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Coxiella burnetii infection in sheep and goats: a public risk health, Colombia

Verónica Contreras[1], Marco Gonzalez[1], Jaime Alvarez[1], Salim Mattar[1,*]

Abstract

Objective. The aim of this study was to provide molecular evidence of *C. burnetii* in sheep and goats from some herds of Valledupar, Cesar, Colombia.
Materials and methods. Fifteen herds of sheep and goats were chosen by convenience to investigate the infection by *C. burnetii,* during March and April of 2013. 328 female goats and 66 sheep from 15 herds were included in this study. Milk from ewes and vaginal mucus samples from goats were analyzed by Polymerase Chain Reaction for DNA detection of transposase gene (*IS*1111) of *C. burnetii.*
Results. DNA of *C. burnetii* in 6% (4/66) of sheep's milk and 0.6% (2/328) vaginal mucus from goats was found. 13% (2/15) of the herds had at least one infected animal.
Discussion. Our findings suggest the circulation of *C. burnetii* in sheep and goats from some herds of Valledupar, Colombia, and it highlights the possibility of occurrence of infections in humans and animals.
Conclusions. The detection of *C. burnetii* in sheep milk could represent a public health risk factor for people who consuming raw milk, cheeses or people associated to agriculture and livestock handling. Further studies are necessary to evaluate other routes such as tick's bite, feces, milk from goats and vaginal mucus from sheep of this region of Colombia.

Key words: Q fever, Zoonoses, Milk ejection, Disease Vectors, Health Services Research, Education, Communicable diseases.

Infección por *Coxiella burnetii* en ovinos y caprinos: un riesgo en salud publica en Colombia

Resumen

Objetivo. El objetivo de este estudio fue proporcionar evidencia molecular de infección por *C. burnetii* en ovinos y caprinos de algunos rebaños de Valledupar, Cesar, Colombia.
Materiales y métodos. Quince rebaños de ovinos y caprinos fueron seleccionados a conveniencia para investigar la infección por *C. burnetii,* durante marzo y abril de 2013. En este estudio se incluyeron 328 caprinos y 66 ovinos de 15 rebaños. La leche procedente de ovinos y muestras de moco vaginal de caprinos fueron analizados mediante PCR (Reacción en Cadena de Polimerasa) para la detección de ADN del gen transposasa (*IS*1111) de *C. burnetii*.
Resultados. Se encontró ADN de *C. burnetii* en 6% (4/66) de leche de oveja y 0,6% (2/328) de moco vaginal de cabras. El 13% (2/15) de los rebaños tenían al menos un animal infectado.
Discusión. Nuestros hallazgos sugieren la circulación de *C. burnetii* en ovinos y caprinos de algunos rebaños de Valledupar, Colombia, y destaca la posibilidad de ocurrencia de infecciones en humanos y animales.
Conclusiones. La detección de *C. burnetii* en la leche de oveja podría representar un factor de riesgo para la salud pública de las personas que consumen con frecuencia leche cruda, quesos o personas que trabajan en la agricultura y manipulación de ganado. Otros estudios son necesarios para evaluar otras rutas como la mordedura de la garrapata, las heces, la leche de las cabras y el moco vaginal de las ovejas de esta región de Colombia.

Palabras clave: Fiebre Q, Zoonosis, Secreción de leche, Vectores de enfermedades, Investigación de servicios de salud, Educación, Enfermedades contagiosas

Introduction

Coxiella burnetii is a Gram-negative bacterium causing of Q fever, a zoonosis that concerns public health throughout the world. Mammals, birds and arthropods, mainly ticks can be infected, but domestic ruminants (sheep, goats and cattle) are the main reservoirs[1], in which *C. burnetii* is shed through placenta, vaginal discharges, urine, feces and milk[2,3]. *C. burnetii* is a frequent cause of reproductive disorders mainly in minor ruminants[3-5]. In goats, it has been reported as a cause of abortions and stillbirths[6,7] and in cows, infection is asymptomatic, but metritis and subclinical mastitis have been described[8].

The risk of *C. burnetii* transmission between animals and from animals to humans depends of the prevalence of shedders and excretion's levels in ruminants[5]. *C. burnetii* is able to persist for long time adverse conditions[9] and it is easily transported by the wind[10]. Inhalation of contaminated dust with the

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bacterium from products of infected animals is the main source of infection in humans. Nevertheless, infection by consuming raw milk has also been reported[11]. Clinical spectrum in humans is very broad; patients may experience asymptomatic seroconversion, nonspecific febrile syndrome, atypical pneumonia or hepatitis. Chronic Q fever is rare (<5%), but it occurs in patients with underlying conditions such as immunosuppression, vascular disease, aneurysm, etc., and is mainly expressed with endocarditis that could have a fatal course[12].

The epidemiology of Q fever is characterized by a complex interaction of factors like variety of hosts, low infectious dose, nonspecific symptoms, difficult access to diagnostic tools and the lack of epidemiological association, and they provide a masking with others febrile syndromes[1]. However, in the Q fever epidemic occurred in the Netherlands (2007-2012), more than 4,000 human cases related to farms with infected goats were reported, and it has highlighted its potential impact on human health. The confluence of concentration of infected goat farms near areas with high human density and a favorable meteorological context were among the facilitating factors of bacterial spreading[13].

In Colombia, seroprevalence studies of *C. burnetii* and some human cases of Q fever has been reported[14-16] Additionally, DNA of *C. burnetii* was detected in 45% of 11 bulk cow milk samples[17]. Furthermore, in Colombia there is report describing a case of a 56-year-old patient with an associated in agriculture and livestock handling, the diagnose was made using an indirect immunofluorescence assay showed high titers of IgG for *C. burnetii* anti-phase I (1: 256) and anti-phase II (1:1024)[18]. However, there is a lack of information of *C. burnetii* infection in minor ruminants, which are able to transmit the infection. The aim of this study was to provide molecular evidence of *C. burnetii* infection in sheep and goats from some herds of municipality of Valledupar, Cesar, Colombia.

Materials and Methods

Type of study, geographical area, ruminant's population, size sample and specimens.
A descriptive, prospective and transversal study was performed. Fifteen herds of small ruminants from six villages of municipality of Valledupar, department of Cesar (Colombia) (10º 27´N y 73º 15´O) were chosen at convenience (Figure 1), during March and April 2013. The number of herds and animals per farm were calculated by using of Free-Calc., software[19]. It was taking to account the population of 20,000 sheep and 8,000 goats and a regional register of 100 herds from Valledupar; a confidence level of 95%, maximum error of 5%, and an expected proportion of infected animals and herds according to previous reports were chosen[20]. The obtained results were analyzed through descriptive statistic.

328 female goats and 66 sheep with at least one birth were included in this study. Goats belonged to all 15 herds, whereas sheep came from 11 of them. 15 ml of milk's ewes and vaginal swabs were collected from goats using sterile cotton swabs. They were placed in sterile plastic tubes, transported to the laboratory at 4°C and subsequently preserved at -20°C. Milk samples showed normal physical characteristics (color, pH and density). The state of *C. burnetii* infection in herds was unknown at baseline. A herd was considered positive if at least one animal (sheep or goat) was found infected with *C. burnetii* (milk or vaginal mucus positive by PCR).

DNA extraction and molecular detection of *C. burnetii*.
Milk samples and vaginal swabs were subjected to DNA extraction using the DNA mini kit Purelink (Invitrogen, CA, USA). Milk's specimens directly from 300 µl of whole homogenized milk were analyzed. 300 µl of TE buffer (10 mM Tris Base, 1 mM EDTA and pH 8) was added to vaginal swabs and mixed by vortex. To ensure no contamination, negative controls (sterile water) were included. DNA was purified in a final volume of 100 µl; according to the manufacturer's conditions. Sample was stored at -20°C until use as template for Polymerase Chain Reaction (PCR).

A conventional PCR was performed using oligonucleotides CoxP4 (5'-GGCTGCGTGGTGATGG) (Gen bank accession number: M80806) and CoxM9 (GTCCCGGTTCAACAATTCG), previously described with some modifications (21), which amplify a fragment of (435 bp) transposase gene (*IS*1111) of *C. burnetii*. All amplified products were visualized in electrophoresis of agarose gel (1.5%); it was purified and sequenced by Macrogen, Korea services. The obtained sequences were edited with MEGA program (version 6.0) and analyzed in BLAST.

Results

Four of 66 (6%) sheep milk and two of 328 (0.6%) vaginal swabs of goats yielded *C. burnetii* DNA. The transposase gene sequences (*IS*1111) generated in this study had a percentage of identity of 100 and 99% with *C. burnetii* strain CbuK Q_154 (Genbank access number CP001020) and *C. burnetii* strain Guiana Cb175, respectively.

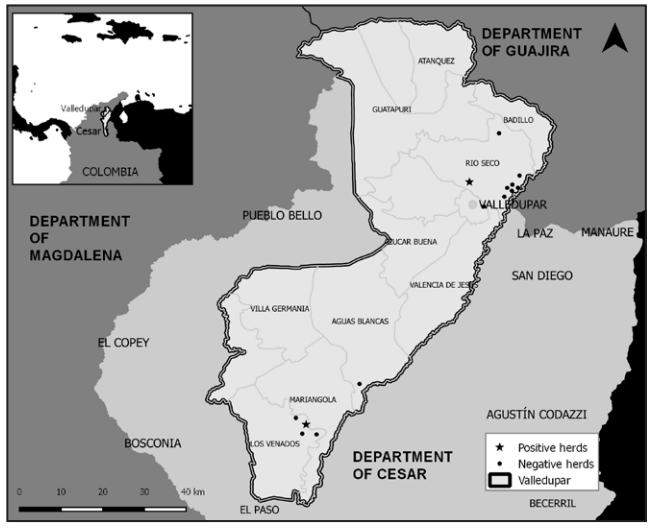

Figure 1. Geographical location of goats and sheep herds in the municipality of Valledupar, department of Cesar, Colombia.

Table 1. Description of studied herds.

Villages of Valledupar	Herds Code	Reproductive background´s	Herds size (n)		Positive PCR / Total obtained samples		Frequency of infection
			Sheep	Goats	Sheep's milk	Goat's vaginal swabs	
Los Venados	**A**	A*	102	30	4/11	1/20	16%
	B	A, M	26	29	0/1	0/25	0
	C	A, M	60	23	0/10	0/23	0
	D	M**	60	35	0/1	0/24	0
	E	A, M	35	27	0/1	0/23	0
Mariangola	**F**	A, M	56	29	0/2	0/23	0
El Jabo	**G**	A, M	130	28	0/9	0/26	0
Río Seco	**H**	A, M	22	27	0	1/16	6%
	I	M	28	47	0/1	0/16	0
Guacoche	**J**	M	25	28	0/1	0/16	0
	K	A	47	38	0/4	0/24	0
	L	A, M	38	25	0	0/24	0
	M	M	22	38	0/20	0/24	0
Guacochito	**N**	M	40	30	0/5	0/28	0
Valledupar	**O**	M	60	40	0	0/16	0
Total					4/66 (6%)	2/328 (0,6%)	13% (2/15)

* Abortions; **Mastitis

Thirteen percent (n=2) of 15 herds had at least one infected animal (Figure 1). Four milk's sheep (4/11; 36%) and one vaginal swab from goats (1/16; 5%) were found infected with *C. burnetii* from a same herd (Table 1). The average of animals in the herds was 77.2 (SD = 33.47, range 1-158). Sheep and goats were mixed in all herds and these latter were predominant. The type of production in sampled herds was 60% (9/15) of ruminants of double purpose (meat and milk production). Farms and its herds were not handled by technical means, and they did not have identification records and reproductive history from animals. However, the occurrence of mastitis, abortions or both were informed in all herds in previous deliveries. Positive herds had records of abortions (Table 1), but the causal agent of such conditions was never identified.

Discussion

The circulation of *C. burnetii* has been reported in some regions of Colombia. In 2006, a seroprevalence in rural workers from Cordoba and Sucre departments was informed[14]. A case of endocarditis and one of pneumonia by Q fever were reported in 2012[15,16], and a case of infection by *C. burnetii* was reported in a patient with a background in agriculture and livestock handling from a rural area of Monteria, Cordoba[18]. Furthermore, in a study performed in cattle farms from Monteria, *C. burnetii* DNA was found in 45% of 11-bulk milk, and 61% of farm workers and residents of the farms had antibodies against *C. burnetii*[17]. Herein, we report the infection by *C. burnetii* in sheep and goats from some herds of Valledupar, Colombia.

The shedding of *C. burnetii* in sheep and goats is a major issue for public health[22]. However, studies of frequency of infection in ruminants based on PCR analysis are uncommon[23] and this knowledge is important to determine the risk of transmission between animals and from animals to humans[5]. In the present study, 6% (n=4) of 66 sheep's milk and 0.6% (n=2) of 328 vaginal swabs from goats yielded *C. burnetii* DNA. In a study performed in Turkey, out of the 350 bovine milk samples and 250 ovine milk samples collected, 1,42% and 0,4% were found to be positive using the PCR technique, respectively[24]. The amplification of transposase gene (*IS*1111)[1] allowed for the sensitivity of the assay to be increased, because this a multi-copy gene (7-110 copies)[25]. DNA sequences generated in the present study confirm that *C. burnetii* is circulating in goats and sheep from some herds of Valledupar, Colombia.

As it was above described, shedding of *C. burnetii* can be intermittent, it could increase during postpartum periods, be different between species and vary according to the type of sample. *C. burnetii* in vaginal mucus of goats is less frequent, but more frequent in milk[2]. In the present work, this result is in according to the proportion of vaginal mucus samples from goats that yielded *C. burnetii* DNA (0.6%). Therefore, it is likely that the sample collection in the present study may have coincided with the shedding period for some individuals and not with others. Additionally, only one type of sample was obtained from each ruminant species (ovine milk and mucus vaginal from goats), and taken together these results, we suggest that a concomitant analysis of various

and different types of samples from the same animal might increase the probability of finding an infected animal with *C. burnetii*. Moreover, Guatteo *et al.,*[5] reported that infected animals shed *C. burnetii* mainly during parturition; in our study, samples were collected several months after delivery in herds. However, in a study carried out by Rodolakis *et al.,*[2], it was found that shedding of *C. burnetii* could not be related with the parturition. *C. burnetii* DNA was found in samples of milk, mucus vaginal and feces taken from 0 to 421 days after parturition in bovine herds, from 5 to 119 days in caprine and 11 to 238 days in ovine herds. The results of this study might suggest that the excretion of *C. burnetii* could be higher in the studied animals and could vary in different times. These results also suggest that the excretion of *C. burnetii* in infected animals could create a public health risk for people in the immediate surroundings as well as in surrounding areas. Additional studies are necessary, with a larger number of animals and with several samples in different periods. Likewise, it is important to carry out studies on other species, such as rodents and ticks, which have been described to be included in the epidemiological cycle of *C. burnetii*[26].

In this study, 13% (n=2) of 15 herds had at least one infected animal with *C. burnetii*. One herd had five infected animals, four sheep's shed *C. burnetii* in milk and one goat shed in vaginal mucus, and it showed that this herd had active circulation of *C. burnetii*. In a study performed in Germany, *C. burnetii* DNA was found in 5% of 39 flock's sheep[27]. In the Netherlands, *C. burnetii* DNA was detected in 33% of 292 goat farms[28]. In Italy, *C. burnetii* DNA was amplified in 18% of 199 goats and sheep farms with reproductive history[29]. In the present study, all herd owners reported the occurrence of reproductive problems (abortions and/or mastitis) in previous deliveries, but causal agent of such conditions were undetermined. One of positive herds to *C. burnetii* have a history of abortions and other one have history of abortions and mastitis, however, others studies are necessary to determine causal agent of this disorders.

On the other hand, Q fever is an important zoonosis. However, In Colombia and Latin American countries, Q fever is a neglected disease due to great multiplicity of symptoms, absence of knowledge of the disease and epidemiological data, which most expected lead to underdiagnoses and underreporting of the disease. This is the first report in the Caribbean area of Colombia, which the main reservoirs and sources of human infections were above described, however, ticks also are involved and the disease presents with a variety of clinical manifestations as atypical pneumonia, febrile hepatitis and endocarditis may also occur. The variability in the clinical manifestations of Q fever may lead to postponement of diagnosis. Therefore, anamnesis, epidemiological factors and serological tests are tremendously important in Colombia. Being exposed to livestock, living in rural area or living closely to farms are public health risk factors. Above that, the lack of direct contact with animals cannot disregard the diagnosis of Q fever, since airborne transmission of *C. burnetii* is also recurrent.

Conclusion

We report the *C. burnetii* infection in sheep and goats from some herds in Valledupar, Colombia. Due to environmental stability and potential aerosol dispersion of *C. burnetii*, our findings highlight the possibility of occurrence of infections in humans and animals in Valledupar, Colombia. The detection of *C. burnetii* in sheep milk could represent a public health risk factor for people who consuming frequently raw milk, cheeses or other products. Further studies are necessary to evaluate other routes such as tick's bite, feces, milk from goats and vaginal mucus from sheep of this region of Colombia. Clinical differential diagnoses including Q fever in high-risk people should be taken into account in Colombia.

Conflict of interest

None of the authors in this study declares having any conflict of interests.

Ethical statement

The research ethics committee of the Institute of Tropical Biological Research at Universidad de Córdoba approved the study through Act 026-2011. Ethical, technique, scientific and administrative standards contained in Resolution No. 008430 de 1993 and Law 84 of 27th of December 1989 were taken into account to their collection, manage and conservation of samples.

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Determinación de la mutación S315T del gen katG en aislados resistentes a Isoniacida de *Mycobacterium tuberculosis* mediante PCR-RFLP

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Resumen

Objetivo: determinar la mutación S315T del gen *katG* en aislados de *Mycobacterium tuberculosis* resistentes a isoniacida mediante la técnica PCR-RFLP.
Materiales y métodos: A partir de 68 aislados de *Mycobacterium tuberculosis* se realizó el análisis de polimorfismo en productos de amplificación de 1054 y 630 pb que contenían la mutación S315T del gen *katG* mediante PCR-RFLP empleando las enzimas de restricción *Msp*I y *Sat*I. Mediante SPSS se determinó sensibilidad, especificidad, valores predictivo positivo y negativo, coeficientes de probabilidad positivo y negativo.
Resultados: El 74,46% de aislados fenotípicamente resistentes y 4,76% fenotípicamente sensibles presentaron la mutación del gen *katG* S315T. La PCR-RFLP para S315T del gen *katG* presentó 85,4% de sensibilidad y 95,2% de especificidad con *Msp*I y 85,4% de sensibilidad y 94,4% de especificidad con *Sat*I.
Discusión: La PCR-RFLP tiene una alta capacidad resolutiva que depende de la enzima que se emplee como se observó en estudios previos. La presencia de la mutación S315T en pacientes vírgenes al tratamiento sugiere la circulación de aislados resistentes a Isoniacida.
Conclusión: La PCR-RFLP resultó una alternativa válida y rápida para el diagnóstico de la resistencia a isoniacida, mediante la detección de la mutación S315T del gen *katG* en comparación con el método convencional de las proporciones.

Palabras Clave: Mutaciones, Polimorfismo, Isoniacida, PCR-RFLP, *Mycobacterium tuberculosis*, *katG*.

Determination of S315T mutation within the katG gene in isoniazid-resistant *Mycobacterium tuberculosis* isolate by PCR-RFLP

Abstract

Objetive: To determine the S315T mutation of the *katG* gene in *Mycobacterium tuberculosis* isoniazid-resistant isolates by PCR-RFLP.
Materials and Methods: Polymorphism analysis of 1054 and 630 bp products containing the S315T mutation of the *katG* gene was performed by PCR-RFLP using the *Msp*I and *Sat*I restriction enzymes from 68 *Mycobacterium tuberculosis* isolates. Sensitivity, specificity, positive and negative predictive values, positive and negative likelihood ratio were determined using SPSS.
Results: 74.46% of isoniazid-resistant and 4.76% of isoniazid-sensitive isolates showed the S315T mutation in *katG* gene. The PCR-RFLP for S315T of the *katG* gene had 85.4% sensitivity and 95.2% specificity with *Msp*I and 85.4% sensitivity and 94.4% specificity with *Sat*I.
Discussion: The PCR-RFLP has a high resolutive capacity that depends on the enzyme that is used as it was observed in previous studies. The presence of the S315T mutation in treatment-naive patients suggests the circulation of isolates resistant to isoniazid.
Conclusion: PCR-RFLP is a valid and rapid alternative for the diagnosis of isoniazid resistance, by detection of S315T mutation in the katG gene compared to the conventional method of proportions.

Keywords: Mutation, Polymorphism, Isoniazid, PCR-RFLP, *Mycobacterium tuberculosis, katG*.

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Introducción

La Tuberculosis (TB) en la actualidad es considerada como una de las principales causas de mortalidad a nivel mundial, generando cada vez más preocupación por el incremento en la prevalencia de variantes genéticas con resistencia a los fármacos antituberculosos[1]. La Organización Mundial de la Salud (OMS) reportó que, en el año 2015 cerca de 10,4 millones de personas contrajeron la enfermedad a nivel mundial, de las cuales 1,8 millones de personas fallecieron, y el 20% eran TB-MDR (Multidrogorresistencia) y de estos un 9,7% de las personas con TB-MDR presentan XDR-TB[2]. Tratamientos subóptimos y la mala adherencia a las terapias de drogas antituberculosas contribuyen al aumento de la resistencia a fármacos; mientras que tratamientos inadecuados aumentan el riesgo de transmisión directa de *Mycobacterium tuberculosis* resistente a los medicamentos[3-5]. Ecuador se encuentra entre los países priorizados dada la gran carga de la enfermedad y el elevado índice de TB-MDR, reportándose un 4,6% de TB-MDR en 2015[6].

