main goal of this study was to investigate the prevalence of antimicrobial susceptibility of *E. coli* strains derived from organic pork as compared to conventional pork. The implications of these results in terms of microbiological safety, especially concerning the development and spread of antimicrobial resistance to the food chain, are also discussed.

Materials and methods

Collection of pork meat samples

A total of 121 loin boneless samples were taken during 2005 from supermarkets and butcher shops: 54 fresh pre-packaged organic-reared pork samples, and 67 fresh pre-packaged conventionally-reared pork samples. For the case of conventional pork, 4–5 samples of 14 commercial brands were used. All samples were taken from different lots in different supermarkets and butcher shops. For the case of organic pork, only three commercial brands certified as organic by a official agency were found. Thus, 18 samples were taken from each brand, all of them in different days. Samples were taken to the laboratory in an ice chest in less than an hour for immediate processing. All supermarkets and butcher shops were located in Galicia (north-western Spain).

Microbiological analyses

A total of 25 g portions was obtained from each pork meat sample, placed in a sterile masticator bag together with an appropriate volume (1/9) (w/v) of sterile 0.1% peptone water (Merck, Darmstadt, Germany), and homogenized in a masticator (AES, Combourg, France) for 1 min. After homogenization, samples were cultured for the presence of *E. coli*. About 1 ml of 10^{-1} to 10^{-4} dilutions of meat extracts were processed on plates of Fluorocult® Agar prepared following the manufacturers' instructions (Merck). Once the agar had solidified, the plates were overlaid with 3–4 ml of melted Fluorocult® and incubated at 44°C for 24 h. After incubation, pink to red colonies exhibiting blue florescence after exposure to a 365 nm ultraviolet lamp (Vilbert Lourmat, Marne, France) were identified as *E. coli* and counted.

Once bacterial counts were determined, one-to-three typical *E. coli* colonies isolated from each pork sample were picked, transferred onto Columbia agar supplemented with 5% sheep blood (BioMérieux, Marcy l'Etoile, France) and incubated at 44°C for 24 h in order to obtain pure cultures. Such pure cultures were identified by colony and cell morphology, Gram stain, methyl red stain, oxidase and catalase activity and indole production. Positive strains preliminary identified as *E. coli* were confirmed by the API 20E miniaturized identification tests (BioMérieux). All 180 *E. coli* isolates (90 derived from organic pork meat and 90 from conventional pork meat) were stored at –80°C in maintenance freeze medium units (Oxoid, Basingstoke, UK) until antimicrobial susceptibility was tested.

All meat samples were processed in triplicate. Sampling and processing of pork meat samples were always carried out by the same laboratory personnel. Agar media plates were prepared by the same research assistant throughout the study.

Antimicrobial susceptibility testing of bacteria

Antimicrobial susceptibility testing was performed for a total of 180 isolates of *E. coli*. Antimicrobial susceptibility testing was carried out by agar disk diffusion on Müeller–Hinton agar plates (Oxoid) according to Clinical and Laboratory Standards Institute (CLSI,

formerly NCCLS) guidelines [13]. Antimicrobial disks considered were ampicillin (10 μg), cephalotin (30 μg), chloramphenicol (30 μg), doxycycline (30 μg), enrofloxacin (5 μg), gentamicin (10 μg), nitrofurantoin (300 μg), streptomycin (10 μg) and sulfisoxazole (300 μg) (Oxoid). Antibiotic resistance breakpoints considered were those recommended by CLSI for veterinary pathogens [12], except for the case of nitrofurantoin and streptomycin, in which the CLSI interpretative criteria for Enterobacteriaceae were followed [13]. *E. coli* ATCC 25922 was used as a reference strain for this study.

Antimicrobial agents were selected in terms of their different structure and mechanism of action. *E. coli* isolates were classified as sensitive, intermediate (moderately sensitive) or resistant according to the criteria of the inhibition diameter zones established by the CLSI. Isolates exhibiting resistance to at least two of the antimicrobial agents tested were considered to be multi-resistant strains.

Statistical analysis

E. coli counts were grouped into three categories (<1 log CFU g^{-1}; 1–2 log CFU g^{-1} and >2 log CFU g^{-1}) prior to the performance of statistical analyses. The distributions of *E. coli* counts (log CFU g^{-1}), as well as distributions of resistant strains were compared by means of the X^2 test and Fisher's exact test. The differences were considered to be significant when P was lower than 0.05. All statistical analyses were performed by means of the Statgraphics version 5.0.1. software (SAS Institute, North Carolina, USA).

Results

The results indicated that the special characteristics of organic production provided a higher prevalence of *E. coli* than conventional farming. Thus, for organic pork meat, *E. coli* was detected (>1 log CFU g^{-1}) in 35 of the 54 samples tested (64.8% of the total). The counts determined were >2 log CFU g^{-1} for 9 of the 54 samples tested (16.7%), while 26 samples (48.1%) harboured *E. coli* in the 1–2 log CFU g^{-1} range. In the case of pork reared conventionally, *E. coli* was detected in only 33 of the 67 samples investigated (47.8% of the total). The counts determined in such samples were >2 log CFU g^{-1} in 2 of the 67 samples tested (3%), while 32 samples (47.8%) reached counts in the 1–2 log CFU g^{-1} range. According to these results, the *E. coli* counts obtained for organic pork samples were significantly higher ($P = 0.0231$) than those obtained for conventional pork.

With respect to antimicrobial resistance of *E. coli* isolates, different patterns were observed in the strains isolated from organic pork samples as compared to their counterparts isolated from conventional pork (Table 1). Antimicrobial resistance was significantly higher in *E. coli* isolates derived from conventional pork samples for the antibiotics ampicillin ($P < 0.0001$), doxycycline ($P < 0.0001$), and sulfisoxazole ($P < 0.0001$), as compared to *E. coli* counterparts isolated from organic pork (Table 1). In addition, althought significant differences between batches were observed with respect to cephalotin-resistance ($P = 0.0046$), the results obtained for this antimicrobial agent were ambiguous. Thus, although a higher percentage of cephalotin-resistant microorganisms were isolated from conventional pork meat as compared to organic pork meat (14.4 vs. 6.7%), the latter products exhibited a lower presence of cephalotin-sensitive bacteria than the former (47.8 vs. 61.1%).

Similarly, organic pork meat showed a lower isolation rate of multi-resistant *E. coli* strains than conventional pork samples (Table 2). The percentage of *E. coli* isolates that exhibited resistance to at least two antimicrobial agents was lower ($P < 0.0001$) in organic pork (41.1%) than in strains isolated from conventional pork (90%).

Table 1 Percentages of *Escherichia coli* strains isolated from conventional and organic pork that exhibited a sensitive (S), intermediate (I) or resistant (R) phenotype with respect to antimicrobial agents, as determined by agar disk diffusion method

Antimicrobial agent (μg)	Conventional pork meat ($n = 90$)			Organic pork meat ($n = 90$)			P
	S	I	R	S	I	R	
Ampicillin (10)	10	8.9	81.1	60	16.7	23.3	<0.0001
Cephalotin (30)	61.1	24.4	14.4	47.8	45.6	6.7	0.0046
Chloranphenicol (30)	94.4	3.3	2.2	93.3	1.1	5.6	0.2231
Doxycycline (30)	11.1	18.9	70	30	38.9	31.1	<0.0001
Enrofloxacin (5)	96.7	3.3	0	91.1	8.9	0	0.1366
Gentamicin (10)	91.1	8.9	0	91.1	7.8	1.1	0.5890
Nitrofurantoin (300)	93.3	5.6	1.1	91.1	6.7	2.2	0.8057
Sulfisoxazole (300)	28.9	1.1	70	72.2	7.8	20	<0.0001
Streptomycin (10)	4.4	68.9	26.7	3.3	53.3	43.3	0.0525

Table 2 Resistance patterns in *Escherichia coli* strains isolated from conventional and organic pork

Number of antimicrobials*	Conventional pork (n = 90) No. (%) of isolates	Organic pork (n = 90) No. (%) of isolates
0	2 (2.2)	26 (28.9)
1	7 (7.8)	27 (30)
2	28 (31.1)	21 (23.3)
3	39 (43.3)	12 (13.3)
4	11 (12.2)	2 (2.2)
≥5	3 (3.3)	2 (2.2)
Multi-resistant strains (%)	81 (90)	37 (41.1)

*The percentage of multi-resistant strains is expressed between brackets. Differences between conventional and organic pork were considered significant ($P < 0.0001$)

Discussion

Results obtained in the present work indicated a relationship between the levels of antimicrobial resistance in *E. coli* and the tolerance of antimicrobial agents use in the pork production systems considered in this study. Thus, as expected for conventional pork, in which rearing procedures require more intensive antimicrobial consumption than organic pork farming, the average counts of *E. coli* were significantly lower than in the organic pork samples, in which the use of antimicrobial agents is seriously restricted. Recently, in function of the country of origin, widely variable rates of antimicrobial resistance were reported in *E. coli* isolated from pigs and pork meat conventionally farmed [3, 8, 9, 15, 18, 20]. The antimicrobial resistance rates determined in our study referred to conventionally-farmed pork are compatible with the results reported by these authors.