La isoniacida (INH: Isonicotinic Acid Hydrazide) una de las drogas de primera línea junto a la Rifampicina (RFM), son consideradas las drogas de mayor eficacia frente a una infección por *Mycobacterium tuberculosis*[7]. La INH actúa inhibiendo la biosíntesis de los ácidos micólicos de la pared celular, e ingresa en el *Mycobacterium tuberculosis* como un profármaco por difusión pasiva y es activada por la enzima catalasa-peroxidasa codificada por *katG,* para generar radicales libres, que atacan a continuación múltiples objetivos en las células[8]. Se ha reportado en varios estudios que la resistencia a isoniacida está relacionada con mutaciones específicas en varios genes importantes de *Mycobacterium tuberculosis,* existiendo variantes genéticas tales como: *katG* (S315T, S315N, S315D, S315I, S315R, V61G, Q247H, A62T, G383A, C701G, G1388T)*, inhA* (S94A, I21V, I21T, I47T, I194T, 3, 258, 190) y su región promotora (-15 C-T, -8 T-C, T-A ó T-G,-17 G-T), *ahpC, nhd, kasA, oxyR, furA, fabG1*[9-21], de igual manera, se ha podido mostrar que la aplicación de PCR-RFLP del gen *katG* permite la identificación eficiente de aislados resistentes a isoniacida pudiendo emplearse como una prueba rápida de detección[22-25].

Aún existiendo evidencia de la aplicación de pruebas moleculares en búsqueda de la resistencia en *Mycobacterium tuberculosis,* en el Ecuador no existen mayores estudios relacionados a la identificación de mutaciones que interfieren en la acción de las drogas antituberculosas, ni las mutaciones presentes en los genes relacionados a la resistencia a fármacos, limitando la detección de la resistencia a análisis microbiológicos, o escasamente se emplea métodos genotípicos comerciales centrándose solo a la detección de resistencia a Rifampicina (GeneXpert MTB/RIF), excluyendo otras tecnologías que pueden detectar genotipicamente resistencias a otros medicamentos y que se emplean actualmente en varios países[2]. Por lo antes expuesto, el presente estudio propone determinar la mutación S315T del gen *katG* en aislados de *Mycobacterium tuberculosis* resistentes a isoniacida mediante la técnica PCR-RFLP utilizando las enzimas de restricción *Msp*I y *Sat*I, estrategia que permitiría caracterizar en menor tiempo la resistencia a Isoniacida comparado con el método de las proporciones, sugiriendo su aplicación como método rápido de tamizaje, complementario de las pruebas fenotípicas, que permitan mejorar la vigilancia epidemiológica de esta enfermedad a nivel país por el conocimiento de variantes genéticas circulantes, personalizando el tratamiento y disminuyendo los índices de TB-MDR.

Materiales y métodos

Un total de 68 aislados de *Mycobacterium tuberculosis* a los que se les había determinado previamente la resistencia mediante el método de las proporciones de Canetti, Rist y Grosset[23] fueron obtenidos del banco de cepas del Centro de Referencia Nacional de Micobacterias del Instituto Nacional de Investigación en Salud Pública INSPI "Dr Leopoldo Izquieta Pérez". El protocolo de investigación fue aprobado por el Comité de Ética de Investigación de Seres Humanos de la Universidad San Francisco de Quito bajo código 2017-018E.

Extracción de ADN

La extracción del ADN se realizó de acuerdo al protocolo descrito por van Soolingen *et al*.[24], con ligeras modificaciones. A partir de 100 µL de aislado de *Mycobacterium tuberculosis* se adicionó 50 µL de lisozima (10mg/ml), incubando a 37ºC por una hora, luego se adicionó 80 µL de SDS 10% y 5 µL de proteinasa K (10mg/ml), e incubó a 65ºC por 10 minutos; seguido se adicionó 100 µL de NaCl 5M y 100 µL CTAB/NaCl, se mezcló e incubo a 65ºC por 10 minutos; posteriormente se agregó 100 µL de Cloroformo: Alcohol Isoamilico (24:1), se agitó y centrifugó por 20 minutos a 14.000 r.p.m., se transfirió la fase acuosa y adicionó 60 µL de isopropanol al 90% y 2 µL de RNAsa, se mezcló e incubó a -20ºC hasta el siguiente día. Se centrifugó 20 minutos a 14.000 r.p.m a 4ºC. Se descartó el sobrenadante, se realizaron dos lavados con etanol al 70% centrifugando cada vez por 20 minutos a 14.000 r.p.m., a 4ºC. El ADN se resuspendió en 200 µL de buffer TE 1X y se almacenó a -20ºC hasta su utilización.

Amplificación del gen *katG*

Para determinar el polimorfismo de la región 315 del gen *katG*, dos productos fueron amplificados por reacción en cadena de la polimerasa (PCR) con los iniciadores *katG*500fUT1 5´ACCCAACTGAATGGAGCATGGGCGGAC3´ (1054 pb), *katG*904: 5-AGCTCGTATGGCACCGGAAC-3 (630 pb), respectivamente y *katG*1533rUT2: 5-ACGCACTTGACTTGTCTTCGTCGGGGTCGT-GACCTCCCA-3 (modificado de Herrera-León *et al.*[20]) los cuales reconocen una región circundante del codón 315. Las amplificaciones se realizaron en un volumen de reacción final de 50µL conteniendo 1X de GoTaq Colorless Master Mix (Promega, Madison, WI 53711 USA) el cual contiene Taq DNA Polimerasa, 400µM dNTPs y 3mM $MgCl_2$, 0,25 µM de cada iniciador y 150 ng de ADN extraído. Las amplificaciones fueron llevadas a cabo en un termociclador C1000 (Bio-Rad Laboratories, CA,

USA) y las condiciones de PCR fueron desnaturalización inicial a 94°C por 4 min, seguido de 40 ciclos a 94°C por 45 s, hibridación a 65°C por 45 s, extensión a 72°C por 1 min y una extensión final a 72°C por 5 min. Los productos de PCR fueron analizados por electroforesis en gel de agarosa al 2% con SYBR safe bajo luz ultravioleta.

Pcr-rflp del gen *katG* con *Msp*I y *Sat*I

Para la generación de los perfiles de digestión del fragmento amplificado del gen *katG* se utilizó el procedimiento descrito por Leung *et al.*[19]. Los perfiles teóricos fueron analizados previamente "*in silico*" utilizando el programa online Webcutter 2.0. Los productos amplificados fueron digeridos por separado con las enzimas de restricción *MspI* (Promega, Madison, WI 53711 USA) y *SatI* (Thermo Fisher Scientific, Waltham, MA USA). La enzima *Msp*I reconoce la secuencia de corte C↑CGG, y *SatI* GC↑NGC, permitiendo detectar la mutación en la posición nucleotídica 945, codón 315 del gen *katG* (AGC →ACC).

Las reacciones de digestión fueron preparadas para un volumen final de 20 µL, conteniendo 3,0 U de enzima *MspI,* 1× del Buffer 10X (suplementado con la enzima), 2 µg de BSA acetilado, 10µg µL-1, y 2,0 U de enzima *Sat I,* 1X Buffer Anza™ 10X acorde a las instrucciones del proveedor. Las mezclas de reacciones fueron incubadas a 37◦C por 4 horas en un equipo de PCR C1000 (Bio-Rad Laboratories, CA, USA). Los productos fueron separados por electroforesis en gel de agarosa al 3% en buffer TAE 1X durante 3 horas a 100 voltios, y se visualizó los fragmentos obtenidos bajo luz ultravioleta y digitalizados en un fotodocumentador GelDoc XR (Bio-Rad Laboratories, CA, USA). Los resultados de los perfiles PCR-RFLP de cada enzima fueron interpretados acordes a estudios publicados por Leung *et al.*[19] y Herrera-León *et al.*[21].

Análisis de datos

Los datos fueron sistematizados en una hoja de cálculos de Excel diseñada para el estudio la cual incluyó datos epidemiológicos como edad, sexo, procedencia, tipo de muestra, fase de tratamiento, susceptibilidad a isoniacida proporcionados por el Centro de Referencia Nacional de Micobacterias del Instituto Nacional de Investigación en Salud Pública INSPI "Dr Leopoldo Izquieta Pérez". Los análisis de sensibilidad (S), especificidad (E), valor predictivo positivo (VPP) y negativo (VPN), razón probabilística positiva (LR+) y negativa (LR-) fueron realizados mediante tablas cruzadas de 2 x 2 considerando como prueba estándar los resultados del método de las proporciones empleando el programa para Windows SPSS versión 22 (SPSS Inc, Chicago, IL, USA).

Resultados

De los 68 aislados, 69,1% (47) correspondió a aislados fenotípicamente resistentes y 30,9% (21) a aislados fenotípicamente sensibles de acuerdo al método de las proporciones. 77,9% (53) provenía de hombres y 22,1% (15) de mujeres. 48,0% eran de pacientes vírgenes al tratamiento (VT), 41,0% habían abandonado el tratamiento (AT) y 11,0% se encontraba en control del tratamiento (CT). La mayoría de los aislados provenían de Guayaquil (70,6%), seguidos por los obtenidos en Babahoyo (8,8%), Quito (7,4%), Portoviejo (5,9%), Machala (4,4%), Esmeraldas (1,5%) y Santa Elena (1,5%) (Figura 1).

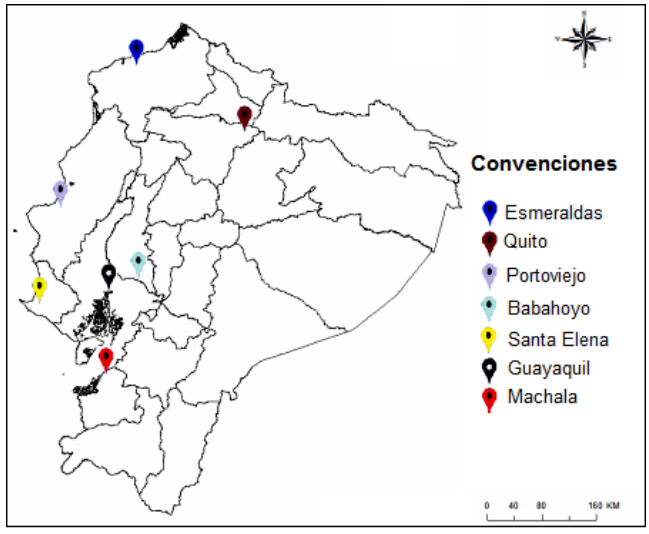

Figura 1. Distribución geográfica de los aislados resistentes y sensibles a Isoniacida (INH) de Mycobacterium tuberculosis.
Convenciones presentan la procedencia de los aislados en cada una de las provincias: negro: Guayaquil; marrón: Quito; rojo: Machala; celeste: Babahoyo; amarillo: Santa Elena; lila claro: Portoviejo; azul oscuro: Esmeraldas.

Se observó mediante electroforesis que al emplear PCR-RFLP en el producto de 1054 pb con la enzima *Msp*I y *Sat*I los aislados resistentes y sensibles fenotípicamente presentaron similar perfil de restricción. A partir del producto de 630 pb se pudo evidenciar la presencia de un fragmento de aproximadamente 132 pb al utilizar *Msp*I y 205 pb con *Sat*I, en los aislados que presentaban la mutación en el codón 315. Los productos que presentaron bandas diferenciables fueron confirmados por secuenciación (datos no mostrados).

Al emplear las enzimas *Msp*I y *Sat*I se determinó que un 52,9% de los aislados (35 resistente y 1 sensible) presentó el codón mutado en la posición S315T; un 38,2% y 33,8% no presentaron la mutación; mientras un 8,8% y 13,2% presentaron un perfil no diferenciable para *Msp*I y *Sat*I, respectivamente.

Los aislados resistentes presentaron similar porcentaje del perfil con mutación (74,5%) con la enzimas *Msp*I y *Sat*I, mientras que en los aislados sensibles el 4,8% presentó el perfil con mutación para ambas enzimas (Tabla 1).

Al analizar la incidencia de los perfiles con mutación S315T y su relación con los tratamientos antifímicos recibidos, se puede observar que los aislados con mutación presentan igual porcentaje de incidencia entre los obtenidos de personas antes tratadas (AT) y vírgenes al tratamiento (VT) con 23,5% respectivamente, para ambas enzimas utilizadas. En los aislados resistentes fenotípicamente, aquellos que provienen de personas vírgenes al tratamiento, un 34,0% de

Tabla 1 . Resultado comparativo de la susceptibilidad de Isoniacida (INH) en aislados de Mycobacterium tuberculosis analizados mediante métodos genotípicos y fenotípicos.

N° de aislados (%)	Métodos fenotípicos y genotípicos		
	Método de las Proporciones	PCR-RFLP (n=68)	
		MspI (*n;* %)	SatI (*n;* %)
47 (69,1)	R	R (35/47; 74,5)	R (35/47; 74,5)
	R	S (6/47; 12,8)	S (6/47; 12,8)
	R	ND (6/47; 12,8)	ND (6/47; 12,8)
21 (30,9)	S	R (1/21; 4,8)	R (1/21; 4,8)
	S	S (20/21; 95,2)	S (17/21; 81,0)
	S	ND (-)	ND (3/21; 14,3)

R, resistente; S, sensible; ND, patrones no diferenciado.

estos aislados presentó la mutación, seguido de los antes tratados con 31,9%, para ambas enzimas. En el caso de los aislados sensibles fenotípicamente, un 4,8% de los aislados con la mutación provienen de personas antes tratados, para ambas enzimas utilizadas (Tabla 2).

Para determinar la especificidad y sensibilidad de la PCR-RFLP, el método de las proporciones fue utilizado como la prueba de referencia considerando que es el método estándar empleado en la actualidad para evaluar la resistencia en Ecuador. Se obtuvo 85,4% de sensibilidad y 95,2% de especificidad con *Msp*I y 85,4% de sensibilidad y 94,4% de especificidad con *Sat*I para la detección de la resistencia a Isoniacida en *Mycobacterium tuberculosis* (Tabla 3).

Discusión

La detección de la resistencia a drogas en pacientes con TB es necesaria para combatir la creciente prevalencia de TB-MDR. En los últimos años se han realizado estudios relacionados con la resistencia a medicamentos que han permitido el desarrollo de métodos fenotípicos y genotípicos[25].

La secuenciación del genoma de *Mycobacterium tuberculosis* y la identificación de genes asociados a drogo-resistencia, ha permitido la aplicación de métodos de diagnóstico genotípicos tales como PCR-RFLP que ofrecen la posibilidad de identificar mutaciones relacionadas a resistencia[26]. Las mutaciones en los genes de *Mycobacterium tuberculosis katG* e *inhA*, han sido reportados como los responsables del 60 - 80% de los aislados resistentes a isoniacida[27-30].

En nuestro estudio se observó que la PCR-RFLP tiene una aceptable capacidad resolutiva para la detección de resistencia a isoniacida en aislados de *Mycobacterium tuberculosis,* pero se debe tener presente que existen aislados que no presentan la mutación y tienen un patrón fenotípico resistente el cual podría estar relacionado a otros genes que confieren resistencia a isoniacida, de igual manera, dependiendo de las variantes geneticas de la mutación 315 que sean más frecuentes, la capacidad resolutiva dependerá de la enzima empleada (*Msp*I: 95,2 (83,8-100,0), *Sat*I: 94,4 (81,1- 100,0). Similares resultados han sido descritos por Herrera-León *et al.,*[16],y Mathuria *et al.,*[18], esto sería debido a que *Msp*I solo detecta la sustitución de Ser→Thr (AGC→ACC), mientras que *Sat*I puede detectar las otras sustituciones (AGC→ACC, ACA, ACT, ACG) que ocurren en el codón 315 que afectan los nucleótidos GC en esta posición.

Nuestros resultados mostraron que al emplear las enzimas *Msp*I y *Sat*I, el 74,5% de los aislados resistentes presentaron perfil con la mutación S315T, mientras que en los aislados sensibles el 4,8% la presentó. Estos resultados difieren de trabajos previos publicados en los cuales solo se reporta la presencia de perfiles de mutación en la posición S315T en aislados resistentes[7,19,20,22,31], el 25,5% de aislados fenotipicamente resistentes que no presentaron la mutación estudiada, pudo deberse a la presencia de polimorfismos en regiones diferente a la posición S315T[15-20] en el gen *kat*G u otros genes que aporten resistencia a Isoniacida[9-14] indiciendo a la sobreexpresión que superen el poder inhibitorio de INH[26,31]; otra causa puede ser la presencia de mecanismos de resistencia diferente a las mutaciones tales como mecanismos de barrera (reducción de la permeabilidad[32-33] y bomba de eflujo[34-37]). En cuanto a la presencia de un aislado sensible con la mutación S315T, posiblemente se deba a que el límite de la detección por PCR-RFLP empleada se encuentre cercano al 1% de resistentes que basado en el método de las proporciones

Tabla 2. Resultado comparativo de la susceptibilidad de Isoniacida (INH) en aislados de Mycobacterium tuberculosis analizados mediante PCR-RFLP MspI y SatI entre los diferentes tratamientos recibidos.

Método de las proporciones	Tratamiento	PCR -RFLP *Msp*I		PCR -RFLP *Sat*I	
		Mutante *n* (%)	Normal *n* (%)	Mutante *n* (%)	Normal *n* (%)
Resistente (*n*= 47)	**AT**	15 (31,9)	4 (8,5)	15 (31,9)	4 (8,5)
	VT	16 (34,0)	2 (4,3)	16 (34,0)	2 (4,3)
	CT	4 (8,5)	-	4 (8,5)	-
Sensible (*n*= 21)	**AT**	1(4,8)	6 (28,6)	1(4,8)	5 (23,8)
	VT	-	14 (66,7)	-	12 (57,1)
	CT	-	-	-	-

VT, virgen al tratamiento; AT, antes tratados; CT, con tratamiento; ND, no perfil determinado.

Tabla 3. Parámetro estadístico de la digestión por PCR-RFLP con MspI y PCR-RFLP con SatI en relación al método de las proporciones para la detección de la mutación del gen katG en aislados resistentes y sensibles a Isoniacida de Mycobacterium tuberculosis.

Parámetros estadísticos	PCR-RFLP *Msp*I (*n* =68, 95% IC)	PCR-RFLP *Sat*I (*n* =68, 95% IC)
Sensibilidad (%)	85,4 (73,3-97,4)	85,4 (73,3-87,4)
Especificidad (%)	95,2 (83,8-100,0)	94,4(81,1- 100,0)
Valor Predictivo Positivo (%)	97,2	97,2
Valor Predictivo Negativo (%)	76,9	73,9
Razón Probabilistica Positiva	17,9	15,4
Razón Probabilistica Negativa	0,15	0,15

pasa imperceptible y se considera como sensible, tambien podría deberse a una inadecuada identificación del perfil de resistencia mediante la prueba de las proporciones en el aislado sensible, y/o contaminación del aislado después de su conservación.

Al analizar los diferentes casos de pacientes con TB, se puedo observar que, 46,05% de muestras con el perfil mutado S315T mediante PCR-RFLP correspondió a pacientes antes tratados (AT) y vírgenes al tratamiento (VT), esto sugiere que posiblemente estos pacientes no cumplieron el tratamiento correctamente y/o abandonaron el mismo generando cepas resistentes a esta droga, adicionalmente, que existan paciente sin antecedentes de tratamiento (VT) y que presenten la mutación S315T sugiere que están circulando dentro de la población, aislados de *M. tuberculosis* que presentan resistencia previa a la isoniacida, haciendo poco efectiva la medicación recibida.

La sensibilidad general fue del 85,4% (IC95%:73,3-97,4), en contraste a otros estudios que mostraron 88,2%[23], 91,5%[38], 95,0%[39], sin embargo, presenta un alto valor predictivo positivo (0,972) cuando se lo compara con el método de las proporciones, lo que aseguraría que un 97,2% de los aislados en los cuales se ha detectado mediante PCR-RFLP la mutación S315T tendrían la resistencia al medicamiento, siendo un porcentaje útil para la toma de decisiones terapéuticas mientras se obtiene el resultado por cultivo microbiológico, de igual manera, es bien conocido que los valores predictivos de las pruebas son directamente dependientes de la prevalencia de la caracteristica de la población particular[44], lo cual al considerar la prevalencia de resistentes a Isoniacida de nuestro estudio (69,12%) impacta en la dimsinución del VPP del test empleado, lo que se mejoraría ampliando la población de estudio.

En relación a las muestras que presentaron patrones no diferenciables (8,8% para *Msp*I y 13,2% para *Sat*I), es probable que se deba a causas como: la presencia de mecanismos de resistencia diferente a las mutaciones que afectan la permeabilidad de la membrana y bomba de eflujo, la calidad de la muestra, errores técnicos y presencia de inhibidores los cuales pueden afectar la calidad de amplificación y posterior generación del perfil de restricción.

El contar con buenas practicas de laboratorio, que garanticen condiciones pre-analíticas adecuadas, es primordial con la finalidad de evitar contaminaciones y/o falsos positivos.

Una desventaja de cualquier prueba genotípica es la incapacidad de detectar mutaciones silenciosas (mutaciones que no conducen a ningún cambio de aminoácido). Mutaciones en otras regiones diferentes a la S315T dentro del mismo gen[15-20] u otros genes parte del genoma de *Mycobacterium tuberculosis* pueden contribuir al desarrollo de la resistencia a isoniacida[9-14], por lo cual realizar estudios con poblaciones mayores en las que se involucre la identificación de mutaciones de otros genes relacionados a la resistencia en isoniacida u otros medicamentos mejoraría la caracterización de las variantes genéticas presentes en los linajes de *Mycobacterium tuberculosis,* adicionalmente, al conocer las nuevas variantes presentes en los diferentes genes relacionados a la resistencia se aportaría al mejoramiento de los sistemas de detección comerciales actuales, considerando que los aislados presentan variaciones genéticas propias de su distribución geográfica[13-18, 40-43].