Other authors have described a correlation between percentages of antimicrobial resistant isolates and the use of antimicrobial agents in pork farming [1, 3]. The results obtained in our study provide evidence supporting the idea that prevalence and antimicrobial resistance of *E. coli* isolates differ depending on the type of animal production system (conventionally raised vs. organically raised) considered. Thus, while organic pork meat resulted to be more contaminated with *E. coli*, these isolates were more sensitive to certain antimicrobial agents than the counterpart *E. coli* strains isolated from conventionally-farmed animals. In this sense, the differences for the antimicrobial agents more commonly used in pork medicine, such as β-lactams (ampicillin), tetracyclines (doxycycline) and sulphonamides (sulfisozaxol) [3, 9, 20, 22, 23] should be noted. Moreover, the fact that no significant differences were observed for the antimicrobial agents banned for pork medicine in the EC such as chloranphenicol and nitrofurantoin provides additional support to the hypothesis that the development of antimicrobial resistance may be a direct consequence of clinical usage of certain antimicrobial agents.

Cross-resistance between β-lactams and tetracyclines or sulphonamides has been previously described [11, 25]. In the present work, the most frequent multiresistance patterns found corresponded to combinations of ampicillin, sulfisozaxol and/or doxycycline.

Organic meat can demand higher prices in the market than conventional meat, and the sales of organic foods has increased in the recent years [27]. For this reason, the regulations concerning organic food production should ensure that meat derived from organic farming present in the markets are truly originated from organically reared animals. In this sense, the investigation of antimicrobial resistance could be a useful tool to detect fraudulent practices, since the detection of bacterial resistance to antimicrobial agents such as ampicillin, sulfisoxazol or doxycycline might point to the fraudulent substitution of organic pork meat with meat derived from conventional farming.

In summary, this work investigated the presence of antimicrobial resistance in microbial isolates obtained from pork meat derived from organic farming and conventional practices. The lower percentage of antimicrobial resistant *E. coli* isolates in organically raised pork supports organic pork rearing as a method to limit the presence of antibiotic resistance bacteria in food animals.

Acknowledgements The authors wish to thank the Xunta de Galicia for granting two research projects (PGIDIT05TAL017E PGIDIT05TAL019E). The authors also thank Carmen Carreira for her expert technical assistance.

References

1. Aarestrup FM (1999) Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. Int J Antimicrob Agents 12:279–285
2. Aarestrup FM (2000) Occurrence, selection and spread of resistance to antimicrobial agents used for growth promotion for food animals in Denmark. APMIS 108(Suppl 101):5–48
3. Asai T, Kojima A, Harada K, Ishihara K, Takahashi T, Tamura Y (2005) Correlation betweeen the usage volume of veterinary therapeutic antimicrobials and resistance in *Escherichia coli* isolated from the feces of food-producing animals in Japan. Jpn J Infect Dis 58:369–372
4. Bager F, Helmuth R (2000) Epidemiology of resistance to quinolones in *Salmonella*. Vet Res 32:285–290
5. Bailey JS, Cosby DE (2005) *Salmonella* prevalence in free-range and certified organic chikens. J Food Prot 68:2451–2453

6. Cui S, Ge B, Zheng J, Jianghong M (2005) Prevalence and antimicrobial resistance of *Campylobacter* spp. and *Salmonella* serovars in organic chickens in Maryland retail stores. Appl Environ Microbiol 71:4108–4111
7. Dransfield E, Ngapo TM, Nielsen NA, Bredahl L, Sjödén PO, Magnusson M, Campo MM, Nute GR (2005) Consumer choice and suggested price for pork as influenced by its appearance, taste and information concerning country of origin and organic pig production. Meat Sci 69:61–70
8. El-Shibiny A, Connerton PL, Connerton IF (2005) Enumeration and diversity of Campylobacters and bacteriophages isolated during the farming cycles of free-range and organic chickens. Appl Environ Microbiol 71:1259–1266
9. Emborg HD, Heuer OE (2003) DANMAP 2002—use of antimicrobial agents and occurence of antimicrobial resistance in bacteria from food producing animals, foods and humans in Demmark. Statens Serum Institut, Danish Veterinary and Food Administration, Danish Medicines Agency, Danish Veterinary Institute, Copenhagen, Demmark
10. Kijima-Tanaka M, Ishihara K, Morioka A, Kojima A, Ozono T, Ogikubo K, Takahashi T, Tamura Y (2003) A national surveillance of antimicrobial resistance in *Escherichia coli* isolated from food-producing animals in Japan. J Antimicrob Chemother 51:447–451
11. Meunier D, Jouy E, Lazizzera C, Kobisch M, Madec JY (2006) CTX-M-1- and CTX-M-15-type β-lactamases in clinical Escherichia coli isolates recovered from food-producing animals in France. Int J Antimicrob Agents 28:402–407
12. National Committee for Clinical Laboratory Standards (NCCLS) (2002) Performance standards for antimicrobial disk and dilution susceptibility test for bacteria isolated from animals: Approved Standard M31-A2, 2nd edn. NCCLS, Wayne
13. National Committee for Clinical Laboratory Standards (NCCLS) (2002) Performance standards for antimicrobial susceptibility testing, 12th informational supplement. M100-S12. NCCLS, Wayne
14. Phillips I, Casewell M, Cox T, De Groot B, Friis C, Jones R, Nightingale C, Preston R, Waddell J (2004) Does the use of antimicrobials in food animals pose a risk to human health? A critical review of published data. J Antimicrob Chemother 53:28–52
15. Sáenz Y, Zarazaga M, Briñas L, Lantero M, Ruiz-Larrea F, Torres C (2001) Antibiotic resistance in *Escherichia coli* isolates obtained from animals, foods and humans in Spain. Int J Antimicrob Agents 18:353–358
16. Sato K, Barlett PC, Kaneene JB, Downes FP (2004) Prevalence and antimicrobial susceptibilities of *Campylobacter* spp. Isolates from organic and conventional dairy hens in Wisconsin. Appl Environ Microbiol 70:1442–1447
17. Sato K, Bennedsgaard TW, Barlett PC, Erskine RJ, Kaneene JB (2004) Comparison of antimicrobial susceptibility of *Sthapylococcus aureus* isolated from bulk tank milk in organic and conventional dairy herds in the Midwestern United States and Denmark. J Food Prot 67:1104–1110
18. Schroeder CM, White DG, Ge B, Zhang Y, McDermott PF, Ayvers S, Zhao S, Meng J (2003) Isolation of antimicrobial-resistant *E. coli* from retail meats purchased in greater Washinton, DC, USA. Int J Food Microbiol 85:197–202
19. Soonthornchaikul N, Garelick H, Jones H, Jacobs J, Ball D, Choudhury M (2006) Resistance to three antimicrobial agents of *Campylobacter* isolated from organically- and intensively-reared chickens purchased from retail outlets. Int J Antimicrob Agents 27:125–130
20. Teshager T, Herrero IA, Porrero MC, Garde J, Moreno MA, Domínguez L (2000) Surveillance of antimicrobials resistance in *Escherichia coli* strains from pigs at Spanish slaughterhouses. Int J Antimicrob Agents 15:137–142
21. Tikofsky LL, Barlow JW, Santiesteban C, Schukken YH (2003) A comparison of antimicrobial susceptibility patterns for *Staphylococcus aureus* in organic and conventional dairy herds. Microb Drug Resist 9:39–45
22. Timmerman T, Dewulf J, Catry B, Feyen B, Opsomer G, de Kruif A, Maes D (2006) Quantification and evaluation of antimicrobial drug use in group treatments for fattening pigs in Belgium. Prev Vet Med 74:251–263
23. Ungemach FR, Müller-Brahrdt D, Abraham G (2006) Guidelines for prudent use of antimicrobials and their implications on antibiotic usage in veterinary medicine. Int J Med Microbiol 296:33–38
24. Van den Bogaard AE, London N, Driessen C, Stobberingh EE (2001) Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. J Antimicrob Chemother 47:763–771
25. Von Baum H, Marre R (2005) Antimicrobial resistance of *Escherichia coli* and therapeutic implications. Int J Med Microbiol 295:503–511
26. Vose D, Acar J, Anthony F, Franklin A, Gupta R, Nicholls T, Tamura Y, Thompson S, Threlfall EJ, Van Vuuren M, White DG, Wegener HC, Costarrica ML, Office International des Epizooties Ad hoc Group (2001) Antimicrobial resistance: risk analysis methodology for the potential impact on public health of antimicrobial resistant bacteria of animal origin. Rev Sci Tech 20:811–827
27. Walshe BE, Sheeman EM, Delahunty CM, Morrisey PA, Kerry JP (2005) Composition, sensoty and shelf life stability analyses of *Longissimus dorsi* muscle from strees reared under organic and conventional production systems. Meat Sci 73:319–325
28. Wiuff C, Lykkesfeldt J, Svendsen O, Aarestrup FM (2003) The effects of oral and intramuscular administration and dose escalation of enrofloxacin on the selection of quinolone resistance among *Salmonella* and coliforms in pigs. Res Vet Sci 75:185–193