En conclusión, las pruebas moleculares no reemplazan los métodos convencionales, sin embargo, son un eficiente método de tamizaje cuando se combinan con datos clínicos y epidemiológicos, impactando significativamente en una diagnosis temprana de la resistencia, lo que contribuye a estrategias de control de la resistencia de TB en los países[44]. De igual manera, investigaciones dirigidas al estudio de la resistencia por secuenciamiento del genoma completo ayudarían en el futuro a dilucidar los mecanismos moleculares de resistencia a isoniacida u otros medicamentos en aislados de *Mycobacterium tuberculosis* circulantes en el Ecuador[45-47]. La PCR-RFLP resulta una alternativa confiable y rápida para el diagnóstico de la resistencia a isoniacida, mediante la detección de mutación S315T del gen *katG,* otros codones mutados del mismo gen y/o demás genes que producen resistencia, en comparación con el método convencional de las proporciones.

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Responsabilidades éticas

Protección de personas y animales. Los autores declaran que para esta investigación no se han realizado experimentos en seres humanos ni en animales.

Confidencialidad de los datos. Los autores declaran que no han publicado datos de pacientes.

Derecho a la privacidad y consentimiento informado. Los autores declaran que en este artículo no aparecen datos de pacientes y poseen el consentimiento informado de los pacientes.

Conflicto de interés

Los autores del presente manuscrito declaran no tener conflicto de interés

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ARTÍCULO ORIGINAL

Costo-efectividad de pruebas para el diagnóstico de geohelmintiasis: Revisión sistemática de la literatura

Jaiberth Antonio Cardona-Arias[1,*], Deisy Cristina Restrepo Posada[2]

Resumen

Introducción: Existen diversas pruebas para el diagnóstico de las geohelmintiasis, con múltiples estudios que demuestran la heterogeneidad en su validez diagnóstica y pocos que aluden su costo-efectividad.
Objetivo: Sistematizar las evaluaciones económicas sobre las pruebas aplicadas en el diagnóstico de geohelmintos.
Métodos: Revisión sistemática en seis bases de datos con 24 estrategias de búsqueda. Se aplicó un protocolo de selección de estudios, garantizando exhaustividad, reproducibilidad y evaluación de la calidad metodológica. Se realizó síntesis cualitativa de la información.
Resultados: En la literatura científica mundial sólo se dispone de cuatro evaluaciones económicas completas para el diagnóstico de geo-helmintos, en la modalidad de estudios de costo-efectividad; en éstas se evaluaron cinco pruebas en 247 adultos y 6.708 niños. En los desenlaces en salud empleados se encontraron la proporción de pacientes positivos y algunos parámetros de validez diagnóstica como la sensibilidad y la especificidad, mientras que los costos se circunscribieron a insumos y salarios. Estos hallazgos ponen de manifiesto la baja aplicación de los recursos teóricos y metodológicos de la economía de la salud en el diagnóstico de las geohelmintiasis.
Conclusión: Las evaluaciones económicas de pruebas para el diagnóstico de geohelmintos y parásitos intestinales en general son exiguas en el ámbito mundial y los pocos estudios disponibles presentan limitaciones de validez interna y externa que impiden la aplicación de sus resultados a otros ámbitos.

Palabras clave: Evaluación económica, costo-efectividad, geohelmintos, pruebas diagnósticas, revisión sistemática

Cost-effectiveness of diagnostic tests in soil-transmitted helminth infections: a systematic review of literature

Abstract

Introduction: The tests for diagnosis of the geohelmintiasis are diverse, with multiple studies that demonstrate heterogeneity in its diagnostic validity and few about its cost-effectiveness.
Objective: To systematize the economic evaluations on the tests applied in the diagnosis of geohelminths.
Methods: Systematic review in six databases with 24 search strategies. A protocol for the selection of the studies was applied, guaranteeing completeness, reproducibility and evaluation of methodological quality. Qualitative synthesis of the information was made.
Results: In the scientific literature there are four complete economic evaluations (cost-effectiveness studies) for the diagnosis of geo-helminths; in these, five tests were evaluated in 247 adults and 6,708 children. In the health outcomes the studies employed the proportion of positive patients and some diagnostic validity parameters such as sensitivity and specificity, while costs were limited to inputs and salaries. These findings show the low application of the theoretical and methodological resources of health economics in the diagnosis of soil-transmitted helminth infections.
Conclusion: The economic evaluations of tests for the diagnosis of geohelminths and intestinal parasites in general are meager worldwide and the few available studies have limitations of internal and external validity that prevent the application of their results to other areas.

Keywords: Economic evaluation, cost-effectiveness, geohelminths, diagnostic tests, systematic review.

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Introducción

Los helmintos trasmitidos por el suelo (geohelmintos) son los causantes de las parasitosis intestinales más comunes en el mundo; ocasionadas principalmente por *Ascaris lumbricoides, Trichuris trichiura*, uncinarias (*Ancylostoma duodenale* y *Necator americanus*) y *Strongyloides stercoralis*. Según la Organización Mundial de la Salud (OMS), cerca de 1.500 millones de personas están infectadas por geohelmintos (24% de la población mundial) con más de 267 millones de niños en edad preescolar y más de 568 millones en edad escolar viviendo en zonas con intensa transmisión de estos parásitos. Afectan principalmente las regiones más pobres, ampliamente distribuidas en zonas tropicales y subtropicales como África subsahariana, América, China y Asia oriental. En las Américas, las geohelmintiasis están en toda la región y se estima que una de cada tres personas está infectada[1].

Los geohelmintos en estadio adulto viven en el intestino humano donde excretan miles de huevos o larvas a través de las heces de los infectados, contaminan el suelo y agua de las regiones con sistemas de saneamiento inadecuado, infectando otras personas que consumen dicha agua o alimentos, o a través de la piel (uncinarias y de *S. stercoralis*). Aunque se presentan en toda la población, los grupos más vulnerables son los niños en edad preescolar y escolar, así como las gestantes, presentándose la mayor incidencia en niños de 5 a 15 años. En este grupo, las infecciones graves pueden causar manifestaciones intestinales (diarrea y dolor abdominal), malnutrición, malestar general y debilidad, problemas de desarrollo cognitivo, retardo del crecimiento, anemia, y en infecciones intensas, obstrucción intestinal que requiere tratamiento quirúrgico[2,3].

Lo expuesto evidencia que este grupo de infecciones constituye un problema clínico, epidemiológico y de salud pública, principalmente en países de bajos ingresos donde existe déficit de atención sanitaria, infraestructura de saneamiento inadecuado, consumo de agua no potable, hacinamiento y altos índices de pobreza[1-3].

Por otra parte, las decisiones clínicas y de salud pública frente a múltiples problemas, incluidas las parasitosis, se basan en los resultados de las ayudas de laboratorio, siendo las pruebas diagnósticas una de las herramientas que más información aportan en la etiología y el perfil epidemiológico de muchas enfermedades. Por tanto, es imperativo conocer la correlación real entre los resultados de dichas pruebas y el estado patológico del paciente; en este sentido, los estudios de evaluación diagnóstica tienen alta relevancia ya que aportan evidencia científica sobre la validez, la eficiencia y el desempeño de las pruebas en la práctica clínica y en programas de tamización[4].

No obstante, el déficit en recursos económicos obliga a los sistemas de salud a implementar pruebas diagnósticas válidas, cuya aplicación sea factible frente a los recursos económicos existentes. En este orden de ideas, los estudios de costo-efectividad correlacionan los costos con los beneficios obtenidos en la implementación de tecnologías en salud, brindando evidencia científica que permita mejorar el bienestar de la población y optimizar los recursos, bajo el imperativo de lograr la eficiencia en la asignación de los recursos del sector salud. En el caso concreto del diagnóstico de las geohelmintiasis, los estudios de costo-efectividad permiten dar solución a posibles disyuntivas entre utilidad diagnóstica y costos de la implementación de las pruebas[5,6].

En este contexto, el diagnóstico de la parasitosis intestinal en general, y las geohelmintiasis en particular, se realiza mediante examen directo de las heces, aunque se recomiendan métodos de concentración que aumentan la sensibilidad hasta en un 30% y facilitan la observación de huevos de helmintos. La técnica más utilizada es la sedimentación de Ritchie modificada (formol-eter), por estar disponible en los laboratorios de todos los niveles de atención y ser efectiva en su diagnóstico; inclusive por encima de técnicas de flotación como el método de Willis (cloruro de sodio) y el método de Faust (sulfato de zinc), dado que en éstas, el peso de los huevos pueden provocar su pérdida en el procesamiento de la muestra, dando lugar a falsos negativos[7,8].

Específicamente para *Strongyloides stercoralis* se realiza coprológico directo o aislamiento de larvas con la técnica de Bearmann, método de Harada Mori y aislamiento en placa de agar[9]. Un metanálisis sobre métodos diagnósticos para este agente reportó que el cultivo en placa de agar es la técnica con mejor validez, desempeño, eficacia y rendimiento diagnóstico, seguido de la técnica de Baermann; mientras que el examen directo presentaba baja capacidad para discriminar individuos infectados y sanos[10]. En el caso de geohelmintos como *Ascaris lumbricoides,* uncinarias y *Trichuris trichiura*, además de determinar su presencia o ausencia, se realiza cuantificación de la carga parasitaria mediante el recuento de huevos con la técnica de Kato Katz recomendada por la OMS, por ser un método sencillo y no necesitar equipos sofisticados. Sin embargo, presenta baja sensibilidad si se analiza una sola muestra, por lo que es necesario realizar lecturas por duplicado y recolección de muestras en días consecutivos, aumentando sus costos[11,12].

En adición a lo anterior, se han desarrollado otras técnicas como el FLOTAC que, comparado con Kato Katz, sedimentación con éter y el cultivo en placa de agar, tiene una mayor sensibilidad para la detección de helmintos, aunque presenta recuentos más bajos de huevos, requiere equipos que restringen su uso a algunos laboratorios y mayor cantidad de reactivos que aumentan su costo. El mini FLOTAC supera algunas limitaciones de la técnica original y permite su uso en todos los laboratorios[12,13]. Una investigación previa en el 2013 reportó una mayor sensibilidad del mini FLOTAC (90%) para el diagnóstico de helmintiasis, en comparación con la técnica de concentración formol-eter (60%) o el examen directo (30%), mientras que otro estudio comparó dicho mé-

todo con la técnica de Kato Katz y el examen directo, encontrando mayor sensibilidad para el diagnóstico de uncinarias, sin diferencias estadísticamente significativas en el conteo de huevos de helmintos frente al Kato-Katz[14,15].

Lo expuesto evidencia una alta diversidad de pruebas disponibles para el diagnóstico de las geohelmintiasis y heterogeneidad en su validez diagnóstica según especie infectante, estadio evolutivo, experiencia del personal que realiza el diagnóstico, el tiempo y forma de conservación de la muestra, el método de recolección que utiliza el paciente, entre otros factores. Este contexto se complejiza, al tener presente que en la actualidad es necesario que las pruebas de detección de los parásitos, además de presentar validez diagnóstica, demuestren utilidad o eficiencia económica de acuerdo a su costo y beneficios en salud obtenidos mediante su implementación[16,17]. Pese a la relevancia de las evaluaciones económicas en los programas de tamización de geohelmintiasis, las publicaciones en este campo son exiguas.

El objetivo de este estudio fue sistematizar las evaluaciones económicas sobre las pruebas aplicadas en el diagnóstico de geohelmintos, publicadas en la literatura científica mundial.

Material y métodos

Tipo de estudio. Revisión sistemática de la literatura.

Protocolo de búsqueda y selección de estudio

Identificación o búsqueda de información: Se realizó una búsqueda de artículos originales publicados en las bases de datos Pubmed, Scielo, Science direct, en la Biblioteca Cochrane para revisiones sistemáticas (EBM Reviews-Cochrane Database of Systematic Reviews) y en las bases de datos especializadas para evaluaciones económicas Health Technology Assessment y NHS Economic Evaluation Database. Se aplicaron las siguientes estrategias de búsqueda: cost effectiveness AND kato katz, cost effectiveness AND coproscopic techniques, cost effectiveness AND parasitic techniques, cost effectiveness AND direct examination parasitic techniques, cost effectiveness AND Ritchie concentration method, cost effectiveness AND formol ether concentration, cost effectiveness AND faecal flotation techniques, cost effectiveness AND faecal Faust method, cost effectiveness AND Zinc sulphate flotation, cost effectiveness AND Willis flotation, cost effectiveness AND Baermann technique, cost effectiveness AND agar plate culture method, cost effectiveness AND Harada Mori, cost effectiveness AND Helminths, cost effectiveness AND Ascaris, cost effectiveness AND Strongyloides, cost effectiveness AND Trichuris, cost effectiveness AND Ancylostoma, cost effectiveness AND Necator, cost effectiveness AND hookworm, economic evaluation AND helminth, cost assessment AND helminth, cost-utility AND helminth, cost-benefit AND helminth.

Tamización o aplicación de los criterios de inclusión: Estudios originales, en humanos, con términos de búsqueda en título, resumen o palabras clave, estudios de evaluación económica (parcial o completa) de pruebas diagnósticas en geohelmintos, sin restricción por año de publicación ni idioma. En esta etapa se realizó la eliminación de los artículos duplicados en EndNote Web.

Elección o aplicación de los criterios de exclusión: Estudios no disponibles en las bases de datos y sin respuesta de autores ante su solicitud, evaluaciones económicas que no hacen explícita la perspectiva de análisis (institucional o social), los costos incluidos ni las medidas de efecto en salud evaluadas, evaluaciones económicas de programas de control o tratamiento antihelmíntico, estudios de economía de la salud en otros parásitos (no intestinales), estudios de prevalencia o sobre el riesgo de infección.

Inclusión: Los estudios que cumplían los criterios de inclusión fueron leídos en su totalidad para determinar el número de investigaciones que se incluirían en la síntesis cualitativa, posterior a la aplicación de los criterios de exclusión. Asimismo, se determinaron las variables a analizar en cada estudio: autor principal, año publicación, tipo de evaluación, lugar de estudio, población, prueba diagnóstica analizada, tipo de costeo, medida de efecto, fuentes de información usadas para el costeo y los desenlaces en salud.

Reproducibilidad y evaluación de la calidad

Se realizó una búsqueda por sensibilidad, realizando una inicial sin límites y posteriormente aplicando los filtros propios de cada base de datos para disminuir la cantidad de artículos identificados que se podría importar desde el programa EndNote web; esto con el fin de obtener el mayor número de estudios posibles indizados en las bases de datos bibliográficas y garantizar la exhaustividad del protocolo. La búsqueda de estudios, así como la extracción de la información la realizaron dos investigadores de forma independiente para garantizar la reproducibilidad en estas dos etapas. Las discrepancias se resolvieron por consenso. En la evaluación de la calidad metodológica de los estudios se aplicaron las recomendaciones de la guía ISPOR (International Society for Pharmacoeconomics and Outcomes Research) CHEERS (Consolidated Health Economic Evaluation Reporting Standards) 2013[18].

Resultados

Con la aplicación de las 24 estrategias de búsqueda se identificaron 24.996, de los cuales se tamizaron 134 y se incluyeron cuatro estudios después de aplicar el protocolo de búsqueda y selección de los artículos (Figura 1).

Un estudio se publicó en 1993 y los tres restantes entre 2010 y 2017; la mayoría de las investigaciones se realizaron en población escolar, los cuatro estudios correspondieron a evaluaciones económicas de costo-efectividad en las cuales se calcularon costos y desenlaces en salud como proporción de pacientes diagnosticados o validez de las pruebas diagnósticas; siendo Kato Katz y FLOTAC las pruebas más estudiadas (Tabla 1).

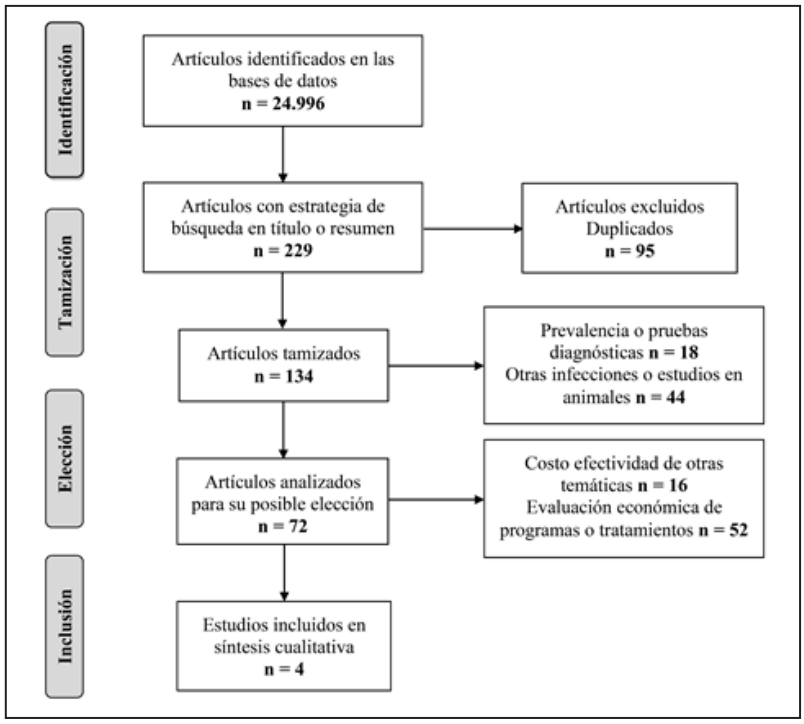

Figura 1. Flujograma de selección de los estudios.

Tabla 1. Descripción de los estudios incluidos en la revisión sistemática.

Estudio	Año	País	Población	Prueba diagnóstica
Kaminsky R[19]	1993	Honduras	427 Adultos	Baermann, Directo, Cultivo en agar
Speich B [12]	2010	Tanzania	1.066 Escolares	Kato Katz, FLOTAC
Assefa L [20]	2014	Kenia	657 Escolares	Kato Katz, miniFLOTAC
Liu C [21]	2017	China	4.985 Escolares	Kato Katz

En el estudio de Kaminsky[19] en 1993, se evaluaron los costos y la efectividad del frotis directo, el método de Baermann y el cultivo en agar para la detección de *Strongyloides stercolaris* en 427 muestras, concluyendo que Baermann fue 3,6 veces más eficiente para la detección del agente que el frotis directo, el cultivo en agar aumentó la eficiencia sobre Baermann 0,8 veces, el uso simultáneo de Baermann y el cultivo en agar resulta 6,7 veces más eficiente frente al directo. En relación con los costos de los insumos, fue más económico el frotis directo, y 15 veces más costoso el cultivo.

En Tanzania, Speich B y colaboradores[12] en el 2010 compararon los costos del diagnóstico de geohelmintos con Kato Katz y FLOTAC, mediante el análisis de heces de 1.066 niños, de forma individual y por duplicado. Encontraron que la sensibilidad de Kato Katz por duplicado para la detección de huevos de *T. trichiura*, uncinarias y *A. lumbricoides* fue de 88%, 81% y 68%, respectivamente, frente a 95%, 54% y 88% por FLOTAC. Mediante la estimación de costos debido a salario, materiales e infraestructura, encontraron que Kato katz individual y por duplicado tiene un costo de US$1.73 y US$2.06, respectivamente; mientras que analizar una vez una muestra por FLOTAC cuesta US$ 2.35 y por duplicado US$ 2.83. Los autores concluyeron que el mayor costo de FLOTAC se debe principalmente al largo tiempo requerido en la preparación y examen microscopico de la muestra.

Posteriormente, Assefa L y colaboradores[20], en 2014 evaluaron la costo-efectividad de Kato Katz y miniFLOTAC en el diagnóstico de geohelmintiasis en general, mediante el análisis de 525 muestras recolectadas un día y 132 muestras recolectadas en dos días consecutivos, en escuelas y hogares de niños escolares. Reportaron una sensibilidad de 52% y 76,9% para Kato katz cuando se analiza una muestra y dos muestras en días consecutivos, respectivamente; y de 49,1% y 74,1% para miniFLOTAC. Asimismo, estimaron la especificidad, valores predictivos, precisión y correlación entre los métodos, encontrando que la validez y el desempeño diagnóstico son similares en ambas técnicas. Mediante la estimación de costos debido al personal, materiales, transporte e instalaciones, estimaron tres resultados en cada escenario:

- En el análisis de muestra única, el costo por muestra examinada para Kato katz fue US$ 10.14 en las muestras de las escuelas, US$ 11.99 en las recolectadas en los hogares y para muestra en dos días consecutvos en las escuelas fue de US$ 20.28; para miniFLOTAC fue de US$ 10.14, US$ 11.99 y US$ 26.22, respectivamente.

- El costo por caso de infección detectado para Kato katz fue de US$ 178.66 con muestra única recolectada en el hogar, US$ 128.24 con muestra unica recolectada en la escuela y US$ 124.65 con dos muestras en días consecutivos; y para miniFLOTAC US$ 234.53, US$ 152.04 y US$ 158.31, respectivamente.

- El costo por infección correctamente clasificado para Kato katz fue de US$ 12.84 con muestra única recolectada en el hogar, US$ 15.18 con muestra unica recolectada en la escuela y US$ 25.68 con dos muestras en días consecutivos; para miniFLOTAC US$ 16.64, US$ 18.98 y US$ 33.27, respectivamente.

Los autores concluyen que la costo-efectividad fue mejor en Kato Katz para los tres escenarios.

La ultima evaluación económica identificada en esta revisión, se publicó en 2017, donde Liu C y colaboradores[21], como un objetivo secundario en su estudio de prevalencia de geohelmintos, evaluaron la costo-efectividad de recolectar una muestra fecal adicional, para el diagnóstico de geohelmintos mediante Kato katz. En éste, reportan que recoger la segunda muestra cuesta US$4,60 y en general, los resultados sugieren que la inversión de 31% más de capital en la recogida de muestras de heces evita una subestimación de la prevalencia en alrededor de 21%, mejorando la sensibilidad diagnóstica del método Kato Katz.