RESUMEN

Se ha demostrado que el uso de antimicrobianos durante la producción animal, puede ser una causa de aparición de cepas resistentes a estos fármacos, y que esta resistencia puede ser diseminada a cepas bacterianas que afectan a los humanos, haciendo que poco a poco los tratamientos terapéuticos con estos medicamentos lleguen a ser ineficaces en su lucha contra las enfermedades. Debido a esta preocupación, por parte de las autoridades sanitarias por una parte se han establecido reglas estrictas sobre el uso de estos fármacos y por otra parte se considera fundamental el realizar estudios sobre la evolución actual de las tasas de resistencias.

En este sentido, se ha descrito a *Escherichia coli* como un biomarcador muy útil para el control de resistencias debido a la alta frecuencia de mutación que manifiesta, en comparación con otros microorganismos.

Como consecuencia de lo expuesto, el objetivo de este trabajo es determinar la presencia de cepas de *E. coli* resistentes a antimicrobianos habitualmente utilizados durante la producción de carne de cerdo y compararla con la presencia encontrada en carnes de producción ecológica, en las que supuestamente no se utilizan estos medicamentos.

Se determinaron las medias de los recuentos microbianos de *Escherichia coli* en 54 muestras de carne de cerdo ecológica y en 67 muestras de carne de cerdo de cría convencional. Las muestras pertenecían a diferentes lotes y fueron recogidas en distintas carnicerías y supermercados de Galicia; aunque cabe señalar que sólo se encontraron 3 marcas diferentes certificadas como carnes ecológicas (de las cuales se recogieron muestras en diferentes días y comercios).

De cada una de estas muestras se aislaron 3 cepas hasta obtener un total de 180 cepas (90 de cada tipo de producción).

De todas las cepas, mediante el método de difusión en disco, se determinó la susceptibilidad a 9 agentes microbianos muy usados actualmente en terapéutica porcina. Los resultados pusieron de manifiesto que la carne de cerdo ecológica está significativamente ($P = 0.0231$) más contaminada por *E. coli* (mayor númerode muestras contaminadas y en mayor grado) que la carne procedente de cerdos de cría

convencional, posiblemente debido a que sufren una menor presión puesto que el uso de medicamentos está estrictamente restringido en las producciones de tipo ecológico.

Cuando se estudió la presencia de cepas resistentes a los agentes antimicrobianos ensayados, se observó que para 3 (ampicilina, doxyciclina y sulfisoxazol) de los 9 antimicrobianos, las tasas entre las aisladas de carne ecológica eran significativamente menores ($P < 0.0001$) que las aisladas de carnes de producción convencional.

Además se detectó una mayor presencia ($P < 0.001$) de cepas multiresistentes (resistentes a combinaciones de ampicilina, doxyciclina y/o sulfisoxazol) entre los aislamientos de carnes procedentes de la producción convencional (90 % frente a 41.1 % en carne ecológica), este hecho coincide con que en la literatura científica ya se había descrito la posibilidad de trasmitir la resistencia de forma cruzada entre los antimicrobianos β-lactámicos y tetraciclinas o sulfamidas.

En función de los resultados obtenidos en el presente trabajo se puede concluir que, aunque la carne de cerdo ecológica presenta una mayor contaminación por *E. coli* (puesto que efectivamente no han sufrido los efectos del uso de antimicrobianos), las cepas aisladas presentan un menor nivel de resistencia a los antimicrobianos ensayados como los más habituales en clínica porcina. Los datos sobre resistencias antimicrobianas determinados para carnes de cerdo de cría convencional coinciden con los de otros autores, que indican que efectivamente el uso y/o abuso de estos fármacos favorece la selección de cepas de *E. coli* resistentes. Por tanto, la producción ecológica porcina pudiera decirse que es un mecanismo que contribuye de forma efectiva a disminuir la diseminación de bacterias resistentes a antimicrobianos hacia el consumidor final. En este sentido, la investigación de las resistencias de *E. coli* a antimicrobianos como la ampicilina, doxyciclina y/o sulfisoxazol, podría utilizarse como herramienta eficaz para detectar las sustituciones fraudulentas de carnes de cerdo ecológicas.

3.9. Evaluación de Chromocult® enterococci agar para el aislamiento y recuento selectivo de *Enterococcus* spp.

Evaluation of Chromocult® enterococci agar for the isolation and selective enumeration of *Enterococcus* spp. in broilers

Miranda, J.M., Franco, C.M., Vázquez, B.I., Fente, C.A., Barros-Velázquez, J., Cepeda A.

Letters in Applied Microbiology, 41 (2005) 153-156.

Letters in Applied Microbiology 2005, **41**, 153–156 doi:10.1111/j.1472-765X.2005.01728.x

Evaluation of Chromocult® enterococci agar for the isolation and selective enumeration of *Enterococcus* spp. in broilers

J.M. Miranda[1], C.M. Franco[1], B.I. Vázquez[1], C.A. Fente[1], J. Barros-Velázquez[2] and A. Cepeda[1]

[1]Laboratorio de Higiene Inspección y Control de Alimentos, Dpto de Química Analítica, Nutrición y Bromatología, Facultad de Veterinaria, Universidad de Santiago de Compostela, Lugo, and [2]Area de Tecnología de los Alimentos, Dpto de Química Analítica, Nutrición y Bromatología, Facultad de Veterinaria, Universidad de Santiago de Compostela, Lugo, Spain

2005/0063: received 21 January 2005, revised 15 March 2005 and accepted 17 March 2005

ABSTRACT

J.M. MIRANDA, C.M. FRANCO, B.I. VÁZQUEZ, C.A. FENTE, J. BARROS-VELÁZQUEZ AND A. CEPEDA. 2005.

Aims: To investigate the productivity and specificity of a new chromogenic enterococci selective medium (Chromocult® enterococci agar) recently developed by Merck.

Methods and Results: The study was carried out comparing Chromocult® enterococci agar with MRS agar (Merck), a basal lactic acid bacteria medium in current use. A total of 216 faecal samples from poultry were collected and enterococci populations were counted. Likewise, 100 randomly selected strains were identified for each medium. The differences found between the two media were analysed and discussed.

Conclusions: A good sensitivity of 98% was obtained for Chromocult® agar and all false-positive isolates obtained were identified as *Leuconostoc* spp. However significant differences ($P < 0{\cdot}01$) were obtained between the enterococci species isolation rates identified from these two media, suggesting the poor growth of some species in Chromocult® enterococci agar. Viable counts of *Enterococcus* spp. obtained with MRS agar were significantly higher than those obtained with Chromocult® enterococci agar.

Significance and Impact of the Study: The use of chromogenic media for microbiological analysis is increasing. Independent studies are important to evaluate newly developed chromogenic media.

Keywords: Chromocult®, chromogenic media, enterococci, evaluation, poultry.

INTRODUCTION

Enterococci are found in a variety of environments. They frequently occur in large numbers in dairy and food products (Giraffa and Sisto 1997) and they are very resistant to adverse environmental conditions and heat treatment. Therefore, they are good indicators of the degree of faecal contamination of foods (Stiles and Holzapfel 1997). From the metabolic point of view, *Enterococcus* spp. produce lactic acid as the main product of carbohydrate fermentation. Thus, they may be considered to be lactic bacteria (Doming *et al.* 2003). Some strains are enterotoxigenic, especially those belonging to the species *Enterococcus faecium* and *Enterococcus faecalis* (Batish *et al.* 1984), and several authors have included this genus as a food-borne agent of disease (Grag and Mital 1991). Among all the enterococcal species, *E. faecalis* and *E. faecium* are the most important in human infection (Facklam *et al.* 2002).

The ability to detect the presence of a specific and exclusive enzyme using suitable substrates, in particular fluorogenic and chromogenic enzyme substrates, has led to the development of many methods for the presumptive identification of micro-organisms in their primary isolation media. The incorporation of such substrates into a selective medium may dispense with the need for subculture and further biochemical tests to establish the identity of certain micro-organisms.

The recent introduction of many of these media has led to improved accuracy and faster detection of target micro-

Correspondence to: C.M. Franco, Laboratorio de Higiene Inspección y Control de Alimentos, Facultad de Veterinaria pabellón 4 p.b., Campus Universitario, 27002-Lugo, Spain (e-mail: cmfranco@lugo.usc.es).

organisms (Manafi 1996). In this sense, a new selective and differential medium (Chromocult® enterococci agar; Merck, Darmstadt, Germany) has been designed by Merck for the isolation and enumeration of *Enterococcus* spp., using a chromogenic mix in a selective agar. Enterococci cleave chromogenic substrates in this medium. Owing to the β-D-glucosidase activity present in enterococci, the chromogenic mix is cleaved and the red colour of the colonies indicates the presence of enterococci. Other β-D-glucosidase-producing organisms are suppressed by the sodium azide content of the media (Manafi 2000), sodium azide being an inhibitor of enzyme systems (catalase, cytochrome c oxidase) in electron transport (Hartman *et al.* 1966). Although many studies designed to test different chromogenic media have been carried out in recent years (Manafi 2000; Finney *et al.* 2003; Cárdenes *et al.* 2004), to our knowledge little work has been done concerning the evaluation and comparison of Chromocult® enterococci agar in highly contaminated samples. The purpose of the present work was to test the use of Chromocult® enterococci agar medium as compared with MRS medium, a nonspecific lactic bacteria medium in current use (De Man *et al.* 1960; Doming *et al.* 2003), using faecal samples in order to evaluate its use as an appropriate alternative medium in the processing of highly contaminated samples.

MATERIAL AND METHODS

Culture media

Chromocult® enterococci agar was prepared by suspending 33·0 g of medium in 1 l of distilled water and heating in a boiling water bath. The contents were stirred to aid dissolution for about 45 min, after which the medium was allowed to cool to 45–50°C and was then poured into plates, following the manufacturer's instructions. Chromocult® enterococci agar is composed of (g l^{-1}): peptones 10·0; sodium chloride 5·0; sodium azide 0·2; dipotassium hydrogenphosphate 3·4; potassium dihydrogenphosphate 1·6; ox bile 0·5; Tween®-80 1·0; chomogenic mixture 0·25; Agar-agar 11·0.

The MRS agar was prepared by suspending 66·2 g in 1 l of distilled water and autoclaving for 15 min at 118°C. The medium was allowed to cool to 45–50°C, and used before the surface of the plates had dried, following the manufacturer's instructions. MRS agar is composed of (g l^{-1}): peptone from casein 10·0; meat extract 8·0; yeast extract 4·0; D(+)-glucose 20·0; dipotassium hydrogen phosphate 2·0; Tween®-80 1·0; diammonium hydrogen citrate 2·0; sodium acetate 5·0; magnesium sulfate 0·2; manganese sulfate 0·04; Agar-agar 14·0. Agar medium types were prepared by the same research assistant throughout the study.

Sample preparation and processing

A total of 216 faecal samples were obtained from 18 different batches of six broilers aged 6–10 weeks, using the same feed for all birds. Faecal samples were obtained by swabbing the cloacae of the birds with sterile swabs. A minimum of 1 g of faeces was obtained from each bird and placed aseptically in a sterile tube. Samples were carried to the laboratory in an ice chest in less than half an hour after collection for immediate processing. Samples from three animals were placed in a sterile masticator bag with an appropriate volume (1 : 10) in sterile buffered peptone water (Merck) and subsequently homogenized with a masticator (Aes, Combourg, France) for a maximum of 1 min. After homogenization, 0·1 ml of appropriate dilutions of the homogenates from the masticator bags were surface-plated in triplicate onto Chromocult® enterococci agar and MRS agar plates. Chromocult® plates were incubated at 37 ± 1°C for 48 ± 2 h and MRS agar plates were incubated at 35 ± 1°C for 72 ± 2 h, as specified by the manufacturer. The sampling and processing procedures of poultry faeces described in this paper were always carried out by the same laboratory personnel.

Enterococcus enumeration and identification

Red colonies in Chromocult® enterococci agar with a diameter of 0·5–2 mm were considered to belong to *Enterococcus* spp. The occasional non-enterococcal strains developing on the medium were colourless, blue, violet, turquoise or green. Circular white colonies with varying diameters were considered to be *Enterococcus* spp. in MRS agar. *Enterococcus faecalis* ATCC 29212 was used as control of colony morphology in both media. Only plates containing 20 to 250 colonies were counted. The numbers of *Enterococcus* spp. were converted to $\log_{10}$ values and expressed as $\log_{10}$ CFU g^{-1} faeces, after calculating the enterococci population obtained from triplicate assays for each culture media. After incubation, presumptive enterococci colonies were harvested, transferred onto Columbia agar with 5% sheep blood (Biomerieux, Marcy L'Etoile, France), and incubated inverted at 37 ± 1°C for 48 ± 2 h to obtain pure cultures.

One hundred pure cultures of presumptive enterococcus colonies from each medium were characterized by colony and cell morphology, their haemolytic character, Gram stain, and oxidase and catalase activity. Following this preliminary identification, pure cultures were subcultured in Columbia agar with 5% sheep blood, harvested, and used to inoculate galleries of API 20 Strep (Biomerieux) (Appleman *et al.* 2004) for identification purposes. Readings were taken after 4 and 24 h, as specified by the manufacturer. In the identifications with a reliability of <95%, API 50 CH (Biomerieux) was also used to complete bacterial identifica-

© 2005 The Society for Applied Microbiology, *Letters in Applied Microbiology*, **41**, 153–156, doi:10.1111/j.1472-765X.2005.01728.x

tion. Readings were taken after 24 and 48 h, as specified by the manufacturer. Data for the API galleries were collected using the Apilab Plus software® (Biomerieux).

Statistical analysis

A paired Student's *t*-test was used to compare the overall mean values in both media. The chi-square test was used to compare the different percentages of appearance of the enterococci species in each medium. Differences were considered significant when probabilities were lower than 0·01. All statistical analyses were carried out using Statview version 5.0.1 (SAS Institute, Cary, NC, USA).

RESULTS

Enterococcus spp. colonies appearing on Chromocult® enterococci agar were small (1–1·5 mm diameter), their diameters being slightly larger than those specified by the manufacturer and bright red in colour. These colonies could be readily distinguished from other apparent contaminants. No contaminating colonies with colours related to red were detected, and only in two or three cases were some small pink colonies observed.