La calidad metodológica de los estudios fue similar, aunque sólo Speich B[12] y Assefa L[20] hacen alusión a la forma de establecer el precio o la tarifa de los insumos requeridos en las diferentes pruebas, desde la perspectiva del proveedor, el gobierno, opiniones de expertos y mediciones de tiempos para los salarios. Asimismo, estos dos estudios fueron los únicos que aplicaron análisis de sensibilidad para evaluar los escenarios en que los resultados de costos y efectos en salud podría extrapolarse; mientras que los estudios de Kaminsky y Liu presentaron una baja calidad metodológica en general, con limitaciones que impiden la generalización de sus resultados (Tabla 2).

Tabla 2. Evaluación de la calidad metodológica de los estudios incluidos.

Criterio de calidad metodológica CHEERS	Estudio incluido en la revisión			
	Kaminsky	**Speich**	**Assefa**	**Liu**
Título y Resumen	Si	Si	Si	Si
Introducción	Si	Si	Si	Si
Población de estudio	Si	Si	Si	Si
Perspectiva del estudio	Si	Si	Si	Si
Comparador	Si	Si	Si	Si
Horizonte temporal	Si	Si	Si	Si
Tasa de descuento	Si	Si	Si	Si
Selección del desenlace en salud	Si	Si	Si	Si
Medición de la efectividad	Si	Si	Si	Si
Estimación de recursos, costos y precio	No	Si	Si	No
Elección del modelo de decisión	No	Si	Si	No
Supuesto del modelo y método analítico	No	Si	Si	No
Resultados de los parámetros	No	Si	Si	No
Costos y efectividad incremental	No	Si	Si	No
Análisis de incertidumbre o sensibilidad	No	Si	Si	No
Discusión de resultados, generalización y limitaciones	No	Si	Si	Ni
Fuente de financiación	No	Si	No	No
Conflicto de intereses	No	Si	No	No

Discusión

En esta revisión sistemática se analizaron cuatro evaluaciones económicas completas, en la modalidad de estudios de costo-efectividad, con cinco pruebas usadas para el diagnóstico de geohelmitos, en 247 adultos y 6.708 niños. En los desenlaces en salud empleados se encontraron la proporción de pacientes positivos y algunos parámetros de validez diagnóstica como la sensibilidad y la especificidad, mientras que los costos se circunscribieron a insumos y salarios. Estos hallazgos ponen de manifiesto la baja aplicación de los recursos teóricos y metodológicos de la economía de la salud en el diagnóstico de estos agentes.

En relación con los desenlaces en salud hallados en esta revisión (proporción de pacientes positivos, sensibilidad, especificidad), se concluye que éstas resultan insuficientes dado que no dan cuenta de todos los parámetros necesario para determinar la reproducibilidad, la validez, el desempeño y la eficacia de las pruebas diagnósticas, como ejes para una buena evaluación económica, que brinde mayor eficiencia en la gestión de los recursos destinados al diagnóstico y tamización de las geohelmintiasis[22].

La reproducibilidad alude el grado de concordancia entre dos o más pruebas, es decir, no evalúa la validez de las observaciones respecto a un estándar de referencia, sino cuán acordes están entre sí observaciones sobre el mismo fenómeno[23]. Por su parte, la validez es la capacidad de la prueba de cumplir con su objetivo, es decir, medir lo que debe medir, determinada por la *sensibilidad*, que es la capacidad de detectar la enfermedad (probabilidad de que una persona enferma tenga un resultado positivo) y la *especificidad*, que es la capacidad de detectar sanos (probabilidad de que una persona sin la enfermedad tenga un resultado negativo). Tanto la sensibilidad como la especificidad reflejan características intrínsecas de las pruebas, sin verse afectados por la prevalencia de la enfermedad; aun así, tienen poca utilidad clínica a la hora de tomar una decisión frente a un resultado obtenido, por lo que resulta necesario evaluar otros parámetros como la seguridad y la eficiencia[24].

El desempeño o seguridad es la capacidad de la prueba de predecir la ausencia o presencia de la enfermedad dado un resultado de laboratorio; está determinada por el *valor predictivo positivo*, que es la probabilidad que un paciente con un resultado positivo esté realmente enfermo y el *valor predictivo negativo* o probabilidad que un resultado negativo corresponda a un verdadero sano. Estos parámetros son útiles para evaluar la prueba en el ámbito clínico pero se ven

afectados por la prevalencia de la enfermedad en la población [25]; por tanto, no permiten la comparación de diferentes pruebas diagnósticas o extrapolar resultados de varios estudios, haciendo necesario el uso de otros parámetros que no estén afectados por la prevalencia y que dan cuenta de la eficiencia pronóstica de la prueba[24].

La eficiencia pronóstica o cocientes de probabilidad son independientes de la prevalencia de la enfermedad y tiene utilidad clínica. Los *cocientes de probabilidades positivo* y *negativo* miden cuánto más probable es un resultado positivo o negativo según la presencia o ausencia de enfermedad, respectivamente[25]. También se calcula la razón de odds diagnóstica que es la fuerza de asociación entre el resultado de la prueba y la enfermedad, es la razón entre la probabilidad de estar enfermo si la prueba es positiva o sano si la prueba es negativa; sin embargo, al obtener un único valor se pierde el valor relativo de los parámetros comparados, por ejemplo esta razón en una prueba con alta sensibilidad y baja especificidad puede ser igual si sus valores se invierten[26].

En adición, se determina los índices de exactitud diagnóstica mediante un análisis de curvas ROC (Receiver Operating Characteristic), una representación gráfica de la sensibilidad frente a la especificidad, que unifica el proceso de evaluación de las pruebas, determinando el punto de corte en el que se alcanza la sensibilidad y especificidad más alta (índice de Youden) y comparando la capacidad discriminativa entre varias pruebas o varios estudios en el caso de los metanálisis[22].

Por otra parte, en esta revisión todos los estudios correspondieron a evaluaciones de costo-efectividad, los cuales proporcionan información relevante al momento de incorporar las pruebas diagnósticas a los planes de beneficios y los servicios de salud, en la medida que este tipo de análisis revela el costo aproximado que tendría el mejoramiento de la validez, el desempeño, la eficacia pronóstica y la exactitud de las pruebas de detección de parásitos intestinales, y cómo éstos aumentarían la eficiencia de las intervenciones derivadas de un diagnóstico oportuno[27].

El análisis de costo-efectividad determina los costos en términos monetarios y los beneficios obtenidos se estiman en las unidades naturales que se utilizan en la práctica clínica como los parámetros diagnósticos descritos. De esta forma se establece como principal ventaja sobre otras evaluaciones económicas, el hecho de valorar indicadores "*naturales*" relacionados con la calidad de vida humana y la efectividad de los sistemas de salud[29,30]. Dado que los recursos en salud son muy limitados, este tipo de evaluación económica ofrece otra ventaja en el momento de determinar qué tecnologías tienen mayor prioridad para la población, ya que compara diferentes intervenciones (procedimientos rutinarios y nuevas alternativas diagnósticas o terapéuticas) y permite ordenarlas de acuerdo a la relación existente entre su coste y su efectividad (o ganancias en salud)[5,28,29,31].

Sin embargo, se debe precisar que la estimación de las razones de costo efectividad en los estudios de esta revisión presentaron múltiples limitaciones metodológicas en la elección del parámetro de validez diagnóstica, la especificación de los costos incluidos, los análisis de sensibilidad probabilística de la evaluación económica, entre otras que impiden la extrapolación de los resultados, al tiempo que evidencian la necesidad de desarrollar evaluaciones económicas en cada lugar y población de interés. En este caso, resulta importante determinar la razón de costo-efectividad como base de la estimación de la inversión monetaria por parte del sistema de salud para mejorar el diagnóstico de las enfermedades parasitarias, principalmente las geohelmintiasis; dado que se trata de infecciones consideradas por la OMS como Enfermedades Tropicales Descuidadas (ETD) y que, pese a que son un problema de salud pública en países de bajos ingresos, los gobiernos les prestan poca atención y la inversión económica frente a éstas es deficiente[27].

Entre las limitaciones de la revisión destaca el bajo número de estudios disponibles en la literatura científica, la baja calidad metodológica de la mitad de las evaluaciones económicas sistematizadas, la heterogeneidad en las pruebas evaluadas y los agentes etiológicos reportados; aspectos que se constituyen en líneas de trabajo para estudios posteriores.

Conclusiones

Las evaluaciones económicas en pruebas para el diagnóstico de geohelmintos y parásitos intestinales en general, es exigua en el ámbito mundial y los pocos estudios disponibles presentan limitaciones de validez interna y externa que impiden la aplicación de sus resultados a otros ámbitos. Estudios posteriores deberían mejorar la elección de los parámetros de validez diagnóstica a incluir en la evaluación económica, así como el sistema de costeo; de tal forma que sus hallazgos contribuyan a distribuir mejor los recursos del sector, aumentar la cobertura de servicios e identificar las pruebas diagnósticas que maximizan los beneficios en salud generados con los recursos económicos disponibles[5,28]. Esto resulta de mayor importancia en las geohelmintiasis que representan una alta carga de morbilidad en los paises de bajos ingresos, y constituyen un evento relegado por los gobiernos, a pesar de que la OMS, las incluyó en los objetivos de eliminación para 2020 [36], mediante tratamiento periódico a la población con alta prevalencia, ya que el tratamiento adecuado ha demostrado ser eficaz y reduce las secuelas producidas por la enfermdad; siendo necesario realizar un diagnóstico oportuno y confiable, y seguimiento a los progrmas de control mediante pruebas válidas y seguras que permitan alcanzar los máximos beneficios en términos sanitarios y económicos.

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ARTÍCULO ORIGINAL

Comparación de la prueba tridimensional con PCR múltiple, para detección de carbapenemasas

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Resumen

Objetivo: Comparar la prueba fenotípica para la detección de carbapenemasas (Prueba Tridimensional -THT), con la prueba de biología molecular, reacción de polimerasa en cadena (PCR múltiple), para la detección de genes de resistencia.

Materiales y métodos: De un total de 118 aislamientos de cepas de bacterias Gram negativas, del programa de vigilancia de multirresistencia en un hospital de tercer nivel, fueron evaluadas para la detección de carbapenemasas por Test de Hodge tridimensional (THT) y PCR Múltiple. Se hicieron cálculos de sensibilidad, especificidad, VPP, VPN, índice de validez e índice de Youden (IY), con sus intervalos de confianza.

Resultados: Se observó que la prueba THT en comparación con PCR, presentó una sensibilidad de 98,41% (IC 95% 94,53 - 100), la especificidad fue de 83,64% (IC 95% 72,95 – 94,32) y los valores predictivos positivo y negativo fueron respectivamente 87,32 (IC 95% 78,88 – 95,77) y 97,87 (IC 95% 92,68 - 100). El índice de Youden fue 0,82 (IC 95% 0,72 – 0,92) y el índice de validez 91,53% (IC 95% 86,08 – 96,97)

Conclusión: La prueba tridimensional de Hodge (THT), para detección de resistencia a carbapenémicos, puede ser una prueba de rutina útil en el laboratorio para sugerir resistencia por carbapenemasas

Palabras clave: Carbapenémicos, beta-lactamasas, Reacción en Cadena de la Polimerasa, diagnóstico

---Título inglés---

Abstract

Objective: To compare the phenotypic test for detection of carbapenemases (Three-dimensional Test - THT), with molecular biology test (Multiplex PCR), for the detection of resistance genes.

Methods: A total of 118 isolates of Gram-negative bacteria strains from the multiresistant surveillance program at a third-level hospital were evaluated for the detection of carbapenemases by three-dimensional test (THT) and the molecular biology PCR Multiple method . We calculated sensitivity, specificity, PPV, NPV, validity index and Youden index (IY), with their confidence intervals.

Results: THT test compared to the multiple PCR test had a sensitivity of 98.41% (95% CI 94.53 - 100), specificity was 83.64% (95% CI 72.95 - 94 , 32) and positive and negative predictive values were respectively 87.32 (95% CI 78.88 - 95.77) and 97.87 (95% CI 92.68 - 100).

Conclusion: The Hodge three-dimensional test (THT), for detection of carbapenem resistance, may be a useful routine laboratory test to suggest resistance by carbapenemases.

Key words: Carbapenems, beta-lactamases, Polymerase Chain Reaction, diagnostic test

Introducción

En 2015 la Asamblea Mundial de la Salud aprobó el plan de acción global para enfrentar la resistencia a los antimicrobianos (RAM), que tiene entre sus objetivos fortalecer los conocimientos y la base de evidencia científica a través de la vigilancia y la investigación.[1] Según la Organización Mundial de Salud (OMS), la resistencia a antibióticos es un problema en aumento; y reporta que la resistencia de enterobacterias al tratamiento con carbapenémicos se ha diseminado mundialmente; lo que ha llevado a que en algunos países este grupo de antibióticos ya no sean eficaces en más de la mitad de los pacientes con infecciones por *Klebsiella pneumoniae*.[2] Por ello, las infecciones bacterianas a causa de enterobacterias resistentes a carbapenémicos, son de interés tanto desde el punto de vista clínico como en salud pública, debido

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a que actualmente los carbapenémicos hacen parte de los antibióticos de primera línea de tratamiento para infecciones severas por enterobacterias productoras de betalactamasas de espectro extendido (BLEES).[3]

Dada la relevancia del problema para la salud pública, la vigilancia de la RAM es una prioridad a nivel mundial, puesto que permite establecer las medidas de intervención de manera oportuna, así como orientar el tratamiento de los pacientes y definir estrategias para el control de la resistencia; por lo tanto el aumento de infecciones por microorganismos multirresistentes conlleva un reto en la rápida identificación de cepas resistentes, mediante métodos costo efectivos, como las pruebas fenotípicas; que permitan tomar decisiones seguras para los pacientes[4] y que sean una alternativa frente al mayor costo que demandan las pruebas de biología molecular.

Por ello, Colombia en el Plan Decenal de Salud Pública (PDSP), propone entre sus metas la disminución de la morbilidad, mortalidad y discapacidad general por enfermedades transmisibles, y entre sus objetivos plantea la vigilancia de las mismas, así como reducir la carga de Infecciones Asociadas a la Atención en Salud (IAAS), y contener la resistencia a los antimicrobianos.[5]

En la Vigilancia de la RAM, el Instituto Nacional de Salud (INS) reportó que en Colombia en el primer semestre de 2016 en los servicios de hospitalización diferentes a cuidados intensivos (no UCI), hubo aumento en la resistencia a carbapenémicos para *Klebsiella pneumoniae* en comparación con 2015; mientras que para *Escherichia coli y Enterobacter cloacae* la resistencia aumentó tanto en los servicios no UCI como en los UCI. Con base en estos hallazgos, el INS recomienda realizar pruebas de tamizaje como el test de Hodge Modificado (MHT por su sigla en inglés), así como las pruebas de sinergismo con ácido etilen-diamino-tetra-acético (EDTA) y ácido fenil-borónico (PBA), para fortalecer la capacidad de los laboratorios en la detección de mecanismos de resistencia.[6]

Aunque las pruebas de biología molecular son los métodos de referencia para la detección de genes de resistencia, las pruebas fenótipicas para el tamizaje como el Test de Hodge Modificado (MHT)[7] pueden ser útiles en lugares de recursos limitados, para la identificación de microorganismos productores de carbepenemasas (CPM), los cuales tienen capacidad de hidrolizar dichos antibióticos y casi todos los betalactámicos de uso clínico.[8] Entre las pruebas fenotípicas, se encuentra el THT, que permite la detección de bacterias Gram negativas (Enterobacterias y/o bacilos Gram negativos no fermentadores - BGNNF) productoras de carbapenemasas, y se ha utilizado en Colombia, por su bajo costo y facilidad de implementación.[9,10]

La prueba THT, es una variante del Test de Hodge, propuesta para la confirmación de betalactamasas de tipo AmpC en enterobacterias, con 2 variantes según la forma de obtención de las enzimas: En el método directo se aplica la suspensión de bacterias mientras que en el método indirecto se hace previamente la extracción de las betalactamasas a partir de la cepa, mediante 5 a 7 ciclos de congelación-descongelación, seguido de centrifugación para obtener el sedimento.[11,12] En ambos casos se puede aplicar la preparación de las colonias o el extracto enzimático sobre la superficie del agar o en una zona de incisión a partir del disco con antibiótico.[13,14,15]

Con el tiempo se han incorporado otras variaciones para la detección de CPM, tales como la utilización de un carbapenémico en lugar de Cefoxitina y utilización de otros métodos de lisis bacteriana de tipo mecánico o físico, tales como sonicación, ciclos de congelación - descongelación o el uso de buffer de lisis. Diferentes autores han utilizado la prueba THT para la detección de CPM en BGNNF, con resultados de sensibilidad *en Acinetobacter baumannii* del 99,1% (IC 95% 94,6%-100,0%) en la detección de enzimas tipo OXA-23-like. Del mismo modo, en *Pseudomonas aeruginosa* se han descrito resultados de sensibilidad del 75,0 al 100,0% y de especificidad desde 71,4% a 98,4%, además en este germen se ha reportado buena concordancia en comparación con PCR múltiple, para la detección de bla_{VIM}.[10]

El objetivo de este trabajo fue comparar la prueba THT para detección de carbapenemasas, con la prueba PCR múltiple (prueba de referencia), en cepas de bacterias Gram negativas, del programa de vigilancia de multirresistencia en un hospital de tercer nivel, con el propósito de aportar evidencia de la posible utilidad de este método como prueba de tamizaje, en la detección de resistencia a carbapenémicos.

Materiales y métodos

Se hizo un estudio descriptivo para comparar la pruebas THT y PCR múltiple en la detección de resistencia a antibióticos carbapenémicos, en bacterias Gram negativas de la colección de cepas del programa de vigilancia de multiresistencia bacteriana de un Hospital de tercel nivel de la cuidad de Medellín, aisladas de pacientes infectados entre 2012 y 2014. El estudio fue considerado sin riesgo, con base en lo establecido en la resolución 8430/1993 del Ministerio de salud.

Las cepas de bacterias fueron previamente caracterizadas por PCR múltiple, para detección de genes de resistencia bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{OXA-48}, bla_{OXA-23} y bla_{OXA-51}; según la técnica descrita por Poirel.[16] En la reacción de amplificación se utilizó un termociclador C1000Touch™ (BIO-RAD Laboratories, Inc., Francia). Los productos de PCR fueron visualizados en gel de agarosa al 1,5%, teñidos con bromuro de etidio. El patrón de Bandas fue analizado con Fotodocumentador (BIO-RAD Laboratories, Inc., Francia).

Procedimientos: A partir de las cepas previamente caracterizadas por PCR múltiple, para detección de genes de resistencia a carbapenémicos (método de referencia), el personal del laboratorio sin conocimiento de dichos resultados y previamente estandarizados en la prueba THT (prueba a eva-

luar), procesó las cepas como se describe a continuación: Se preparó una suspensión 0,5 McFarland de la cepa indicadora (*Escherichia coli* ATCC® 25922™) y se sembró en una caja de Muller-Hinton Agar™(Becton Dickinson®), se adicionó un disco de Imipenem con concentración de 10µg (bioMérieux Clinical Diagnostics[MR] - Marcy L´Etoile France) en el centro de la caja. A continuación se hizo una incisión de 5mm de profundidad a partir del disco, hasta el borde del medio de cultivo. De cada cepa en estudio, se tomaron 2 porciones de la colonia con asa calibrada de 10µL y re-suspendieron en un vial con 200 µL de buffer de lisis (B-PER™ Bacterial Protein Extraction Reagent -Thermo Scientific Rockford, Il USA), la mezcla se agitó con vortex por 1 minuto y luego se incubó a temperatura ambiente durante 30 minutos y se centrifugó a 13.000 g por cuatro minutos. A partir de la suspensión se dispensaron 15 a 20 µL en la incisión del agar y se incubó la caja a 37°C por 18 a 22 horas. Se utilizaron como controles de la prueba las siguientes cepas de referencia de la "American Type Culture Collection" (ATCC): *Klebsiella pneumoniae* ATCC® BAA-1705™ (expresa gen bla_{KPC}), como control positivo, y *Klebsiella pneumoniae subsp. pneumoniae* ATCC® 700603™ (expresa bla_{SHV-18} no productora de carbapenemasas), como control negativo.

En la identificación de los aislamientos y las pruebas de sensibilidad a carbapenémicos, se utilizó el Sistema Automatizado Vitek® Compact 2 (bioMérieux Clinical Diagnostics™ - Marcy L´Etoile France), según las recomendaciones del fabricante y acorde con estándares internacionales de control de calidad, del Instituto de estándares clínicos y de laboratorio - CLSI M100-S24.[17] En la interpretación de los resultados para el método de referencia, se consideraron positivos los aislamientos con presencia de al menos uno de los genes de resistencia evaluados; mientras que en la prueba THT, los resultados fueron positivos cuando se observó deformación del halo de inhibición en la intersección con la cepa en estudio y la observación del halo de inhibición sin deformidad fue interpretado como negativo.

En el análsis de los datos se utilizaron el programa SPSS® Stadistics 19,0 para el analísis univariado, y el Programa EPIDAT 3.1 para cálculos de sensibilidad y especificidad, el índice de Youden y los valores predictivos.

Resultados

Las cepas evaluadas provenían de aislamientos de pacientes infectados o colonizados, identificados como sospechosos de resistencia a antibióticos betalactámicos del grupo de los carbapenémicos, con base en los criterios CLSI M100-S24. La mayoría de los aislamientos provenían de hombres (66,1%), y por tipo de ubicación del paciente al momento de toma de la muestra, el 44,8% estaban en servicios de hospitalización diferentes de UCI/UCE, el 28,4% en UCI/UCE y el 26,7% en urgencias. El tipo de muestra y la edad de los pacientes se presentan en la tabla 1.