One hundred red colonies were randomly selected as described above from Chromocult® agar, as well as another 100 white colonies with varying diameters for the case of MRS agar. The results (Table 1) revealed that 98% (98/100) of the isolates from Chromocult® were *Enterococcus* spp., and 2% (2/100) were false-positive red colonies on Chromocult® enterococci agar and were always identified as belonging to the genus *Leuconostoc*. Of all the colonies isolated from MRS agar, 49% (49/100) were *Enterococcus* spp. There were significant differences between the isolation rates found for each species of *Enterococcus* in both media ($P < 0·01$).

From the above results, if it is considered that 98% of the red colonies obtained on Chromocult® enterococci agar were indeed enterococci as well as 49% of the colonies counted in MRS agar, these percentages can be applied to the counts obtained with the 216 bird faecal samples, the results concerning the enterococci populations being shown in Table 2. Thus, the true enterococci recovery values obtained using this correction were significantly greater in MRS agar than the values obtained in Chromocult® enterococci agar ($P < 0·01$).

Table 1 Distribution and species identities of *Enterococcus* spp. isolates in Chromocult® agar and MRS agar

Species type	Chromocult®, *n* (%)	MRS agar, *n* (%)
Enterococcus faecalis	65 (66·33)	21 (42·86)
Enterococcus faecium	3 (3·06)	18 (36·74)
Enterococcus durans/hirae	26 (26·53)	2 (4·08)
Enterococcus gallinarum	2 (2·04)	0 (0)
Enterococcus casseliflavus	1 (1·02)	4 (8·16)
Enterococcus avium	1 (1·02)	4 (8·16)
Total enterococci	98	49
Other genera	2	51
Total strains	100	100

Table 2 Estimation of *Enterococcus* populations in bird faeces obtained with Chromocult® enterococci agar and MRS agar from the specificity determined for each of the two culture media

	Chromocult®	MRS
No. samples	216	216
Max value (log CFU g^{-1})	8·0	8·8
Min value (log CFU g^{-1})	4·87	5·17
Median	6·52	7·36
Standard deviation	0·6146	0·6479

DISCUSSION

Target bacterial colonies obtained from Chromocult® enterococci agar are more easily distinguished than those obtained from other chromogenic media used, mainly those used for the isolation of *Salmonella*, which shows a higher number of contaminating colonies with colours similar to those produced by the bacteria for which the medium was designed (Dush and Altwegg 1995). This is probably due to the presence in Chromocult® enterococci agar of sodium azide, a powerful inhibitory agent for Gram-negative bacteria as well as for catalase-positive organisms, while in the case of *Salmonella*, most current chromogenic media do not have such potent selective agents in their formulation.

The results of this study indicate that the specificity of Chromocult® enterococi agar (98%) is good and similar to the best chromogenic media (Manafi 2000). However, the isolation rate for *E. faecium* was significantly higher for MRS agar than that obtained with Chromocult® enterococci agar. This is important because *Enterococcus* spp. are the third most common cause of hospital-acquired bacteraemia (Garnier *et al.* 2000) and *E. faecium* is the species that has the broadest spectrum of natural and acquired antibiotic resistance (Klare *et al.* 2003). Therefore, this must be taken into consideration when using this medium for clinical purposes or when performing specific research on *E. faecium*. Nevertheless, *E. faecalis* and *Enterococcus durans/hirae* isolation rates were significantly ($P < 0·01$) higher in Chromocult® enterococci agar than in MRS.

According to the literature consulted, although the species distribution of the genus *Enterococcus* in faecal samples from poultry shows important differences, depending on the country where the study was performed or on the authors of the individual work, the species distribution found for MRS

© 2005 The Society for Applied Microbiology, *Letters in Applied Microbiology*, 41, 153–156, doi:10.1111/j.1472-765X.2005.01728.x

in the present work can be said to be more related to the data reported in the literature (Devriese *et al.* 1991; Butaye *et al.* 1999; Kühn *et al.* 2003) than in the case of Chromocult® enterococci agar. Careful scrutiny of these data from the literature reveals that *E. faecium* isolation rates among enterococci must probably be higher than the 3·06% found for Chromocult® enterococci agar in the present study. Thus, the latter authors reported *E. faecium* isolation rates in chicken faeces of 37·6, 42 and 15% respectively.

Total enterococci counts obtained from MRS were more in accordance with the reports of other authors such as Netherwood *et al.* (1999) than those obtained from Chromocult® enterococci agar. To date, there are no data available concerning the use of Chromocult® enterococci agar to process highly contaminated samples, although Manafi and Windhager (1997) have reported that the use of this medium could be a good alternative for the enumeration of enterococci in water samples. According to the present findings, although Chromocult® enterococci agar is an interesting alternative for routine purposes owing to the fact that 98% of the red colonies were finally identified as enterococci, it is necessary to consider that perhaps the whole of the *Enterococcus* spp. population might not be estimated in very contaminated samples. The lower *Enterococcus* counts obtained in Chromocult® enterococci agar in comparison with the counts obtained with MRS could derive from the more reduced development of *E. faecium* observed in Chromocult® enterococci agar.

ACKNOWLEDGEMENTS

Special appreciation is extended to Encarnación González and Begoña Sierra for technical assistance.

REFERENCES

Appleman, M., Citron, D. and Kwok, R. (2004) Evaluation of the velogene genomic assay for detection of VanA and VanB genes in vancomycin-resistant *Enterococus* species. *J Clin Microbiol* **42**, 1751–1752.

Batish, V.K., Chander, H. and Ranganathan, B. (1984) Prevalence of enterococci in milk products. II. Identification and characterization of prevalent types. *N Z J Dairy Sci* **19**, 189–196.

Butaye, P., Devriese, L.A., Goossens, H., Ieven, M. and Haesebrouck, F. (1999) Enterococci with acquired vancomycin resistance in pigs and chickens of different age groups. *Antimicrob Agents Chemother* **43**, 365–366.

Cárdenes, C.D., Carrillo-Muñoz, A.J., Arias, A., Rodríguez-Alvarez, C., Torres-Lana, A., Sierra, A. and Arévalo, M.P. (2004) Comparative evaluation of four commercial test for presumptive identification of *Candida albicans*. *J Microbiol Methods* **59**, 293–297.

De Man, J.D., Rogosa, M. and Sharpe, M.E. (1960) Medium for the cultivation of lactobacilli. *J Appl Bacteriol* **23**, 130–135.

Devriese, L.A., Hommez, J., Wijfels, R. and Haesebrouck, F. (1991) Composition of the enterococcal and streptococcal intestinal flora of chickens. *J Appl Bacteriol* **71**, 46–50.

Doming, K.J., Mayer, H.K. and Kneifel, W. (2003) Methods used for the isolation, enumeration, characterisation and identification of *Enterococcus* spp.1. Media for isolation and enumeration. *Int J Food Microbiol* **88**, 147–164.

Dush, H. and Altwegg, M. (1995) Evaluation of five new platting media for isolation of *Salmonella* species. *J Clin Microbiol* **33**, 802–804.

Facklam, R.R., Carvalho, M.G.S. and Teixeira, L.M. (2002) History, taxonomy, biochemical characteristics, and antibiotic susceptibility testing of enterococci. In *The Enterococci. Pathogenesis, Molecular Biology, and Antibiotic Resistance* ed. Gilmore, M.S., Clewell, D.B., Courvalin, P., Dunny, G.M., Murray, B.E. and Rice, L.B. pp. 1–54. Washington, DC: ASM Press.

Finney, M., Smullen, J., Foster, H.A., Brokx, S. and Storey, D.M. (2003) Evaluation of Chromocult coliform agar for the detection and enumeration of Enterobacteriaceae from faecal samples from healthy subjects. *J Microbiol Methods* **54**, 353–358.

Garnier, F., Taourit, S., Glaser, P., Courvalin, P. and Galimand, M. (2000) Characterization of transposon Tn 1549, conferring VanB-type resistance in *Enterococcus* spp. *Microbiology* **146**, 1481–1489.

Giraffa, G. and Sisto, F. (1997) Susceptibility to vancomycin isolated from dairy products. *Lett Appl Microbiol* **25**, 335–338.