Tabla 1. Distribución del grupo de estudio según procedencia de los aislamientos.

Variable	Categoría	n	%
Edad (años)	<15	17	14,4
	15 a 24	11	9,3
	25-44	23	19,5
	45-64	51	43,2
	65 y más	16	13,6
Tipo de muestra	Orina	27	22,9
	Sangre	21	17,8
	Abdominales	19	16,1
	Piel y tej blandos	19	16,1
	Tejido osteomuscular	14	11,9
	Respiratorias	10	8,5
	Frotis rectal	8	6,8
Total		118	100

Las cepas aisladas correspondieron a *Klebsiella pneumoniae* en 53 casos (44,9%); *Pseudomonas aeruginosa*, en 46 (39,0%); *Enterobacter cloacae complex, en 12* (10,2%); *Acinetobacter baumannii, en 5 (*4,2%) y de otros aislamientos se presentaron 2 (1,7%).

En la evaluación de resultados de sensibilidad antibiótica de los aislamientos de bacterias portadoras de genes de resistencia para carbapenémicos, se observó sensibilidad reducida en comparación con aquellas sin producción de CPM.

En general los porcentajes de sensibilidad fueron más altos en los aislamientos sin CPM, excepto en Ceftazidima (CAZ), cuyo comportamiento fue igual en aquellos con y sin CPM. La mayor diferencia en sensibilidad se observó en trimetroprim sulfametoxazol, con alta sensibilidad en enterobacterias no productoras de CPM; mientras que la menor diferencia se encontró en colistina, figura 1.

Las cepas de *Pseudomonas aeruginosa* productoras de CPM no mostraron sensibilidad a ninguno de los betalactámicos evaluados, además en las otras clases de antibióticos la sensibilidad fue baja; excepto en el caso de colistina, figura 2.

En la prueba PCR múltiple, se observó que en 55 de los aislamientos (46,6%) no estaban presentes los genes de resistencia evaluados y en 63 (53,4%) si se detectaron; de estos últimos, la mayoría fue bla_{KPC} (79,4%), seguido de bla_{VIM} (11,1%) y $bla_{OXA-23/+OXA-51}$ (7,9%). Se presentó un caso de *Pseudomonas aeruginosa* con genes tipo bla_{KPC} y bla_{VIM} simultáneamente (1,5%). No se detectaron genes de resistencia tipo bla_{NDM} ni bla_{OXA-48}.

Por otro lado, los valores de concentración inhibitoria mínima (MIC) más altos para imipenem y meropenem se encontraron *en Klebsiella pneumoniae* y en *Enterobacter cloacae,* en los portadores de genes bla_{KPC}; mientras que en los negativos

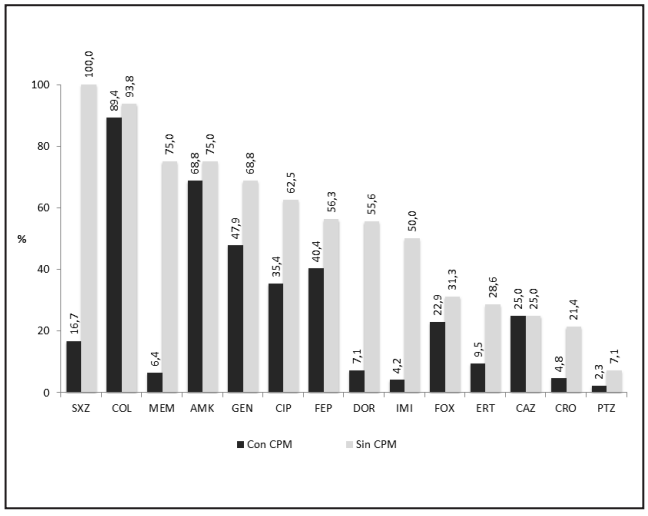

Figura 1. Distribución de la sensibilidad a los antibióticos, en los aislamientos de enterobacterias con y sin carbapenemasas (CPM)
CPM: carbapenemasa, SZX: Trimetoprim Sulfametoxazol, COL: Colistina, MEM: Meropenem, AMK: Amikacina, GEN: Gentamicina, CIP: Ciprofloxacina, FEP: Cefepime, DOR: Doripenen, IMI: Imipenem, FOX: Cefoxitina, ERT: Ertapenen, CAZ: Ceftazidima, CRO: Ceftriaxona, PTZ: Piperacilina-Tazobactam

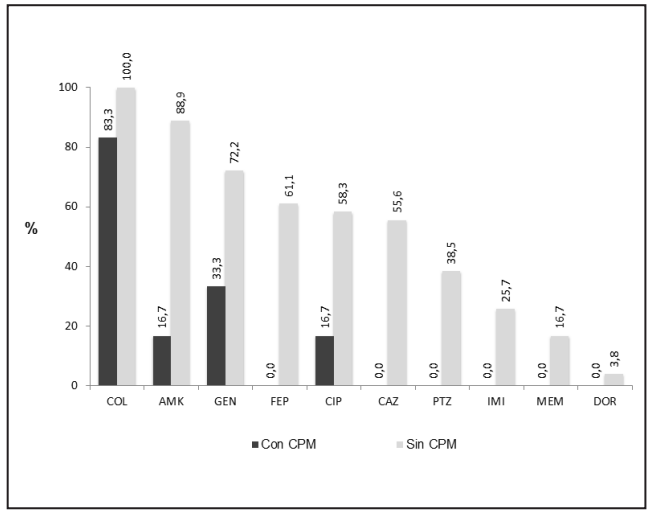

Figura 2. Distribución de la sensibilidad a los antibióticos, en los aislamientos de Pseudomonas aeruginosa con y sin carbapenemasas
CPM: carbapenemasa, COL: Colistina, AMK: Amikacina, GEN: Gentamicina, FEP: Cefepime, CIP: Ciprofloxacina, CAZ: Ceftazidima, PTZ: Piperacilina-Tazobactam, IMI: Imipenen, MEM: Meropenen, DOR: Doripenen

para carbapenemasas se observaron MICs más bajos, tabla 2. De los 118 aislamientos, 63 fueron positivos para los genes de resistencia evaluados con la prueba PCR múltiple, de los cuales 62 también fueron positivos por la prueba THT y uno resultó negativo (falso negativo), que correspondía a un aislamiento de hemocultivo identificado como *Pseudomonas aeruginosa* con carbapenemasa tipo VIM. Además, 9 casos que fueron negativos con prueba de biología molecular, presentaron resultado positivo con la prueba THT (probables falsos positivos), todos enterobacterias (6 *Enterobacter cloacae complex* y 3 d e *Klebsiella pneumoniae*), figuras 3, 4 y tabla 2.

Los hallazgos de la prueba THT, en comparación con la prueba PCR múltiple, mostraron una sensibilidad de 98,41% (IC 95% 94,53% - 100,00%), y la especificidad fue del 83,64 % (IC 95 %: 72,95% - 94,32%). Adicionalmente el índice de validez fue 91,53 (IC 95 %: 86,08 - 96,97), el índice de Youden 0,82 (IC 95 %: 0,72 - 0,92) y el coeficiente de Kappa de Cohen 0,828 (IC 95 %: 0,727 - 0,929), tabla 3.

Discusión

El mecanismo de acción de los carbapenémicos es similar al de otros betalactámicos, con algunas particularidades que les permiten un espectro más amplio y mayor estabilidad a la actividad hidrolítica en bacterias Gram negativas. En enterobacterias la resistencia se debe con más frecuencia a la producción de carbapenemasas y en menor proporción a la modificación en la afinidad de la PBPs, disminución de la permeabilidad de la membrana externa y/o la presencia de bombas de expulsión.[18] En *Pseudomonas aeruginosa* son más frecuentes los mecanismos intrínsecos por disminución de la permeabilidad, presencia de bombas de expulsión y sobre-expresión de enzimas modificadoras. La resistencia adquirida ocurre por carbapenemasas y/o betalactamasas de espectro extendido.[19] En *Acinetobacter baumannii* presenta con mayor frecuenta betalactamasas de la Clase D (oxacilinasas), seguido de bombas de expulsión y modificación de porinas.[20]

Tabla 2. Resultados de pruebas y concentración inhibitoria mínima (MIC) para Imipenem y Meropenem , según germen

Germen	THT		PCR	n	%	Rango MIC	
	Positiva	Negativa	Tipo de resistencia			Imipenem (ug/mL	Meropenem (ug/mL)
KPN	46	7	KPC Sin CPM	43 10	36.4 8.5	≤0.25 a >8 0.5 a 2	≤0.25 a >8 ≤0.25 a >4
ECL	10	2	KPC Sin CPM	5 7	4.2 5.9	8 a >8 ≤0.25 a 4	>8 ≤0.25 a >8
PAE	8	38	KPC y/o VIM Sin CPM	9 37	7.6 31.4	>8 <1 a >8	>8 1 a >8
ABA	5	0	OXA-23/OXA-51 Sin CPM	5 0	4.2 0.0	8 a >8 0	>8 0
Otros	2	0	KPC Sin CPM	1 1	0.8 0.8	4 ≤0.25	1 ≤0.25

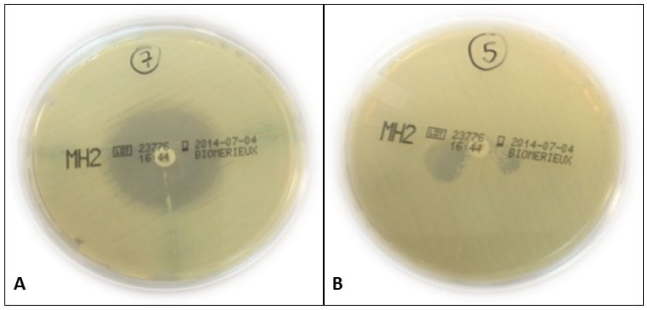

Figura 3. Hallazgos de la prueba Tridimensional en procedimientos de laboratorio en hospital de tercer nivel
A Prueba THT con Resultado negativo: sin producción de carbapenemasas, no se observa deformación del halo de inhibición
B Prueba THT con Resultado positivo: con producción de carbapenemasas, se observa deformación del halo de inhibición

Los métodos fenotípicos para detección de resistencia son los más utilizados de rutina en los laboratorios, entre ellos el test de Hodge modificado (THM), debido a su bajo costo y fácil montaje, además posee buena concordancia en enterobacterias, aunque presenta falsos positivos en aislamientos con enzimas tipo BLEES y AmpC; sin embargo no ha sido recomendado para BGNNF.[17] En los últimos años la atención se ha centrado en las pruebas de hidrólisis de carbapenémicos tales como Carba NP y su versión comercial más reciente: Rapidec Carba NP™ (bioMérieux Clinical Diagnostics - Marcy L´Etoile France), cuyos resultados dependientes del cambio de color de la reacción, pueden generar dificultades en la interpretación, además se han reportado limitaciones para detección de enzimas tipo OXA y GES.[21]

El mayor potencial para pruebas como THT puede estar en la detección de carbapenemasas en BGNNF. Este tipo de bacterias representan una creciente amenaza en la región, en especial *Acinetobacter baumannii* y *Pseudomonas aeruginosa*. En el primero el INS reportó una alta proporción de aislamientos no sensibles a carbapenémicos, de los cuales en 185 de 189 (98,4%) se detectaron genes de resistencia, identificados en su mayoría: 155 (82,8%) como productores de enzimas del grupo D de Ambler, y en alta proporción (75,5%) mediadas por genes tipo $bla_{OXA\text{-}23+OXA\text{-}51}$.[22] En este estudio el 100% de los aislamientos de *Acinetobacter baumanii* presentó resistencia a carbapenémicos, mediada por carbapenamasas tipo $bla_{OXA\text{-}23+OXA\text{-}51.}$ Desde el 2000 se ha reportado la producción de oxacilinasas (enzimas del grupo D de Ambler) como principal responsable de la resistencia a los carbapenémicos en *Acinetobacter baumanii*, desafortunadamente una limitación de diferentes métodos fenotípicos es su baja sensibilidad para la detección de enzimas de este tipo,[23] lo cual aumenta el riesgo de diseminación y presentación de brotes. En el presente estudio en método THT detectó todos los aislamientos de *Acinetobacter baumanii* que eran portadores de genes tipo $bla_{OXA\text{-}23+OXA\text{-}51.}$

La presencia de carbapenemasas en Colombia, en *Pseudomonas aeruginosa*, según los resultados de diferentes estudios se debe en primer lugar a genes tipo bla_{VIM}. El INS reportó este mecanismo en 527 de 1274 (41,4%), seguido de KPC en 276 (21,7%) y en tercer lugar la combinación de VIM+KPC en 81 (6,4%) y en un estudio de Ocampo y col encontraron bla_{VIM} en 14 aislamientos de *Pseudomonas aeruginosa* de los cuales 9 fueron positivas por THT, con 5 resultados falsos negativos. En este estudio, la prueba THT presentó concordancia del 0,91 (IC95% 0,78 a 1,00) frente a PCR múltiple en BGNNF al detectar 13 de 14 cepas productoras de CPM y un resultado falso negativo. En contraste en el estudio de Ocampo-Rios, realizado en aislamientos de *Pseudomonas aeruginosa*: de 20 productoras de CPM confirmadas por PCR múltiple, la prueba THT fue positiva en 15, con resultados de concordancia moderada. En otro estudio realizado en aislamientos clínicos provenientes de 14 hospitales de Colombia, Correa A y colaboradores, de un total de 151 aislamientos de *Pseudomonas aeruginosa* detectaron genes de resistencia bla_{KPC} o bla_{VIM} en 28, de los cuales 26 fueron positivos con la prueba THT, con resultados de Sensibilidad del 100% (IC95% 84-100), y Especificidad del 98,4 (IC95%: 93,8 a 99,7).[9,10,22]

Aunque en la literatura se han evaluado diferentes métodos fenotípicos en comparación con la detección de genes de resistencia y técnicas de secuenciación (métodos moleculares) para BGN, tanto enterobacterias como BGNNF, con el propósito de formular recomendaciones; los resultados sugieren que no existe un método fenotípico con potencial para detectar todos los tipos de CPM documentadas hasta el momento.[24,25]

En el caso particular de las enterobacterias el reto más importante es el riesgo de presencia de carbapenemasas en cepas con MICs en los rangos de sensibilidad con los puntos de corte actuales; en este estudio en 6 de 42 (9,5%) los MICs para meropenem en *Klebsiella pneumoniae* fueron menores o iguales a 2, este tipo de germen podría pasar inadvertido en los programas de vigilancia y ser la fuente de diseminación, incluso si se contara con pruebas moleculares en formato de PCR múltiple, cuya limitación es la incapacidad de detectar bacterias con nuevas mutaciones.[26] Este hallazgo también se ha observado en *Escherichia coli* productora de KPC y OXA-48, según lo reportado por investigadores del Laboratorio de Salud Pública de Ontario (Canadá), entre 14 a 20% de enterobacterias productoras de carbapenemasas se observan resultados con MIC clínico en el rango sensible.[27]

Tabla 3. Resultados de la comparación entre prueba tridimensional (THT) y PCR múltiple

Parámetro	Valor (%)	Intervalo de confianza 95%
Sensibilidad	98,41	94,53 – 100,00
Especificidad	83,64	72,95 - 94,32
VPP	87,32	78,88 - 95,77
VPN	97,87	92,68 - 100,00

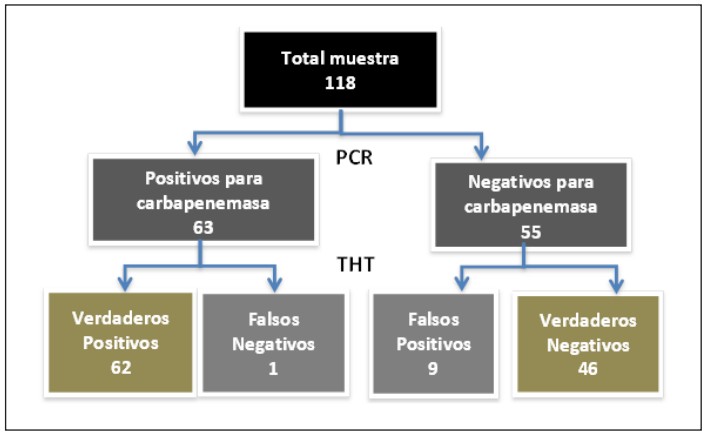

Figura 4. Resultados de la prueba de biología molecular (PCR múltiple) y la prueba Tridimensional (THT)
Distribución de los resultados de las pruebas de PCR Múltiple y THT

El panorama de la detección de resistencia en los bacilos Gram negativos no fermentadores presenta dificultades aún no resueltas, en primer lugar porque la mayoría de métodos fenotípicos disponibles no han sido recomendados en forma explícita en las guías CLSI para detección de carbapenemasas, en segundo lugar porque incluso métodos comerciales más recientes, tales como Rapidec-Carba pueden presentar falsos negativos en aislamientos de *Acinetobacter baumannii* con enzimas tipo OXA-48, OXA-23 y OXA-24,[28] o el método Carba NP, en el que también se reportan falsos negativos en carbapenemasas tipo VIM y KPC en cepas con fenotipo hipermucoide de *Pseudomonas aeruginosa*.[29] En este último germen se ha documentado también la amplia diversidad de mecanismos de resistencia diferentes a la producción de carbapenemasas como causa de resultados discrepantes frente a pruebas moleculares.

Lo antes expuesto, sugiere falta de acuerdo en los métodos de rutina a implementar en los laboratorios, especialmente en países como Colombia, en los que se dispone de pruebas moleculares para confirmación de carbapenemasas solo en laboratorios especializados, debido a su mayor costo y a las dificultades del sistema de salud vigente. En este contexto las pruebas fenotípicas con buen desempeño frente a los métodos moleculares, y con características como el costo menor e implementación simple, pueden ser una opción para la contención de la diseminación de resistencia.

En conclusión, dado que la prueba THT presentó buen desempeño para detección de CPM, tanto en enterobacterias como en BGNNF, en comparación con la prueba PCR múltiple; puede ser una alternativa en laboratorios de microbiología no especializados, dado su bajo costo y fácil implementación por la similitud técnica con el test de Hodge modificado utilizado de rutina por la mayoría de laboratorios, con la diferencia de la inoculación del extracto bacteriano en vez de la siembra directa de las colonias. En el presente estudio se preparó el extracto con el mismo buffer de extracción recomendado por CLSI para la prueba Carba NP, pero se pueden utilizar otros métodos, de tipo mecánico y/o físicos seguidos de centrifugación, utilizados en otras pruebas de rutina en microbiología. Los medios de cultivo, los materiales e instrumentos necesarios para esta prueba hacen parte de los elementos mínimos requeridos para implementación de Laboratorios de Microbiología en Colombia y como todo procedimiento de laboratorio, se requiere entrenamiento y estandarización de la técnica.

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ARTÍCULO ORIGINAL

Artemeter-Lumefantrine therapeutic efficacy, safety and plasma levels in patients with uncomplicated falciparum malaria from the Colombian Pacific region

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Abstract

Introduction: In Colombia, the published studies for the treatment of uncomplicated Plasmodium falciparum malaria with Artemether-Lumefantrine are scarce. The aim of the study was to evaluate the therapeutic efficacy and safety profile of this combination.

Methods: A clinical trial was performed in adults with uncomplicated *P. falciparum* malaria using the 28-day World Health Organization validated protocol. Patients received supervised antimalarial treatment and the primary efficacy endpoint was the clinical and parasitological response. Safety was assessed through adverse events surveillance and plasmatic levels of antimalarial drugs were measured.

Results: 88 patients were included. Adequate clinical and parasitological response rate of 100% on day 28 was achieved in 84 patients, diagnosed by thick blood smear examination. There were four parasitological therapeutic failures (5%) detected by polymerase chain reaction.

Discusion: Therapeutic efficacy similar to previous studies was established with a slight increase in therapeutic failure. The serum levels of the antimalarials were adequate and the few cases of therapeutic failure were not related.

Conclusion: Treatment of uncomplicated *P. falciparum* malaria with Artemeter-Lumefantrine was effective and safe in the study population. All patients reached adequate plasma concentrations of the drugs; therapeutic failures were not associated with low blood levels of the drug.

Keywords: Malaria, Falciparum, Artemeter-Lumefantrine, Efficacy, Safety, Plasmatic levels,

Eficacia terapéutica, seguridad y niveles plasmáticos del Artemeter-Lumefantrine en pacientes con malaria falciparum no complicada de la región del Pacifico Colombiano

Resumen

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Introduction

Malaria is widespread in tropical areas worldwide and has a high morbidity burden. The emergence of parasite resistance to antimalarial medicines is considered part of the challenges impeding countries' abilities to advance towards elimination; these include lack of sustainable funding, risks posed by conflict in malaria endemic zones, and mosquito resistance to insecticides[1].

Approximately 216 million malaria cases were reported globally in 2016 and the disease was considered endemic in 91 countries and territories. For this same year, the most prevalent species in Colombia were *P. falciparum* and *P. vivax* with 57,515 cases confirmed with microscopy and 5,655 with rapid diagnostic tests, and a total of 18 reported deaths in 2015[1]. Previous national data about the infections showed a higher incidence of *P. vivax* infections over *P. falciparum* infections from years 2000 to 2013[2]. In the last years the proportion of *P. falciparum* cases reported is increasing; 50.6% were *P. falciparum* infections between 40,763 cases in 2014, and 59.8% between 51,594 cases in 2015[2]. In the recent years, malaria transmission was focalized in Chocó region.

Artemisinin Combination Therapies (ACTs) have been integrated into the recent success of global malaria control, and protecting their efficacy for the treatment of *P. falciparum* malaria is a global health priority. In Colombia, antimalarial treatment for uncomplicated falciparum malaria is based on ACTs in accordance with the World Health Organization recommendation for regions with resistance to antimalarials. First line treatment is based on fixed-dose co-formulation tablets of Artemeter-Lumefantrine (AL)[3]. The majority of the studies analyzing efficacy of this antimalarial regimen are conducted in high transmission regions, and selection of mutations that could possibly confer *P. falciparum* resistance to ACT has been reported both in vivo and ex-vivo[4-8]. The advice for government health programs is to closely monitor parasite genotypic, phenotypic and clinical dynamics of *P. falciparum* infections in response to ACTs, despite its continued efficacy.

Although multidrug resistance, including Artemisinin, has been reported in five countries of the Greater Mekong sub region (GMS)[1] in America efficacy studies are scarce. A study conducted in Suriname in 2011 detected a 9% treatment failure rate with AL[7]. Other studies conducted in the period 2010–2016 showed effective first-line treatment for *P. falciparum* (treatment failure rates 0%) (1). In Colombia a study in 2008 with Artesunate-Amodiaquine (AS-AQ) regimen vs. AL regimen, found no early treatment failure (ETF) or late clinical failures (LCF) in either group and one late parasitological treatment failure (LPTF) in the AL group [9]there are no published studies for the treatment of uncomplicated Plasmodium falciparum malaria comparing artemisinin combination therapies. Hence, it is intended to demonstrate the non-inferior efficacy/safety profiles of artesunate + amodiaquine versus artemether-lumefantrine treatments. METHODS: A randomized, controlled, open-label, noninferiority (Delta</=5%.

Considering the acute and chronic morbidity of falciparum malaria, the national epidemiologic shift towards *P. falciparum* predominance seen in the last years, the lack of studies about AL efficacy in Latin America and reports of emergent resistance around the globe[10-12] it is pertinent to further study the efficacy of the first line treatment used in the country. The objectives of this study were to assess the parasitological and clinical efficacy in patients with non-complicated *P. falciparum* malaria treated with Artemeter-Lumefantrine, to evaluate the safety of this combination and the correlation of the treatment response with both blood antimalarial drug concentrations.

Patients and methods

Study design and site

This was a therapeutic efficacy study with an one-arm prospective evaluation of the clinical and parasitological response to supervised treatment for uncomplicated Plasmodium falciparum malaria. It was based on the 2009 WHO therapeutic efficacy studies protocol[13].

The study was conducted in the municipality of Quibdó, Chocó (Lat. 05°41'41"N; Long. 76°39'40"W); recruitment during 2013 (8 months), laboratory procedures during 2013-January 2014. Quibdó it is located on the Atrato River, in a warm and humid climate between 43 and 53 meters above sea level with an average temperature of 28°C, a total area of 3.337,5 km^2 and near 100,000 inhabitants, and 35% living in rural locations. Malaria is endemic in the state with a 2013 Annual Parasitic Index of 28.4 and 13,095 reported cases, 4,232 of them in the municipality of Quibdó[14].

Study population and inclusion criteria

Patients with uncomplicated falciparum malaria seeking care at the malaria diagnostic post (outpatient service) in the Ismael Roldan Hospital. Patients were eligible if diagnosed with *P. falciparum* monoinfection (confirmed by molecular diagnosis – PCR- in blood collected on day 0), ages 5 to 65 years, parasitemia between 250-50,000/µl of asexual forms, and axillary temperature of ≥37.5°C in the absence of another cause of fever. Exclusion criteria comprised danger signs (Extreme weakness, Signs of respiratory distress, Hyperpyrexia, Repeated vomiting, Repeated Diarrhea, Signs of severe dehydration, Spontaneous bleeding, Dark urine, Hyperparasitemia), severe malnutrition, known underlying chronic or severe diseases (cardiac, renal, hepatic diseases, HIV/AIDS), confirmed pregnancy and hypersensitivity to the medications tested.

Sample size

According WHO recommendation a classical statistical method for determining sample size was applied[13]. In the case of a medicine with an expected failure rate of 5%, a confidence interval of 95% and a precision level of 5%, a minimum of 73 patients should be enrolled. The sample size estimated was 88 subjects; it included a 20% increase to allow for losses to follow-up[13]. Sampling was done for convenience, in order of arrival at the malaria post among patients who met the entry criteria.

Procedures during inclusion and follow-up

After obtaining the informed written consent, a complete medical history (symptoms, current medications and previous use of anti-malarial drugs), biographic and contact details were noted. A complete physical examination was performed and a case record form was filled in for each patient. Body weight was recorded at day 0; temperature was measured using a thermometer with a precision of 0.1°C at baseline and on follow-up days, and additionally measured as clinically indicated. On each follow-up, clinical signs and symptoms were recorded, including vital signs (axillar temperature, hearth and respiratory rate, blood pressure), symptoms (including fever, headache, chills and abdominal pain, among 17 evaluated) and clinical signs (including pallor, jaundice, signs of bleeding and hepatomegaly, among 18 evaluated); blood smears were performed for detecting malaria parasites, and filter-paper spot samples were taken for molecular diagnosis.

Microscopic Blood Examination and quality control. Thick and thin blood films were prepared and stained with Giemsa for screening and subsequent species diagnosis and parasitemia calculation on days 0, 1, 2, 3, 7, 14, 21 and 28 or if reassessment was required. Parasite counts were done on thick films and the number of parasites per 200 white blood cells (WBCs) were counted by light microscopy. Parasite density, expressed as the number of asexual parasites per µL of blood, was calculated by dividing the number of asexual parasites by the number of WBCs counted, and then multiplying it by an assumed WBC density of typically 8,000 per µL. A blood-slide sample was considered negative when examination of 200 fields containing at least ten WBCs per field revealed no asexual parasites. The presence of gametocytes on the day the patient was enrolled or on the day of follow-up was also recorded.

Two independent lecturers examined Blood smears of enrolled patients, one microscopist at the study site and a professional in microbiology at the Malaria Group Laboratory. If the difference in the quantification of parasitemia between the first two readings varied by more than 25% a third independent reading was performed. Each reader was blinded to the results of the others.

Hemoglobin and antimalarial drug blood concentration. Hemoglobin was determined on days 0 and 28 in 10µL of capillary blood sample with the HemoCue blood Hemoglobin system. A 5 ml blood sample was obtained 1 hour and 120 hours after the fifth medication dose to determine AL blood levels. Specimens were be labeled anonymously (study number, day of follow-up, date).

Antimalarial treatment. All enrolled patients were treated with AL on site, by directly observed treatment and monitored for 28 days. Patients were given the fixed-dose AL regimen (Artemether –ARM- 20mg + Lumefantrine –LU- 120mg per tablet, "Coartem®" from NOVARTIS) adjusted to measured weight (1.7/12 mg/kg body weight of ARM and LF, respectively). The AL schedule consisted of twice-daily doses over 3 days (Day 0 to 2). After supervised administration of the drug, patients were observed during 30 minutes for adverse events or vomiting. Patients who vomited within this period were then provided with another dose of the study drugs and were observed for 30 additional minutes. If a second vomiting episode occurred, the patient was excluded from the study and offered parenteral rescue therapy. Concomitant treatment with acetaminophen was permitted to patients presenting axillary temperature ≥38ºC. In case of a first line therapeutic failure, patients received quinine sulfate (10 mg/kg, administered at 8-h intervals during 7 days) plus clindamycin (10 mg/kg administered at 12-h intervals during 7 days).

Follow-up and loss

Follow-up visits and procedures were scheduled per protocol on days 1, 2, 3, 7, 14, 21, and 28. Patients were instructed to return to the health center at any time if they had fever or any general danger signs as described under exclusion criteria. Where clinically indicated, patients were evaluated out of schedule and treated appropriately. The study team made home visits as follow-ups for study participants that were late for their scheduled visits. Patients who failed to return on days 1 or 2 and missed one dose of the treatment or enrolled patients who could not attend scheduled visits were considered lost to follow-up and excluded from study.

Outcomes

Treatment outcomes were assessed based on parasitological and clinical results and were classified according to the WHO protocol as:[13]

- Early Treatment Failure (ETF): Development of danger signs or severe malaria on day 1, day 2 or day 3 in the presence of parasitemia;
  - Parasitemia on day 2 higher than day 0 count irrespective of axillary temperature;
  - Parasitemia on day 3 with axillary temperature ≥ 37.5 ºC;
  - Parasitemia on day 3 ≥ 25% of count on day 0.
- Late Clinical Failure (LCF)
  - Development of danger signs or severe malaria on any day from day 4 to day 28 in the presence of parasitemia, without previously meeting any of the criteria of Early Treatment Failure;
  - Presence of parasitemia and axillary temperature ≥ 37.5 ºC (or history of fever) on any day from day 4 to day 28, without previously meeting any of the criteria of Early Treatment Failure.
- Late Parasitological Failure (LPF)
  - Presence of parasitemia on any day from day 7 to day 28 and axillary temperature < 37.5 ºC, without previously meeting any of the criteria of Early Treatment Failure or Late Clinical Failure.
  - Adequate Clinical and Parasitological Response (ACPR):

Absence of parasitemia on day 28 irrespective of axillary temperature without previously meeting any of the criteria of Early Treatment Failure or Late Clinical Failure or Late Parasitological Failure.

PCR testing for *Plasmodium* infection

We did PCR testing in order to determine parasitemia, confirm the plasmodium species and the monoinfection. Blood spots were collected on filter paper (Whatman No. 3) on days 0 and 28 and on a different day from participants with recurrent parasitemia. Blood samples were dried at ambient temperature before storage in individual plastic bags with silica gel. DNA samples were extracted from filter paper with blood spots by the saponin-Chelex method[15] and they were analyzed using the nested PCR protocol described by Singh et al[16] described briefly; a first amplification reaction with primers rPLU1 126 and rPLU5 for the fragment of the 18s-rRNA ribosomal subunit of the Plasmodium 127 genus parasites. This PCR product was used for the second reaction (nested PCR) 128 with primers rVIV 1 and rVIV 2 for the identification of *P. vivax* and rFAL1 and rFAL 129 2 for the detection of *P. falciparum*.

Antimalarial drugs concentration

Quantification of ARM, LF and Dihydroartemisinin (DHA) in plasma. The separation of DHA, ARM and LF was carried out by liquid chromatography using a chromatographic column ZORBAX Eclipse XDB-Phenyl 4.6x150mm, 5µ and a mobile phase composed of MeOH: CH3COOH 0.2%: H2O: CH3COOH 0, 2% at a constant flow of 1ml/min and with programming by gradient. The detection of the analytes was performed with a simple quadrupole mass detector, monitoring the ratios m/z 323, 307 and 267 for DHA; for ARM m/z 321 and 267, and for LF m/z 528 and 530. Liquid-liquid extraction was performed to obtain the analytes from plasma. Each curve was made in triplicate and Artesunate (ART) was assumed at each level as an internal standard for DHA and ARM at a fixed concentration of 1,500 ng/ml. All the parameters of quantification were checked in this way: linearity, accuracy, precision and recovery[17-20].

Statistical Analysis

IBM's statistical software SPSS (17th version) was used for data management and analysis. Data was analyzed using two methods: the Kaplan-Meier analysis and the per protocol analysis.

The description of demographic and clinical characteristics are shown as proportions for categorical variables and mean (medians) values for continuous variables. The treatment outcomes at 28 day are presented for both thick smear and PCR results as absolute numbers and proportions. The plasmatic concentration of drugs on days 2 and 7 are presented as media (95% C.I.), median and range. The Mann Whitney U-test was done to evaluate differences in the antimalarial drugs blood concentration on day 2 among patients with a negative PCR on day 28 and those with a positive result. Statistical significance was defined as $P < 0.05$

Ethical considerations

The Ethical Committee of the Faculty of Medicine of the University of Antioquia approved the study protocol. The trial was conducted according to good clinical practice guidelines. Written consent was obtained from all adult patients and from the parents or guardians of the children who participated in the study. Children over 12 years of age signed an informed assent form. The principal investigators had no affiliation with any of the malaria diagnostic centers where the study was conducted.

Results

Baseline characteristics of participants

During the study period, 143 patients were screened; 2 participants were excluded because of the parasitemia was above 50,000 parasites/µl, 10 presented clinical danger signs and 43 living outside the study area. Finally were included 88 patients; during follow-up 4 patients were lost and 84 completed the study. Demographic and laboratory baseline data of the participants is summarized in Table 1.

Efficacy results

The treatment outcomes are summarized in Table 2. On day 1, parasitemia was identified in 78 patients, with an average parasite load of 756 parasites/µl (SD = 2.004, median = 157); five patients were positive on day 2, with an average of 49 parasites/µl (SD = 21.9, median = 39. On day 3, all patients had cleared parasitemia. An adequate clinical and parasitological response, defined by a negative thick blood smear on day 28, was found in 100% of cases, and no treatment failures were detected. No serious adverse events were registered.
The PCR results for 4 patients were positive for *P. falciparum*; comparisons between day 0 and 28 were not performed. The PCR-corrected per-protocol analysis (4 lost patients) showed an adequate clinical and parasitological response for 95.2%; the cure rate (Kaplan-Meier) was 90.7–99.8%. The median of the parasitemia on the initial day did not differ statistically ($P > 0.05$; Mann Whitney U test) between patients with ACPR (median=4,235) and those considered with parasitological failure using PCR (median =3,625).

Antimalarial drugs blood concentration

The plasmatic concentrations are presented in Table 3. The amount of blood was not sufficient to perform the measurements in all patients; however, measurements were made for the three analytes in 56 participants (67%) on day 2.

The median values of DHA on day 2 among patients with a negative PCR on day 28 and those with a positive result were 85.9 ng/ml and 113.8 ng/ml, respectively. The median values of ARM on day 2 among patients with a negative PCR on day 28 and those with a positive result were 100.9 ng/ml and 152.9 ng/ml, respectively. For LF concentration, the median values on day 2 among patients with a negative PCR on day 28 and those with positive PCR were 1,389.4 ng/ml

Table 1. Demographic and clinical characteristics of 88 patients included.

		n	%
Age, years, mean (median)	24.7 (20)		
Males		48	54.5
Ethnicity			
African descent		75	85.2
Mestizo		9	10.2
Native Americans		4	4.5
Malaria, episodes last year			
0		71	80.7
1		9	10.2
2-4		8	9.1
Characteristics of current malaria episode			
Malaria evolution time, days, mean (median)	4.5 (4.0)		
Parasitemia day 0, asexual forms/µl, mean (median)	9,873 (4,109)		
Symptoms during current malaria episode			
Fever		88	100.0
Cephalea		88	100.0
Shivers		88	100.0
Diaphoresis		86	97.7
Osteomuscular pain		82	93.2
Abdominal pain		41	46.6
Nausea		41	46.6
Diarrhea		13	14.8

and 1,965.2 ng/ml respectively; in day 7, the respective values were 200.1 and 1,019.5. No significant differences were found between these values (Mann Whitney U-test; p>0.05).

Discussion

A therapeutic efficacy of 100% after a 28 days follow-up was established, evaluated by microscopic examination. The detection of submicroscopic parasitemia in four cases (5%) on day 28 by PCR may reflect a reduction in susceptibility to AL in the region, however reinfections could not be ruled out as the cause for these findings. In the analysis, these cases were considered as therapeutic failures in accordance with findings in the same area in 2009 where a late failure of 1% was evidenced [9]. From the comparison of this data and the findings in the previous study, we infer that the crude and PCR-corrected ACPR rates found in the Colombian Pacific region [9] are similar. Even though AL is the standard treatment for uncomplicated *P. falciparum* malaria in Colombia, the literature discussing its efficacy is scarce [21-22].

Several studies have shown that high parasite density is associated with treatment failure [23-25]; in this study, not statistically significant difference was found in the initial parasitaemia between those who presented ACPR and therapeutic failure determined by PCR.

The treatment was generally well tolerated and no serious adverse events were observed. This finding is consistent with other studies in which no serious adverse effects were reported[22, 26-27].

Antimalarial drug's efficacy depends not only on the parasite susceptibility to the drug and on its blood concentration but also on the host's immunity. All patients reached adequate therapeutic concentrations. The concentration of the analytes was within the normality ranges expected, which include: for ARM a Cmax of 66.2 ± 54.3 ng/ml and Cmin of 6.7 ± 8.5 ng/ml; for DHA a Cmax of 205 ± 102 ng/ml and a Cmin of 13.4 ± 12.1 ng/ml; for LF, pharmacokinetics are reported as highly variable, but a study in Thailand suggests that the maximum concentrations after reaching the maximum absorption time range between 1,100 ng/ml and 19,000 ng/ml [28]. For these reasons, it can be proposed as a hypothesis that the therapeutic failures are due to resistance of the parasite to the AL combination.

Studies conducted in Colombia have shown high efficacy of therapeutic combinations that include artemisinin derivatives. In the region of Urabá, the therapeutic efficacy of different combinations with ACT was evaluated in 2002-2006 through microscopy; ACPR of 96% was found with Artesunate-Sulfadoxina pirimetamina, 100% with Artesunate-Amodiaquine and 100% with Artesunate-Mefloquine[29]. Findings in 2007 have reported similar results, of 100% mean (95% CI: 89.1%-100%) PCR-adjusted ACPR rates for ACT treatment (AS-AQ) in the Colombian Pacific region[30]. Additionally uncorrected Day-42 cure rates for other ACTs were observed in the Pacific region (Tumaco) in 2008: 97.5% for AL and 98.1% for Artesunate-Mefloquine[22].

Recurrences of parasitaemia by *P. falciparum* may be due to a recrudescence (a repeated attack of malaria due to the survival of parasites in red blood cells) or a reinfection. The first ones are due to a therapeutic failure and therefore it is recommended to try to differentiate between both situations, which can be attempted through the genetic characterization of the parasites. In this study, genotyping was not carried out, which constitutes a limitation. However, in Colombia has been described the presence of many identical or closely related genotypes of *P. falciparum*[31] that difficult to achieve this differentiation.

In conclusion this study showed very high therapeutic efficacy and safety of the AL combination in the municipality of

Table 2. Treatment outcomes at 28 day in 84 patients.

Treatment outcomes	Thick blood film		PCR	
	n	%	n	%
Adequate clinical and parasitological response	84	100%	80	95%
Early treatment failure	0	-	0	-
Late clinical failure	0	-	0	-
Late parasitological failure	0	-	4	5%

Table 3. Plasmatic concentration of antimalarials on days 2 and 7

Analyte	n	Media (95% C.I.)	Median	Lower-Upper
DHA Dihydro-artemisinin day 2 ng/ml	57	131.2 (73.9-188.6)	92.2	1.4 – 1,374
ARM Artemisinin day 2 ng/ml	56	131.3 (99.9-162.7)	100.9	14.9 – 640.9
LF Lumefantrine day 2 ng/ml	58	1,565.2 (1,323-1,807)	1,389.4	84.8-4,358.4
LF Lumefantrine day 7 ng/ml	37	293.6 (72.7-1,947.6)	200.1	72.7-1,947.6

Quibdó, for the treatment of uncomplicated malaria by *P. falciparum,* with 100% of adequate clinical and parasitological response evaluated by microscopy and 95% when molecular diagnosis is applied. All patients achieved the therapeutic concentrations of Artemether and Lumefantrine. The therapeutic failures stablished by PCR were not associated with low levels of the drug in blood. As evidenced by other studies in the country, the treatment of uncomplicated malaria by *P. falciparum* with combinations that include derivatives of artemisinin has very high efficacy.

Studies should be carried out to separately evaluate the drugs that are part of the combinations commonly used in order to ensure the useful life of these ACTs, since it is not known if the cases of failure are due to the appearance of parasites resistant to artemisinin derivatives. In addition, genotyping must be done to interpret the type of recurrence that occurs. Regular monitoring of AL is required in view of malaria elimination initiatives, which will be largely dependent on therapeutic interventions and regular surveillance.

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ARTÍCULO ORIGINAL

Presence of anisakid larvae in commercial fishes landed in the Pacific coast of Ecuador and Colombia

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Abstract

Anisakidosis is a zoonotic disease caused by the consumption of raw or undercooked fish or crustaceans parasitized by nematode larvae of the Anisakidae family. In this study, the presence of anisakid larvae was identified in fish species of consumer of the Pacific coast in Ecuador and Colombia.

We obtained 438 samples grouped into twenty species of fish caught in the fishing ports of Manta, Santa Rosa, Buenaventura and Tumaco. The morphological identification of the larvae was made by taxonomy and the percentage of infection, were calculated. For the identification of species, a multiplex PCR was carried. The taxonomic review identified eight species of fish as hosts of the genders *Anisakis* and *Pseudoterranova*. The larvae were isolated mainly from the intestine with a percentage of infection between 18 and 100%. The percentage of infection and identification of anisakids in these fish will aid in the prevention and control of anisakiasis as a possible emerging disease for this area of the Pacific. With the multiplex PCR, *A. pegreffi*, *A. physeteris*, and *P. decipiens* were identified. The identification of these species is reported for the first time in this geographical area, providing the basis for future research into the Anisakidae family.

Key words: Anisakid nematodes, molecular identification, commercial fishing.

Presencia de larvas de anisakidos en peces comercializados y desembarcados en la costa del Pacífico de Ecuador y Colombia

Resumen

La anisakidosis es una enfermedad zoonótica causada por el consumo de pescado o crustáceos crudos o poco cocinados parasitados por las larvas de nematodos de la familia Anisakidae. En este estudio, se identificó la presencia de larvas de anisakidos en especies de peces de consumo de la costa del Pacífico en Ecuador y Colombia.