Grag, S.K. and Mital, B.K. (1991) Enterococci in milk and milk products. *Crit Rev Microbiol* **18**, 15–45.

Hartman, P.A., Reinbold, G.W. and Saraswat, D.S. (1966) Media and methods for isolation and enumeration of the enterococci. *Adv Appl Microbiol* **8**, 253–289.

Klare, I., Konstabel, C., Badstübner, D., Werner, G. and Witte, W. (2003) Occurrence and spread of antibiotic resistances in *Enterococcus faecium*. *Int J Food Microbiol* **88**, 269–290.

Kühn, I., Iversen, A., Burman, L.G., Olsson-Liljequist, B., Franklin, A., Finn, M., Aarestrup, F., Seyfarth, A.M. *et al.* (2003) Comparison of enterococcal populations in animals, humans, and the environment – a European study. *Int J Food Microbiol* **88**, 133–145.

Manafi, M. (1996) Fluorogenic and chromogenic enzyme substrates in culture media and identification test. *Int J Food Microbiol* **31**, 45–58.

Manafi, M. (2000) New developments in chromogenic and fluorogenic culture media. *Int J Food Microbiol* **6**, 205–218.

Manafi, M. and Windhager, K. (1997) Rapid identification of enterococci in water with a new chromogenic assay. *Abstracts of the 97th Meeting of the American Society for Microbiology*, Miami, FL, USA. pp. 453. Abstr. P-107.

Netherwood, T., Gilbert, H.J., Parker, D.S. and O'Donnell, A.G. (1999) Probiotics shown to change bacterial community structure in the avian gastrointestinal tract. *Appl Environ Microbiol* **62**, 1242–1247.

Stiles, M.E. and Holzapfel, W.H. (1997) Lactic acid bacteria of foods and their current taxonomy. *Int J Food Microbiol* **36**, 1–29.

 Letters in Applied Microbiology, **41**, 153–156, doi:10.1111/j.1472-765X.2005.01728.x

RESUMEN

Teniendo en cuenta que el uso de medios cromogénicos para los análisis microbiológicos va cada vez más en aumento, se considera muy importante el realizar estudios independientes que evalúen la efectividad real de estos medios de cultivo que están saliendo al mercado.

Por eso, se ha estudiado la especificidad y eficiencia de un nuevo medio de cultivo cromogénico para el aislamiento y recuento selectivo de *Enterococcus* spp: Chromocult® enterocci agar en muestras altamente contaminadas.

Para llevar a cabo los ensayos, se recogieron un total de 216 muestras de heces de pollo que se cultivaron tanto en el medio de cultivo objeto de estudio, como simultáneamente en otro medio cromogénico que habitualmente se usa para bacterias lácticas, el agar Man Rogase and Sharpe (MRS). A continuación se seleccionaron aleatoriamente 100 cepas crecidas en cada uno de los 2 medios de cultivo, que fueron aisladas y posteriormente identificadas mediante kits bioquímicos. Se analizó y discutió las diferencias en los recuentos y en la identificación de las cepas encontradas en ambos medios.

Se obtuvo una excelente especificidad del 98% para el medio Chromocult® enterocci agar y todos los falsos positivos asilados (2%), que presentaban en este medio una tonalidad rosa y una morfología similar a los enterococos, fueron identificados como *Leuconostoc* spp.

Sin embargo, se encontraron diferencias estadísticamente significativas ($P < 0.01$) entre los recuentos obtenidos en los dos medios de cultivo comparados, siendo éstos mayores en MRS (7.36 log UFC/g) que en el nuevo medio evaluado (6.52 log UFC/g).

Además se encontró que una de las especies más representativa del grupo enterococos, *E. faecium,* se aislaba en una menor proporción en Chromocult® enterocci agar (3.06 %) frente a MRS (36.74 %).

Por tanto se puede indicar que el medio de cultivo Chromocult® enterocci agar es una herramienta bastante eficaz, excepto para muestras en las que se espere una contaminación predominante por *E. faecium*.

4. CONCLUSIONES

1.- Los principales antimicrobianos objeto de estudio en el presente trabajo, enrofloxacina, sulfamidas y doxiciclina van a provocar una alteración significativa de la población bacteriana intestinal de las aves durante su tratamiento vía oral con estas sustancias, siendo las poblaciones de microorganismos gram negativos los que sufren un mayor incremento de las tasas de resistencia frente a los antimicrobianos ensayados.

2.- El período de tiempo indicado en las especialidades farmacológicas probadas como período de supresión se muestra claramente insuficiente para permitir la recuperación de las poblaciones bacterianas sensibles a los antimicrobianos probados y previamente mayoritarias en la microbiota intestinal.

3.- Las carnes frescas procedentes de pollo y cerdo etiquetadas como ecológicas presentan una contaminación por bacterias fecales significativamente más alta que la presente en carne procedente de animales criados de forma convencional.

4.- Las tasas de resistencia, de bacterias aisladas de carnes frescas de pollo y cerdo etiquetadas como ecológicas, frente a antimicrobianos frecuentemente empleados en los tratamientos clínicos de dichos animales son significativamente menores que las obtenidas de bacterias aisladas de carnes procedentes de las mimas especies obtenidas de forma convencional.

5.- La incidencia de *Listeria monocytogenes* y *Staphylococcus aureus* en carnes no parece estar influenciada por el sistema de cría empleado en los animales de procedencia de dichas carnes, ya sea convencional o ecológico.

6.- La determinación de los patrones de resistencia de cepas de *Escherichia coli* presentes en las carnes puede ser una herramienta para el control de tratamientos antimicrobianos no permitidos en los métodos de producción ecológica.

7.-El Chromocult® enterococci agar presenta un nivel de especificidad similar al de los mejores medios cromogénicos presentes en el mercado. No obstante, los recuentos medios obtenidos de poblaciones de enterococcos determinadas mediante este medio

selectivo así como la proporción de *Enterococcus faecium* identificados son significativamente inferiores a los obtenidos en agar MRS, por lo cual este medio cromogénico podría no ser la mejor opción para realizar estudios en muestras en las cuales la determinación de esta especie bacteriana sea muy importante.

Referencias

Aarestrup, F.M., Agerso, Y., Gerner-Smiddt, P., Madsen, M., Jensen, L.B. 2000. Comparison of antimicrobial resistance phenotypes and resistance genes in Enterococcus faecalis and Enterococcus faecium from humans in the community, broilers, and pigs in Denmark. Diagn. Microbiol. Infect. Dis. 37, 127-137

Antunes, P., Réu, C., Sousa, J.C., Peixe, L., Pestana, N. 2003. Incidence of *Salmonella* from poultry products and their susceptibility to antimicrobial agents. Int. J. Food Microbiol. 82, 97-103.

Avrain, L., Humbert, F., L´Hospitalier, R.L., Sanders, P., Vernozy-Rozand, C. and Kempf, I. 2003. Antimicrobial resistance in *Campylobacter* from broilers: association with production type and antimicrobial use. Vet. Microbiol., 96, 267-276.

Ball, P. 1998. The Quinolones: history and overview, 2nd ed. Academic Press, San Diego, USA.

Bager, F., Emborg, H.D., Heder, O.E. 2002. DANMAP 2001- Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Demmark. Statens Serum institute, Danish Veterinary and Food Administration, Danish Medicines Agency, Danish Veterinary Institute, Copenhagen, Denmark. ISSN 1600-2032.

Blanco, J.E., Blanco, M., Mora, A., Blanco, J. 1997. Prevalence of bacterial resistance to quinolones and other antimicrobials among avian *Escherichia coli* isolates from septicemic and healthy chickens in Spain. J. Clin. Microbiol., 35, 2184-2185.

Botana López, L.M., Landoni, M.F., Martín-Jiménez, T. 2002. Farmacología y terapéutica veterinaria. McGraw-Hill Interamericana, Madrid.

Casewell, M., C. Friis, E. Marco, P. McMullin, Philips, I. 2003. The European ban of growth-promothing antibiotics and emerging consequences for human and animal health. J. Antimicrob. Chemother. 52, 159-161.

Castelló Llobet, J.A., Cedo Benet, R., Cepero Briz, R., García Martín, E., Pontes Pontes, M., Vaquerizo Florez, J.Mª. 2002. Producción de carne de pollo (2ª edic.). Real Escuela de Avicultura, Barcelona, España.

Chopra, I., Hawkey, P.M., Hinton, M. 1992. Tetraciclines, molecular and clinical aspects. J. Antimicrob. Chemother. 29, 245-277.

Chopra, I., Roberts, M. 2001. Tetracycline antibiotics: Mode of action, applications, molecular biology and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev. 65, 232-260.

Cornican, M., Buckley, V., Corbett-Feeney, G., Sheridan, F. 2001. Antimicrobial resistance in *Escherichia coli* isolates from turkeys and hens in Ireland. J. Antimicrob. Chemother. 48, 587-595.

Desmonts, M.H., Dufour-Gesbert, F., Avrain, L., Kempf, I. 2004. Antimicrobial resistance in *Campylobacter* strains from French broilers before and after antimicrobial growth promoter bans. J. Antimicrob. Chemother. 54, 1024-1030.

Domagk, G. 1935. Beitrag zur Chemotherapie der bakteriellen infektionen. Dtsch. Med. Wschr. 7, 250-253.

Edlind, T.D. 1991. Protein synthesis as a target for antiprotozoal drugs, p 569-586 In G. Coombs and M. North (ed.), Biochemical protozoology. Taylor & Francis, London, U.K.

Endzt, H.P., Ruijs, G.J., Van Klingeren, B., Jansen, W.H., Van der Reyden T., Mouton, R.P. 1991. Quinolone resistance in *Campylobacter* isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. J. Antimicrob. Chemother. 27, 199-208.

Ermel, G., Laisney, M.J., Rivoal, K., Ragimbeau, C., Gorin, S., Salvat, G., Colin, P. 2000. Les *Campylobacter* et la filière avicole. Sci. Techn. Avicoles, 20, 3–6.

Fairchild, A.S., Smith, J.L., Idris, U., Lu, J., Sanchez, S, Purvis, L.B., Hofacre, C., Lee, M.D. 2005. Effects of orally administered Tetracycline on the intestinal community structure of chickens and on *tet* determinat carriage by commensal bacteria and *Campylobacter jejuni*. Appl. Environ. Microbiol. 71, 5865-5872.

Falkiner, F.R. 1998. The consequences of antibiotic usage in horticulture. J. Antimicrob. Chemother. 41, 429-431.

Finch, R.G. 1997. Tetracyclines, p. 469-484. In O`Grady HP, Lambert RG. Finch, Greenwood D. eds. Antibiotic and chemotherapy, 7th ed. Edingurgh: Churchill Livingstone Ltd., Edinburgh.

Franco Abuín, C.M., Quinto Fernández, E.J., Fente Sampayo, C., Rodríguez Otero, J.L., Domínguez Rodríguez, L., Cepeda Sáez, A. 1994. Susceptibilities of

Listeria species isolated from food to nine antimicrobial agents. Antimicrob. Agents Chemother. 38, 1655-1657.

Freeman, C.D., Nightingale, C.C., Quintiliani, R. 1994. Minocycline: old and new therapeutic uses. Int. J. Antimicrob. Agents. 4, 325-335.

Gibreel, A., Sjögren, E., Kaijser, B., Wretlind, B., and Sköld, O. 1998. Rapid emergence of high-level resistance to quinolones in *Campylobacter jejuni* associated with mutational changes in *gyrA* and *par*C. Antimicrob. Agents Chemother. 42, 3276-3278.

Griggs, D.J., Johnson, M.M., Frost, J.A., Humphrey, T., Jorgensen, F., Piddock, L.J.V. 2005. Incidence and mechanism of ciprofloxacin resistance in *Campylobacter* spp. Isolated from commercial poultry flocks in the United Kingdom before, during and after fluoroquinolone treatment. Antimicrob. Agents Chemother. 49, 699-707.

Gurbay, A., Garrel, C., Osman, M., Richard, M.J., Favier, A., Hincal, F. 2001. Microsomal metabolism of ciprofloxacin generates free radicals. Frre Radical Biology and Medicine. 30, 1118-1121.

Heuer, O.E., Pedersen, K., Andersen, J.S., Madsen, M. 2001. Prevalence and antimicrobial susceptibility of thermophilic *Campylobacter* in organic and conventional broiler flocks. Lett. Appl. Microbiol. 33, 269-274.

Huovinen, P. 2001. Resistance to trimethoprim-sulfamethoxazole. Clin. Infec. Dis. 32, 1608-1614.

Joseph, S.W., Hayes, J.R., English, L.L., Carr, L.E., Wagner, D.D. 2001. Implication of multiple antimicrobial-resistant associated with the poultry environment. Food Add. Contaminants. 18, 1118-1123.

Kijima-Tanaka, M., K. Ishihara, A. Morioka, A. Kojima, T. Ozono, K. Ogikubo, T. Takahashi, and Y. Tamura. 2003. A national surveillance of antimicrobial resistance in *Escherichia coli* isolated from food-producing animals in Japan. J. Antimicrob. Chemother. 51, 447-451.

Köhler, T., Pechere, J.C. 1998. Bacterial resistance to quinolones: mechanisms and clinical implications. In V.T. Andriole (ed.), The quinolones. Academic Press, London, United Kingdom.

Kordick, D.L., Papich, M.G., Breitschwerdt, E.B. 1997. Efficacy of enrofloxacin or doxycycline for treatment of *Bartonella henselae* or *Bartonella clarridgeiae* infection in cats. Antimicrob. Agents Chemother. 41, 2448-2455.

Kucers, A., Bennett, N.M. 1975. The use of antibiotics: a comprehemsive review with clinical emphasis. William Heinemann Medical Books, London.

Ley 25/1990, de 20 de Diciembre, del medicamento. BOE 22 Diciembre 1990 Nº 306, 38228-38279.

Liss, R.H., Batchelor, F.R. 1987. Economic evaluations of antibiotic use and resistance-a perspective: report of task force 6. Rev. Infect. Dis. 9(Suppl. 3): S297-S312.

Luber, P., Wagner, J., Hahn, H., Bartelt, E. 2003. Antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter coli* strains isolated in 1991 and 2001-2002 from poultry and humans in Berlin, Germany. Antimicrob. Agents Chemother. 47, 3825-3830.

Magkos, F., Arvaniti, F., Zampelas, A. 2006. Organic food: buying more safety or just peace of mind? A critical review of the literature. Critical Reviews in Food Science and Nutrition. 46, 23-56.

Mallon, D.J.P., Corkill, J.E., Hazel, S.M, Sian Wilson, J., French, N.P., Bennett, M., Hart, C.A. 2002. Excretion of vancomycin-resistant enterococci by wild animals. Emerg. Infect. Dis. 8, 636-638.

Manafi, M. 1996. Fluorogenic and chromogenic substrates in culture media and identification test. Int. J. Food Microbiol. 31, 45-48.

Manafi, M. 2000. New developments in chromogenic and fluorogenic cultura media. Int. J. Food Microbiol. 60, 205-218.

MAPA,2005:
http://www.mapa.es/notas/documentos/AVICALT_PRESENTACI%C3%93N_DGG_30-06-05.ppt#22. Accedido el 20-3-2007.
www.mapa.es/app/SCH/indicadores/IndicadoresCA.aspx?lng=es. Accedido el 20-3-2007.

Mayrhofer, S., Paulsen, P., Smulders, J.M., Hilbert, F. 2004. Antimicrobial resistance profile of five major food-borne pathogens isolated from beef, pork and poultry. Int. J. Food Microbiol. 97, 23-29.

McDermott, P.F., Bodeis, S.M., English, L.L., White, D.G., Walker, R.D., Zhao, S., Simjee, S. and Wagner, D. 2002. Ciprofloxacin resistance in *Campylobacter jejuni* envolves rapidly in chickens treated with fluoroquinolones. J. Infect. Dis. 185, 837-840.

Monnet, D.L. 2000. Toward multinational antimicrobial resistance surveillance systems in Europe. Int. J. Antimicrob. Agents. 15, 91-101.