Obtuvimos 438 muestras agrupadas en veinte especies de peces capturados en los puertos pesqueros de Manta, Santa Rosa, Buenaventura y Tumaco. La identificación morfológica de las larvas se realizó por taxonomía y se calculó el porcentaje de infección. Para la identificación de las especies, se llevó a cabo una PCR múltiplex. La revisión taxonómica identificó ocho especies de peces como huéspedes de los géneros *Anisakis* y *Pseudoterranova*. Las larvas se aislaron principalmente del intestino con un porcentaje de infección entre 18 y 100%. El porcentaje de infección e identificación de anisakidos en estos peces ayudará a prevenir y controlar la anisakiasis como una posible enfermedad emergente en esta área del Pacífico. Con la PCR múltiplex, se identifico *A. pegreffi*, *A. physeteris* y *P. decipiens*. La identificación de estas especies se informa por primera vez en esta área geográfica, proporcionando la base para futuras investigaciones sobre la familia Anisakidae.

Palabras clave: nematodos anisákidos, identificación molecular, pesca commercial.

Introduction

Fish and fish products are an important source of animal protein, easily digestible, as well as rich in minerals and vitamins. The Pacific region straddling Colombia and Ecuador has a number of important fishing ports that have enabled the development of industrial and artisanal fishing activities[1] According to the Food and Agriculture Organization of the United Nations (FAO), average annual fish consumption per capita in Colombia and Ecuador is 4.73 kg and 5.6 kg, respectively[1,2]. Compared to countries such as Spain (38 kg / year), and Japan (54 kg / year), and even average consumption in Latin América in general (18 kg / year), consumption in Colombia and Ecuador is notably low. In recent years, with the introduction

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of the Mediterranean diet and the benefits of fish consumption for the prevention of cardiovascular diseases now well-documented, fish consumption has been increasing. However, the intake of these products has been connected to various infections in humans when fish is consumed that is minimally processed (i.e. raw, semi-raw, in sushi, salted or marinated)[3]. In some Latin American countries such as Brazil, Chile, México and Perú, most of the infections related to these types of fish preparation are associated with the presence of parasites in fish, a widespread phenomenon and particularly difficult to eliminate in raw and unprocessed fishery products[4].

The nematodes of the family Anisakidae are of particular concern to public health. These parasites cause anisakiasis, a zoonosis caused mainly by the genera *Anisakis* (Dujardin, 1845) and *Pseudoterranova*[5]. In humans, Anisakid larvae can cause gastric, allergic or gastro-allergic symptoms. Allergic reactions, (hypersensitivity type I), are the most common and caused mainly by the genus *Anisakis*, while gastric symptoms are more commonly associated with the genus *Pseudoterranova*, causing pseudoterranovosis[6].

Human anisakidosis has been reported in more than 26 countries on five continents including, Holland, Italy, Egypt, New Zealand, Canada, the United States, Brazil, Peru, and Chile[7–14]. The majority of reported cases have occurred in Spain, Japan and other Asian countries, where epidemiological studies have indicated that anisakiasis (caused by the genus *Anisakis*) is more frequent in coastal populations. In countries such as Brazil, Chile, Peru and Colombia, anisakidosis is considered an emerging public health risk and one that could increase with the introduction of a Mediterranean or Japanese diet[15].

The World Health Organization (WHO) and the FAO have established regulations, recommendations and guidelines to prevent and minimize the negative effect of anisakid nematodes on human health[16]. For its part, the European Community has specific sanitary regulations for foods of animal origin which are applicable to fishery products in relation to minimizing infection by anisakid parasites[17]. In Latin America, countries such as Nicaragua, Mexico and Ecuador follow some of these measures for the prevention and control of fish parasites that affect human health[18,19]. However, Colombia does not have clear legislation in this regard since these pathogens have not been fully identified.

Given that the process of globalization has popularized the consumption of raw or undercooked fish across various regions of the world and that fish parasitized with anisakids have been recorded in countries such as Chile, Peru, Argentina, Brazil, Colombia and Venezuela. Thus, the objective of this study was to determine the presence of anisakid nematodes in fish species that are sold commercially in the fishing ports of Colombia and Ecuador.

In addition, it is important to note that, in Colombia and Ecuador, no cases of anisakidosis in humans have been described to date, though this is most likely due to a lack of clinical and epidemiological information, and consequently an underreporting of the parasitosis as an emerging disease.

Materials and methods

Study area

They were captured commercial fish species of particular economic importance were collected from the following fishing ports (Figure 1):

Ecuadorian Pacific: Manta (0 ° 57'00 "S 80 ° 42'58" W) and Santa Rosa (3 ° 27'08 "S 79 ° 57'42" W). These ports concentrate a catch of 23,000 tons of fish per year (1).

Colombian Pacific: Buenaventura (3 ° 52'38 "N 77 ° 01'36" W) and Tumaco (1 ° 48'24 "N 78 ° 45'53" W), the main ports of the Colombian Pacific coast where 53.89% of the catch comes from artisanal fishing and 46.11% from industrial fishing, which contributed a combined total of 1,099,568 tons of fish between 1998 and 2013[1].

Sampling

The samples were gathered from collection centers, points of sale, or supplied by fishermen in the region according to availability and the species' economic importance. The samples were stored individually in labeled bags, kept in a refrigerator with ice and then transferred to the laboratory.

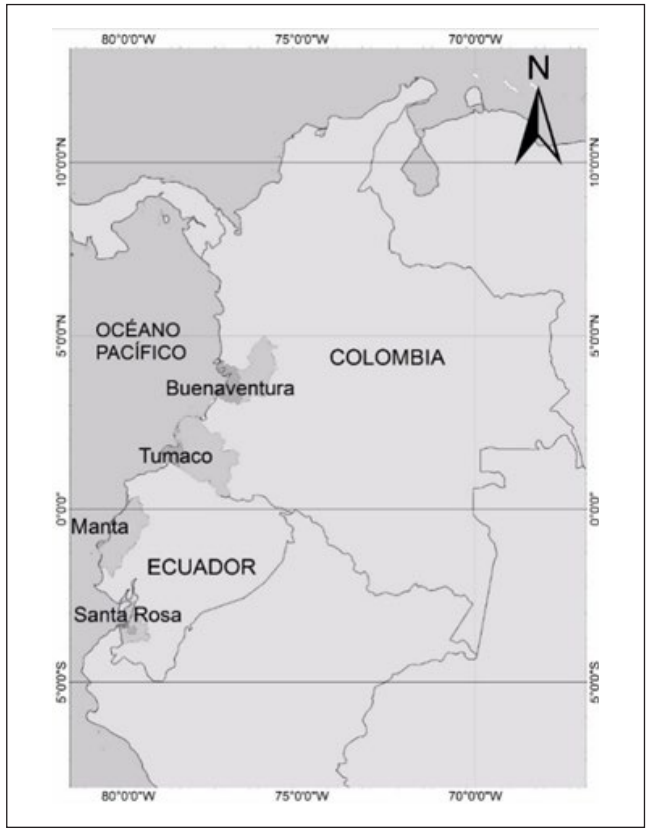

Figure 1. Location of the sampling sites in the Pacific coast of Colombia (Buenaventura and Tumaco) and Ecuador (Manta and Santa Rosa).

The study focused on the identification of anisakids in the viscera of the fish. In most cases, there was not access to the musculature of the fish due to its greater economic value. The nematodes were fixed in warm formalin at 4% (v / v) and immediately transferred to alcohol at 70% (v / v) until their identification by taxonomy. A subsample was stored directly in 96% alcohol (v / v) for subsequent identification by molecular biology.

Morphological identification of nematodes

The nematodes were clarified in gradual solutions of glycerin (20). The observation of their internal structures was carried out under an optical microscope with a built-in clear camera (Leica DM750). Nematodes of the Anisakidae family were identified up to a generic level, taking into account the characteristics described by Shiraki (1974). Images were taken with magnifications of 40x, 100x and 400x (Application Suite LAS v 3.8).

The morphologically identified anisakids were separated, counted and grouped by host. Finally, the percentage of infection for each host was calculated.

Molecular identification

DNA extraction: Thirty-eight anisakid nematodes were processed in the parasitology laboratory in the Faculty of Medicine at the Universidad de Chile. The larvae were sectioned into three and the individual extraction of each section of the nematodes was performed using the Invitrogen kit (Genomic DNA Mini Kit), following manufacturer instructions. The DNA was eluted in elution buffer and kept at -20°C until use.

Primers: Specific primers were used for six anisakid species: *Anisakis physeteris* (Baylis, 1923); *Pseudoterranova decipiens* (Krabbe, 1878); *Anisakis simplex sensu stricto* (Rudolphi, 1809); *Contracaecum osculatum* (Rudolphi, 1802); *Hysterothylacium aduncum* (Rudolphi, 1802) and *Anisakis pegreffii* (Bell-Rouget & Biocca, 1955), (Integrated DNA Technologies)[33].

Identification by multiplex PCR: A multiplex PCR was performed, using six specific primers and a universal primer[21]. The final reaction volume was 25µl: water (9.38µl); buffer 10x (2.5 µl) (HotMaster ™ Taq); dNTPs (0.63 L) (0.2 mM), for each specific primer (1 µl), universal primer (1 µl), HotMaster Taq DNA Polymerase (0.5 µl) (5PRIME) and genomic DNA (5 µl). The thermocycler program (Applied biosystems 2720) consisted of 30 cycles of initial denaturation at 95°C for three minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, hybridization at 52°C for 30 seconds, extension at 72°C for 45 seconds, and a final extension at 72°C for seven minutes. All products were subjected to electrophoresis in 2% (w / v) agarose gel. As a positive control, genomic DNA of L3 larvae of *A. simplex, A. pegreffii* and *P. decipiens* was used, supplied by Dr. Hiroshi Yamasaki of the National Institute of Infectious Diseases in Tokyo, Japan.

Results

A total of 438 commercial fish destined for human consumption were collected and grouped into 20 species. Of those twenty, eight were found to be parasitized by anisakid nematodes with a percentage of infection of between 18 and 100%, and an average abundance between 0.4 and 45.5 (Table 1).

The nematodes were isolated mainly from the intestine (Table 2) and their morphological characteristics examined, resulting in the identification of the *Anisakis* and *Pseudoterranova* genera, both of the Anisakidae family (Table 3), with 90% identified as from the *Anisakis* genus.

The type I (Figure 2) and type II (Figure 3) larvae of the *Anisakis* genus were identified under an optical microscope. Type I larvae are characterized by a rounded posterior and the presence of a mucron (Figures 2c and 2d), while type II larvae present a conical termination with the absence of a mucron (Figures 3c and 3d).

The larvae present characteristics of the *Anisakis* genus; a whitish color, transverse grooves along the entire length of the body that become more pronounced towards its posterior end, (Figures 2b, 3c and 3d), a well-formed mouth composed of three lips surrounding the cuticular tooth, (Figures 2a and 3a), an elongated ventricle with a direct connection to the intestine and well-aligned along the longitudinal axis of the nematode (Figures 2b and 3b).

Multiplex PCR identification confirmed at the species level 26 of the 38 larvae analyzed. *Anisakis physeteris* species were identified in eight hosts and *P. decipiens* and *A. pegreffii* were each identified in one host. *Anisakis physeteris* was also reported in hosts already parasitized by *P. decipiens* and *A. pegreffii* (Table 3).

The specific primers were able to detect *P. decipiens* isolated from the *M. curema* (White mullet), using an amplification product with an expected weight of 370 bp, *A. pegreffii* with 672 bp, isolated from *M. gayi* (Merluza) and *A. physeteris* with 143 bp, isolated from *A. rochei* (Bullet tuna), *C. armatus* (Armed snook), *C. hippurus* (Common dolphinfish), *K. pelamis* (Skipjack tuna), *L. argenteus* (Silver drum), *M. gayi* (Merluza), *M. cephalus* (Flathead grey mullet) and *M. curema* (White mullet) (Figure 4).

Discussion

In the present study we have verified the presence of anisakid nematodes in fish for human consumption that inhabit the waters of the Ecuadorian and Colombian Pacific. Results found a high percentage of infection of *Anisakis* and *Pseudoterranova* parasites, the main causative agents of allergic and gastric anisakidosis respectively[6]. We identified third instar larvae (L3) based on the structural characteristics of the ventricle and the shape of the posterior end of the larvae[22]. In

Table 1: Percentage of infection of anisakid nematodes isolated from fish for human consumption from fishing ports in Colombia and Ecuador.

No	Scientific name	Common name	Country	Location	n	Percentage of infection	Abundance
1	*Auxis rochei*	Bullet tuna	Ecuador	Manta	6	50	0.5
			Ecuador	Santa Rosa	29	28	0.4
2	*Bagre pinnimaculatus*	Red sea catfish	Colombia	Buenaventura	6	0	0.0
3	*Brotula clarkae*	Pacific bearded brotula	Ecuador	Manta	5	0	0.0
4	*Caranx caballus*	Green jack	Ecuador	Manta	7	0	0.0
5	*Centropomus armatus*	Armed snook	Colombia	Buenaventura	12	42	2.8
6	*Centropomus medius*	Blackfin snook	Colombia	Buenaventura	4	0	0.0
7	*Coryphaena hippurus*	Common dolphinfish	Ecuador	Manta	69	30	5.6
8	*Cynoscion phoxocephalus*	Cachema weakfish	Colombia	Buenaventura	9	0	0.0
9	*Cynoscion* sp.		Ecuador	Manta	3	0	0.0
10	*Diplectrum maximun*	Torpedo sand perch	Ecuador	Manta	8	0	0.0
11	*Epinephelu sacanthistius*	Rooster hind	Ecuador	Manta	1	0	0.0
12	*Fistularia corneta*	Pacific cornetfish	Ecuador	Manta	8	0	0.0
13	Katsuwonus pelamis	Skipjack tuna	Ecuador	Manta	100	31	0.7
14	*Larimus argenteus*	Silver drum	Colombia	Buenaventura	2	100	45.5
15	*Lutjanus guttatus*	Spotted rose snapper	Colombia	Buenaventura	3	0	0.0
16	*Macrodon mordax*	Dogteeth weakfish	Colombia	Buenaventura	7	0	0.0
17	*Merluccius gayi*	Merluza	Ecuador	Manta	62	92	9.1
			Ecuador	Santa Rosa	11	18	0.2
18	*Mugil cephalus*	Flathead grey mullet	Colombia	Buenaventura	12	33	0.4
19	*Mugil curema*	White mullet	Colombia	Tumaco	16	94	21.8
20	*Thunnus albacares*	Yellowfin tuna	Ecuador	Manta	58	0	0.0
				Total	**438**		

fish such as *Merluccius* sp., *Centropomus armatus* and *Mugil* sp. - species that are widespread across the Pacific Ocean from the coast of California in the United States to southern Chile[23] - infection with anisakidos could be related to feeding habits. These species inhabit coastal and estuarine waters and they have been classified as detritivores, iliophages, herbivores, omnivores, phytophages and zooplanktons[24], feeding behaviors that favor parasitic infections[4].

In recent years, these fish species have recorded high rates of capture by artisanal fisheries and constitute one of the main sources of protein as an economically important species on the Pacific coast[25]. The identification of these fish species parasitized by *Anisakis* and *Pseudoterranova* can be used to develop measures for the prevention and control of human anisakidosis in Ecuador and Colombia; a disease that can be classified as emergent and easy to manage but whose spread must be addressed.

The location of the larvae is reported in this study, recorded as parasitizing the mesentery, stomach, intestine and liver. Our interpretation is that these results are related to the time that elapsed between the capture of the fish and the parasitological review. Most of the fish were reviewed fresh and it is known that the larvae of the anisakids require a minimum time of thirty minutes to migrate from the digestive system of the fish to the muscle[26]. Our results are similar to those of studies conducted in European countries, where hake (merluza) has a high commercial value and where *A. pegreffii* and *A. simplex* s.s. have been identified in viscera and muscle without significant differences in the values of prevalence between the two species of *Anisakis*[26]. These findings may be of economic interest for Ecuador and Colombia, providing information about the likely locations of larvae in order to eliminate them and therefore develop more effective and sanitary processing of the fish caught in the maritime waters of these countries.

Table 2: Location of anisakid larvae in each of the parasitized hosts.

No	Host species	Location of larvae (%)			
		Mesentery	Stomach	Intestine	Liver
1	*Auxis rochei*	45	27	27	27
2	*Centropomus armatus*	0	0	100	0
3	*Coryphaena hippurus*	14	67	19	5
4	*Katsuwonus pelamis*	16	71	19	0
5	*Larimus argenteus*	0	0	100	0
6	*Merluccius gayi*	32	54	29	31
7	*Mugil cephalus*	25	0	75	0
8	*Mugil curema*	21	7	86	7

The molecular characterization by PCR multiplex enabled the identification of the nematode *A. physeteris* in the fish species *A. rochei*, *C. armatus*, *C. hippurus*, *K. pelamis*, *L. argenteus*, *M. gayi* and *M. cephalus,* as well as *A. pegreffii* in *M. gayi* and *P. decipiens* in *M. curema*. However, it must be taken into account that the use of other molecular methods such as sequencing could vary these results given that these parasites are a complex of species and their taxonomy changes constantly. Nevertheless, these results concur with comparable studies by different authors who previously reported *A. physeteris* parasitizing *C. hippurus* fish in Peru with a prevalence of 33.33%[27–29] and in the *Mugil* sp. fish in Chile where *P. decipiens* was isolated and identified, a parasite associated with cases of gastric infection in that country[5,30]. However, in other investigations, *A. typica* has been recorded in the same hosts examined in our study, as in the *A. rochei* and *K. pelamis* fish in Indonesia[31,32] and the *C. hippurus* fish in the Indian Ocean[33,34].

Hake (merluza) is considered a potential host for all *Anisakis* species[31]. We report *A. pegreffi* and *A. physeteris* for this species, while in Chile it is associated with cases of pseudoterranovosis and in Colombia there is the report of *Contracaecum* sp. parasitizing the fish *Merluccius* sp. from the Caribbean Sea[5,9,30,32].

Additionally, the mugilidae constitute the family of fish with the highest number of anisakidos reports. We identified *P. decipiens* in Lisa fish, as in Chile, Venezuela and Peru, where it has been reported as the causative agent of several cases of anisakidosis[4,27,30,35]. Other authors report this species as a host for *Contracaecum* sp. in Colombia[24,25,36–38] and *A. pegreffii* in the Yellow Sea and in the Mediterranean Sea[31,39]. However, our bibliographic research did not find any reports of anisakids for the *C. armatus* and *Larimus argenteus* fish suggesting our study may be the first report of parasitosis in these species by *A. physeteris*. Similarly, we did not find reports of anisakidos in Ecuador.

Although in the majority of human cases of anisakidosis where molecular biology has been performed *A. simplex s.s.* has been identified as the main etiologic agent[40], in Italy *A. pegreffii* is recognized as the main cause of gastric anisakiasis with the molecular larvae isolated from three clinical cases being identified by molecular techniques[41]. It is important to note that in Japan, where parasitized fish species are widely studied, it is recognized that there are maritime areas of sympatry between *A. simplex* s.s. and *A. pegreffii*, and hybrid individuals have been recorded, while in the western Mediterranean *A. simplex* s.s. is not present, and *A. pegreffii* has been found to parasitize in a broader range of fish species[40]. Such variations underline the importance of conducting further research into fish from the Pacific coast to ascertain whether there are fish parasitized by *A. simplex* in addition to the *A. pegreffii* nematodes identified by this study.

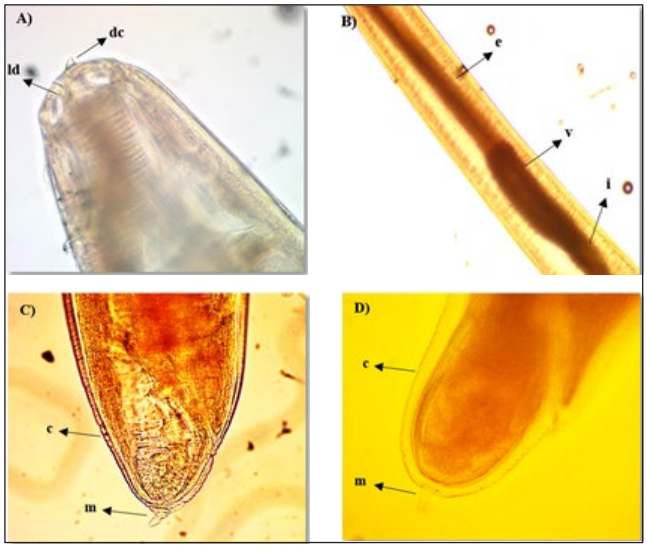

Figure 2. Larva (L3) type I of the Anisakidae family. A. Front end, dc: cuticular tooth, ld: dorsal lip (40x). B. Middle section, e: esophagus, v: ventricle, i: intestine (20x). C and D. Posterior end, m: mucron, c: cuticle (40x).

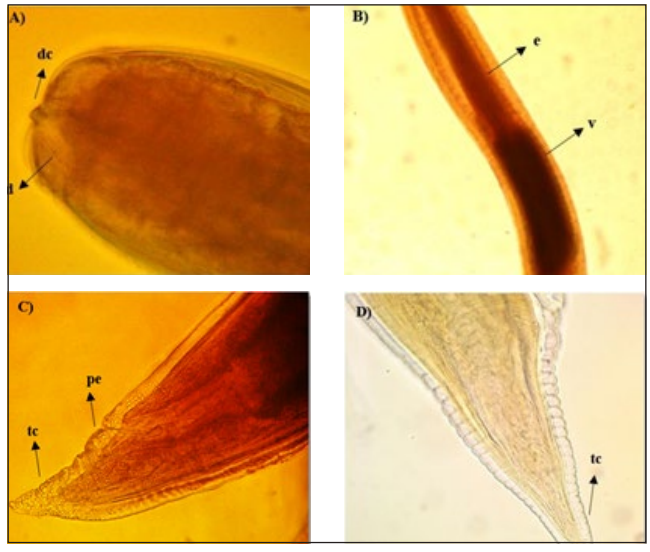

Figure 3. Larva (L3) type II of the Anisakidae family. A. Front end, dc: cuticular tooth, ld: dorsal lip (40x). B. Middle section, e: esophagus, v: ventricle (20x). C and D. Posterior end, tc: conical termination, pa: anal pore (40x).