Pedersen, K., Wedderkopp, A. 2003. Resistance to quinolones in *Campylobacter jejuni* and *Campylobacter coli* from Danish broilers at farm level. J. Appl. Microbiol. 94, 111-119.

Peters, J., Mac, K., Wichman-Schaue, H., Klein, G., Ellerbroek, L. 2003. Species distribution and antibiotic resistance patterns of enterococci isolated from food of animal origin in Germany. Int. J. Food Microbiol. 88, 311-314.

Philips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preston, R., Waddell, J. 2003. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. J. Antimicrob. Chemother. 53, 28-52.

Poole, K. 2000a. Efflux-mediated resistance to fluoroquinolones in Gram-negative bacteria. Antimicrob. Agents Chemother. 44, 2233-2241.

Poole, K. 2000b. Efflux-mediated resistance to fluoroquinolones in Gram-positive bacteria and the mycobacteria. Antimicrob. Agents Chemother. 44, 2595-2599.

Prescott, J.F., Baggot, J.D. 1988. Antimicrobial therapy in veterinary medicine. Blackwell scientific cop., Boston.

Prescott, J.F., Dowling, P.M. 2000. Agriculture`s role in managing antimicrobial resistance: conference report. Canad. Vet. Jour. 41, 191-197.

Real Decreto 109/1995, de 27 de Enero Medicamentos. Regula los medicamentos veterinarios. B.O.E. 3 Marzo 1995, Nº 53, 15660-15664.

Reglamento (CE) 1831/2003 de 15 de febrero de 2005, relativo a la autorización de determinados aditivos en la alimentación animal. D.O.U.E. Nº 268, 29-40.

Reglamento (CE) 2377/1990 de 26 de Junio, por el que se establece un procedimiento comunitario de fijación de los límites máximos de residuos de medicamentos veterinarios en los alimentos de origen animal. D.O.L. 18 Agosto 1990 Nº 224, 1-8.

Reglamento (CE) 2821/1998 de 17 de Diciembre, por el que se modifica la Directiva 70/524/CEE sobre los aditivos en la alimentación animal, en lo que respecta a la revocación de la autorización de determinados antibióticos. D.O.L. 31 Diciembre 2002 Nº 351,1-142.

Reglamento 1804/1999 de 19 de Julio. Producción Agrícola Ecológica. Completa, para incluir las producciones animales, el reglamento (CEE) núm 2092/91, de 24 de Junio de 1991, sobre producción agrícola ecológica y su indicación en los productos agrarios y alimenticios. D.O.L. Nº 222, 1-28

Reglamento 2092/1991 de 24 de Junio, sobre la producción agrícola ecológica y su indicación en los productos alimenticios. D.O.L. Nº 198, 1-15.

Roland, S., Ferone, R., Harvey, R.J., Styles, V.L., Morrison, R.W. 1979. The characteristics and significance of sulfonamides as substrates for *Escherichia coli* dihydropterase synthase. J. Biol. Chem. 254, 10337-10345.

Ross, J.I., Eady, E.A., Cove, J.H., Cunliffe, W.J. 1998. 16S rRNA mutation associated with tetracycline resistance in a gram-positive bacterium. Antimicrob. Agents Chemother. 42, 1702-1705.

Schnappinger, D., Hillen, W. 1996. Tetracyclines: antibiotic action, uptake, and resistance mechanism. Arch. Microbiol. 165, 359-369.

Sköld, O. 2000. Sulfonamide resistance: mechanisms and trends. Drug Resist. Update. 3, 155-160.

Stern, N.J., Robach, M.C. 2003. Enumeration of *Campylobacter* spp. in broiler faeces and in corresponding processed carcasses. J. Food Protect. 66, 1557-1563.

Talens-visconti, R., Garrigues, T.M., Cantón, E. 2002. Mecanismos de resistencia bacteriana a las quinolonas. Revista española de Quimioterapia. 15, 25-31.

Tejedor-Junco, M.T., Afonso-Rodriguez, O., Martín-Barrasa, J.L., González-Martín, M. 2005. Antimicrobial susceptibility of *Enterococcus* strains isolated from poultry faeces. Res. Vet. Sci. 78, 33-38.

Uyttendaele, M., Detroy, P., Debevere, J. 1999. Incidence of *Salmonella*, *Campylobacter jejuni*, *Campylobacter coli*, and *Listeria monocytogenes* in poultry carcasses and different types of poultry products for sale on the Belgian retail market. J. Food Prot. 62, 735–740.

Van den Boogaard, A.E., Willens, R., London, N., Top, J., Stobbering, E.E. 2002. Antibiotic resistance of faecal enterococci in poultry, poultry farmers and poultry slaughterers. J. Antimicrob. Chemother. 49, 497-505.

Van den Bogaard, A.E., London, N., Driessen, C. and Stobberingh, E.E. 2001. Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. J. Antimicrob. Chemother. 47, 763-771.

Vidaver, A.K. 2002. Uses of antimicrobials in plant agriculture. Clin. Infect. Dis. 34, Suppl. 3, 107-110.

Von Baum, H., Marre, R. 2005. Antimicrobial resistance of *Escherichia coli* and therapeutic implications. Int. J. Med. Microbiol. 295, 503-511.

Willians, D.N. 1992. Tetracyclines. *In* Gorbach, S.L. y Blacklow, N.R. (ed.), Infectous Diseases. The W. B. Saunders Co., Philadelphia, USA.

Wiuff, C., Lykkesfeldt, J., Svendsen, O., Aarestrup, F.M. 2003. The effects of oral and intramuscular administration and dose escalation of enrofloxacin on the selection of quinolone resistance among *Salmonella* and coliforms in pigs. Res. Vet. Sci., 75, 185-193.

Yagüe, A. 2005. Carcaterística de la demanda de pollo en España y otros países. Jornadas profesionales de avicultura de carne, Valladolid, 25-27 Abril.

RESUME

Nos derradeiros anos, o uso de antimicrobianos como promotores do crecemento foi obxecto dun arduo debate a nivel mundial. Dunha parte, moitos científicos consideraban excesivo o risco para a saúde pública por mor da posible aparición de cepas resistentes a estes medicamentos. Pero doutra parte, outros especialistas afirmaban que dito risco non estaba o suficientemente demostrado e defendían este uso polos beneficios que xera tanto dende o punto de vista de rendibilidade na producción gandeira como na saúde pública, xa que a súa utilización tamén provoca a eliminación dos microrganismos patóxenos contaminantes dos alimentos de orixe animal. Finalmente, as autoridades comunitarias da Unión Europea dentro dos principios de precaución, optaron por prohibir dende o 1 de Xaneiro de 2006, o uso de calquera tipo de antimicrobiano como promotor do crecemento.

Nembargantes esta prohibición trouxo como consecuencia un notable aumento do uso dos antimicrobianos máis frecuentemente usados con finalidade terapéutica, o cal sospeitase que pode carrexar un aumento nas tasas de aparición de cepas bacterianas resistentes ós antimicrobianos.

No traballo que aquí se presenta, abórdase o estudio da presencia de resistencias bacterianas a determinados grupos de antimicrobianos. Para abordar este problema, primeiro procedeuse á administración dun tratamento terapéutico en animais de granxa (polos, dado que o seu consumo vai cada vez mais en aumento). Os antimicrobianos elixidos pertencen a cada unha das tres familias mais utilizadas en avicultura: enrofloxacina (quinolonas), doxiciclina (tetraciclinas) e unha mestura de sulfamidas (sulfamidas).

Complementariamente, realizouse unha mostraxe de alimentos de orixe animal (carne de polo e de porco) procedentes de sistemas de producción "ecolóxica" e "convencional" (en intensivo, principalmente) en diferentes establecementos de venta ó público. Estas mostras foron analizadas no referente ó grado de contaminación por diversas poblacións bacterianas. Paralelamente estudiouse o grado de resistencia das bacterias illadas, a un amplo grupo de antimicrobianos.

Como parte de este traballo de investigación, levouse a cabo unha evaluación independente dun novo medio cromoxénico selectivo para o cultivo de *Enterococcus* spp. (Chromocult® enterococci agar) usando para elo mostras de feces de polo cunha contaminación presuntamente elevada.