Table 3: Morphological and molecular identification of larvae of the Anisakidae family and their hosts in the Colombian and Ecuadorian Pacific.

Host			Morphological Identification	PCR Multiplex Identification
Scientific name	Country	Location		
Auxis rochei	Ecuador	Santa Rosa	*Anisakis* spp.	*Anisakis physeteris*
Centropomus armatus	Colombia	Buenaventura	Familia Anisakidae	*Anisakis physeteris*
Coryphaena hippurus	Ecuador	Manta	*Anisakis* spp.	*Anisakis physeteris*
Katsuwonus pelamis	Ecuador	Manta	*Anisakis* spp.	*Anisakis physeteris*
Larimus argenteus	Colombia	Buenaventura	Familia Anisakidae	*Anisakis physeteris*
Merluccius gayi	Ecuador	Manta	Familia Anisakidae	*Anisakis pegreffii* *Anisakis physeteris*
Mugil cephalus	Colombia	Buenaventura	*Anisakis* spp.	*Anisakis physeteris*
Mugil curema	Colombia	Tumaco	*Pseudoterranova* sp.	*Pseudoterranova decipiens* *Anisakis physeteris*

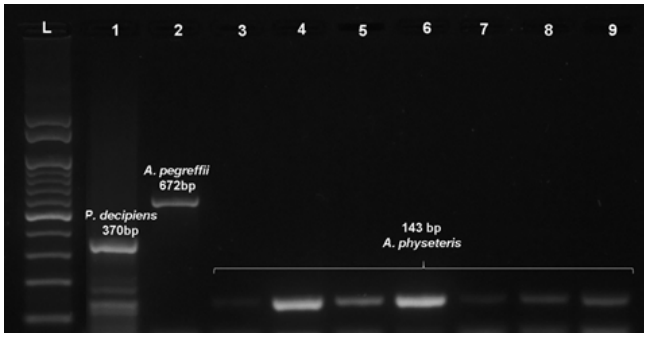

Figure 4. Molecular identification of anisakid nematodes by Multiplex PCR. Line 1: 370 bp, *P. decipiens* (*M. curema*). Line 2: 672 bp, *A. pegreffii* (*M. gayi*) and Lines 3 to 9: 143 bp, *A. physeteris* (*A. rochei, C. armatus, C. hippurus, K. pelamis, L. argenteus, M. gayi, M. cephalus and M. curema*). Line L: molecular weight marker of 100 bp.

We can not rule out that the absence of clinical cases diagnosed in Ecuador and Colombia may be related to a lack of knowledge on the part of physicians about anisakiasis and parasitic allergies, and its confusion with other diseases that present similar clinical pictures.

Our research constitutes the first report of the species *A. pegreffii*, *A.physeteris* and *P. decipiens* in Colombia and Ecuador, complementing the studies of geographical distribution worldwide where the *Anisakis* genus is the most reported in the Mediterranean while *Anisakis simplex* sensu stricto and *H. aduncum* are more frequent in the North Atlantic, and *Pseudoterranova* in the northeastern Atlantic, with no distribution of a particular species apparent in the north of the Pacific Ocean as we have reported.

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Declaration of conflict of interest

The authors declare no conflicts of interest.

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ARTÍCULO DE REVISIÓN

The viruses in the human oncogenesis

Anggi Vélez-Bohórquez[1], Mabel Bohórquez-Lozano[2,*], Magdalena Echeverry-de-Polanco[3]

Abstract

Based on epidemiological associations and experimentation, relationships between viruses and cancer have been established. For more than 14 million new cases of cancer per year, it is estimated that 15% are related to viral agents. Epithelial, hematolymphoid and mesenchymal malignancies related to different viruses have been document such as Epstein Barr, Kaposi's sarcoma, hepatitis B and C, human lymphotropic type 1, Merkel's carcinoma and human papilloma. New virus with oncogenic potential such as cytomegalovirus, JC polyoma virus and BK have been described. The interaction of the viruses with the host induces oncogene activation, inhibition of tumor suppressor genes and activation of miRNAs, as determining factors in the development of cancer. The pathology is initiated with the infection that induces the deregulation of cell signaling. The Epstein Barr virus is the oncogenic prototype, with 1% of the human cancers related to it.

Keywords: Neoplasms, virology, pathogenesis

Los virus en la oncogénesis humana

Resumen

Con base en asociaciones epidemiológicas y experimentación, se ha logrado establecer relaciones entre los virus y el cáncer. Para los más de 14 millones de casos nuevos de cáncer por año, se estima que el 15% se relacionan con agentes virales. Se han documentado malignidades epiteliales, hematolinfoides y mesenquimales, relacionadas con diferentes virus: Epstein Barr, sarcoma de Kaposi, hepatitis B y C, linfotrófico humano tipo 1, carcinoma de Merkel y papiloma humano; se plantean nuevos virus con potencial oncogénico como citomegalovirus, poliomavirus JC y BK. La interacción de los virus con el hospedero muestra activación de oncogenes, inhibición de supresores tumorales y activación de miRNAs, como factores determinantes en el desarrollo de cáncer. La patología se inicia con la infección que induce la desregulación de la señalización celular. El virus de Epstein Barr es el prototipo oncogénico, el 1% de los tipos de cáncer humanos se relacionan con él.

Palabras Clave: Neoplasias, virología, patogénesis.

Introduction

Since the early twentieth century, it was suspected that cell transformation could involve external agents called "filterable agents free of cells", capable of reproducing neoplastic manifestations in experimental animals[1,2]. Later on, these "agents" were classified as viruses, giving rise to the concept of oncogenic viruses in 1970[3].

Viruses are etiological agents of a vast number of human pathologies, from acute self-limiting infections to potentially fatal conditions, among of them cancer which is consider a worldwide public health problem. The rate of incidence of cancer includes 14.1 million new cases and 8.2 million deaths per year; of those, 57% (8 million) of the new cases and 65% (5.3 million) of cancer deaths occur in developing countries[3].

The different types of cancer of viral origin are multifactorial and, like non-infectious ones, seem to be a biological anomaly since the tumors do not increase the transmissibility of the virus, nor do they offer any specific advantage. In short, cancer seems to be the final event of the infection. Due to this, most people exposed to these viruses do not develop malignancy, confirming the need for other factors to trigger oncogenesis[4].

The cause of these malignancies is based on postulates as old as the one attributed to Galileo Galilei: "An agent causes disease when it is necessary and sufficient for the disease to occur". This postulate has evolved through scientist such as Henle, Koch and Hill, that established the criteria needed to declare microorganisms as the causal agents of disease. These criteria include the biological characteristics of the

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microorganism, its effects on the host, the reproducibility of these by different researchers and the epidemiological relationships[5]. Thanks to these scientific methods, the infectious agents that cause cancer have been divided into two categories: direct carcinogens, which express oncoproteins that contribute to the transformation and indirect carcinogens that through the necessary process of infection and inflammation, eventually cause carcinogenic mutations[4].

It is estimated that 15% of cancer cases are related to viral agents, which is why, in this review of literature, readers are offered an update on the subject, which considers the different oncogenic viruses, their pathogenic mechanisms and the interaction with the host cell, which culminates in tumorigenesis.

Materials and methods

Search Strategies

The PRISMA statement (http://www.prisma-statement.org/) was incorporated, a systematic search was made in the electronic databases: NCBI (PuMed), MEDLINE (OvidSP), Scopus (ScienceDirect), combining the terms Search (MesH): "Neoplasm", "Virology", "Related" and "Etiopathogenic", the DOI of the articles was verified at http://www.doi.org/.

Inclusion and exclusion criteria

We included articles that offered a review of the topic in humans in the last 5 years, with the indicated search strategy; The selection of articles is detailed in figure 1.

Types of studies excluded: (I) Clinical trials of drugs and vaccines; (II) Comparison of treatments or diets in patients; case reports; (IV) Syndromes (V) comments and editorials. Data on geographical location, study population, study design and results were extracted, according to the acronym PICO, see table 1.

The full text documents were independently evaluated by two reviewers, the disagreements were resolved by consensus, with the participation of a third party when it was necessary (Figure 1).

Evaluation of quality

The evaluation of the systematic reviews included in the comparative analysis was done through the: "Critical Appraisal Skills Program" (CASP), http://www.casp-uk.net/#!casp-tools-checklists/c18f8; a minimum inclusion score of 6/10 was established, determined by two authors and based on the analysis of the text of the published version.

The methodological quality of the 45.95% of the studies is between 8 and 10 points. The average score is 7.37. No publication showed a methodological quality of less than six.

Results

A total of 34 articles were selected for the comparative analysis, of which 33 (97%), relate the cancer with molecular details of viruses considered oncogenic. The majority of these (29.4%) correspond to journals in the United Kingdom, with

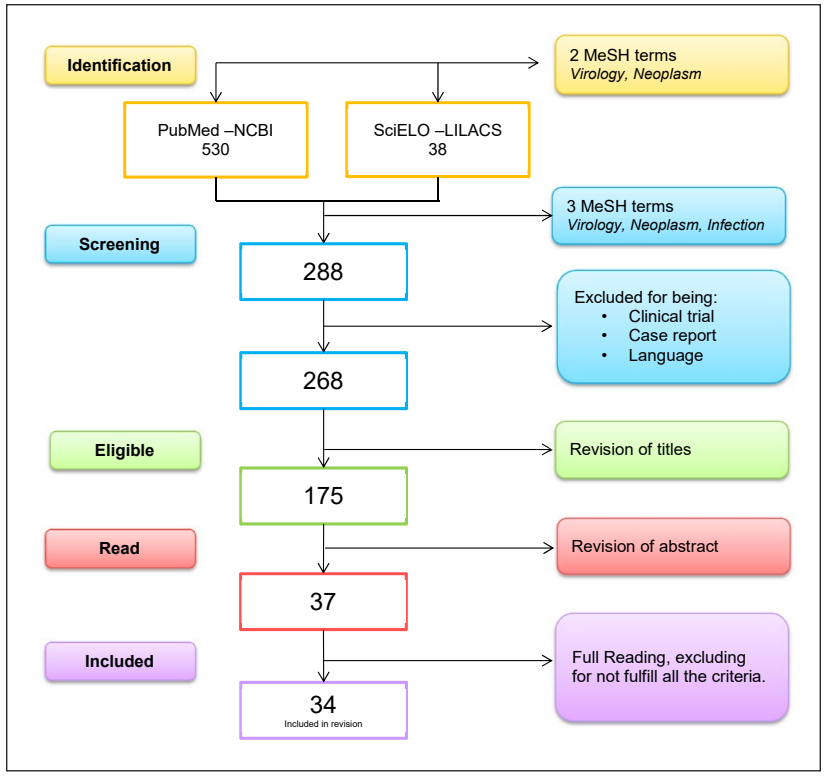

Figure 1.

Table 1. Inclusion criteria according to de acronym PICO *.

Indicators PICO	Results according to PICO
Design	Studies of systematic reviews that include pathogenic aspects in which the interaction of the viruses with the host is circumscribed as a determining factor in the development of human malignancies.
Population	Malignant neoplasm associated to oncogenic viruses
Intervention	None
Comparison	Scientific literature, type: systematic review. "Neoplasm", "Virology", "Related" y "Etiopathogenic"
Outcome	Classification of oncogenic viral particles and their cellular targets related to the infection and its oncogenic effects

*The PICO process (acronyms defining (P) population, (I) intervention, (C) comparison and (O) outcome).

an average H index (IHP) of 133, followed by publications from the United States and China. Of the 34 articles, 10 of them present a review about the Epstein Barr virus (29.4%); 8 of Kaposi's sarcoma virus (23.5%); 6 of human papilloma virus (17.6%); 5 of hepatitis C (14.7%); 4 of hepatitis B (11.7%); 3 of HTLV-1 (8.8%); 2 of Merkel virus (5.8%) and 1 of Cytomegalovirus (2.9%).

Viruses have been identified as etiological agents of cancer since 1909 when Peyton Rous started his research on the transmissibility of sarcoma in hens[6], followed by the description of oncogenic viruses in mammals in the thirties, with accelerated development in tumor virology in the subsequent decades[7]. The twentieth century culminated with Harld zur Hausen's declaration of the human papillomavirus as a high-oncogenic risk and describing it as a "stage setter" for cervical cancer based in long term researchs[2,8]; this announcement was recognized with the Nobel Prize in 2008[9]. With the advances in genomics and the description of the pathogenicity of the different viral families, viral molecules closely related to the development of cancer have been identified[4]. The families of viruses related to cancer in the different publications are: Herpesviridae - double-strand linear DNA, Hepadnaviridae - double-circular DNA chain, Flaviviridae - single-chain RNA, Retroviridae - single-chain RNA, Polymaviridae - double-stranded circular DNA, Papilomaviridae - Double linear DNA[4]. These families have viruses of different genomic and transmissibility characteristics (sexual, oral fecal, fomites, aerial droplets and even blood products transfusions); cellular tropism (immune system, mucous membranes or parenchymal tissues). In addition, they can have diverse pathogenic mechanisms (Table 2) including: infection, inflammation - TNF, IL6, IL8 -, viral persistence and oncogenic mechanisms (Table 3) such as transformation, key in the multiple steps necessary for viral oncogenesis[10,11]. Recently, Lin-Tao, et. al., postulated that after the transformation, there is an uncontrolled proliferation and metabolic reprogramming that facilitate the production of energy and molecular synthesis[12]. In the last four decades, the international agency for cancer research IARC reports to all these families as human carcinogens with different degrees of risk, the highest degree is # 1 in which there is enough evidence linking a certain virus with a malignancy, such as the Epstein Barr virus with lymphomas and epithelial tumors, as detailed in Table 3[13,14].

Virus related to tumors of hemolymphoid origin

Epstein Barr: This virus infects B cells and is expressed in different ways, depending on the oncological pathology it generates: 1. Type I latency (15), characterized by the nuclear antigen EBNA-1. It promotes cell proliferation by activating the c-MYC complex and it prevents the presentation of antigens avoiding the proteosomal degradation characteristic of Burkitt's lymphoma (16); 2. Type II latency, EBNA in conjunction with membrane proteins LMP-1 and 2[15]; characteristic of Hodgkin's lymphoma, in which LMP-1 deregulates the apoptotic pathways by activating p13K and the B cell receptor (BCR), which, in turn, promotes genetic damage by translocation and negative regulation of tumor suppressors through miR21; the immortalization of these cells is attributed to EBNA-3C by interaction with cell cycle control points and apoptotic block[17,18]; 3. Type III latency associated with post-transplant lymphoproliferative disorders is associated with elevation of cellular miR155 that activates BCR, promoting proliferation[15,19].

Hepatitis C (HCV): This virus establishes its oncogenic action in marginal zone lymphoma and others with greater malignancy such as diffuse large B-cell lymphoma by means of the structural protein E2, which, by binding to CD81, activates proliferation upon stimulation CD19, CD21 and BCR[20]; Through NS3 and E7, pro-inflammatory interleukins are activated that increase somatic hypermutation and genetic translocation[21], with the consequent overexpression of BLC-2 and the reduction of caspase action, causing apoptotic dysfunction. MirR122 is a miRNA specific to the liver[18,22], whose mechanism of action is still uncertain. It was described by Lin and Flemington[19] as a positive regulator of virus replication, which causes an increase in the number of copies.

Herpes type 8: It is associated with lymphomas in the serous cavities (PEL). The production of IL-6 induces the production of its human analog, resulting in cell proliferation. In addition, this viral cyclin gene (vCyc), is integrated into the genome of the B lymphocytes, generating the right environment for cell expansion and facilitating transformation[23].

Human lymphotrophic type 1 (HTLV1): HTLV1 is the causal agent of adult T cell leukemia. It infects dendritic cells and T lymphocytes, where the viral protein Tax recruits transcription factors, resulting in accelerated mitosis. The multiple integration of multiple viral copies in the host genome promotes the structural damage of DNA[24]. The HBZ protein activates the alternative pathway NF-kB, promoting proliferation and activation of the transcription of E2F1 that increases the viral load and the action of telomerases[25].

Viruses Related to Tumors of Epithelial Origin

Nasopharyngeal and Oropharyngeal carcinoma: it is a distinctive histological subtype of head and neck tumors, with variants such as keratinized and non-keratinizing carcinoma[26]. This in turn, has a subtype with abundant lymphoid infiltrate, associated with virus infection. EBV infects epithelial cells by transforming beta 1 growth factor, to then activate type II latency and express EBNA1 and LMP1 and LMP2A. This causes dysregulation of proliferation through MAP-kinase, c-MYC and suppression of p13K and p16. EBV expresses BARTs, a family of multi-spliced transcriptional products of the viral genome, with high expression in infected epithelial cells. Its main function seems to be to attack non-coding cellular RNAs responsible for regulating genetic expression. BARTs produce multiple microRNAs that have apoptotic and anti-proliferative mechanisms[26].

Gastric carcinoma: It has been associated with bacterial infection by *Helicobacter pylori*[27], as well as with infection by EBV. Positive tumors for this virus occur in two histological types: conventional adenocarcinomas (16%) and gastric carcinomas with lymphoid and epithelial phenotype (89%)[26]. Tumors positive for EBV are characterized by infecting almost all tumor cells; they are detected by the presence of antigens EBE1 and 2, small non-coding RNAs, with oncogenic properties, such as efficient proliferation of transformed B cells and Activation of insulin-1-like growth factor as an autocrine factor for proliferation[27,28]. The infection is established in its type I or II latency form[15], with expression of EBNA1, BARTs, and LMP2A, which play an essential role in epigenetic abnormalities by promoting the methylation of PTEN (enzyme-suppressor tumor).

Table 2. Viral families, oncogenic viruses, genomics characteristics, transmissibility and pathogenesis.

Genre	Genome	Virus	IARC Group	Replication	Transmission	Cellular tropism	Primary infection
Lympho-crypto-virus	Linear double-stranded DNA	EBV HHV4	1	Lytic and latency. In the core. They produce: immediate mRNAs, they encode proteins that initiate and regulate viral transcription, early mRNAs encode nonstructural proteins involved in DNA replication, and late mRNAs encode structural capsid protein and envelope glycoproteins.	Saliva	B lymphocytes and epithelial cells.	Asymptomatic. Mononucleosis.
Rhadino-virus		KSHV HHV8	1		Sexual contact	B lymphocytes periphery mononuclear cells.	Asymptomatic
Orto-hepadna-virus	Circular double-stranded DNA	HBV	1	In the core. It uses the viral DNA polymerase to generate a complete circular chain and the cellular RNA polymerase for the transcription of the initial products (HBcAg, HBeAg and DNA polymerase of the virus and the pregenomic RNA) and then a reverse transcriptase that encodes the viral DNA.	Sexual, parenteral	Hepatocytes	Acute hepatitis and chronic 10%.
Hepaci-virus	Single chain RNA	HCV	1	In the cytoplasm. Income, denudation followed by translation into a polyprotein that is then fragmented, including a polymerase that directs the transcription and replication, for posteriation in the form of vesicles.	Sexual contact, parenteral	Hepatocytes, B lymphocytes, dendritic cells	Acute hepatitis and chronic 85%.
Deltaretro-virus	RNA	HTLV1	1	Through the reverse transcriptase copies the RNA into double-stranded DNA, which is integrated into the chromosome of the host and replicated with the cell as a provirus by the RNA polymerase of the host, thus producing genomic RNA and Spliced mRNAs, encode envelope glycoproteins and regulatory proteins.	Fluids with cells	T lymphocytes	Asymptomatic.
Polyoma-virus	Circular double-stranded DNA	MC	2A	In the nucleus, the transcription is carried out by the RNA polymerase of the host and leads to the synthesis of viral proteins, for their samplings and release when the cell dies.	Not clear. Maybe respiratory droplets.	Epithelial cells of hair follicles, Merkel´s cells	Asymptomatic.
Papova-virus	Double-stranded DNA	VPH	1	In the core. The RNA polymerase of the host transcribes the early genes, with subsequent synthesis of the early proteins, the DNA synthesis directed by the DNA polymerase of the host cell. The DNA of the virus can be integrated into the chromosomes of the host.	Sexual, mucosal contact.	Stratified epithelial cells	Warts, condyloma acuminatum, oral and laryngeal papillomatosis

Adapted from Sherris Medical Microbiology, 5th edition, Kenneth Ryan-George Ray, Mc Graw Hill editorial. Medical microbiology: Jawetz, Melnick and Adelberg, 25th edition, Mc Graw Hill publishing house. **IARC**: International Agency for Research on Cancer.

Table 3. Oncogenic virus, viral mechanism, cellular target.

Neoplasm	Virus	Oncogenic viral particle	Cellular target/ Cellular effects	Reference
HEMATOLINFOIDE				
B-cell Lymphoma	Epstein Barr	EBNA 1	Protooncogene translocation c-MYC (8-14), proliferation.	15, 16, 19, 30
Plasmablastic lymphoma			Proinflammatory cytokines	
Burkitt lymphoma			It prevents proteosomal degradation and presentation of antigens.	
			Facilitates the union and degradation of p53	
Hodgkin lymphoma		EBNA 3C	Cell immortalization	17
T/NK-cell lymphoma		LMP1	Active p13K y BCR. Deregulation of apoptotic pathways: JAK y JNK	15, 16, 17, 18
			Deregulation of proliferation: MAP-Kinasa	17
			NK cell activator	15, 16, 30
Leukemia/ Adult T-cell lymphoma			miR155, miR146: induces uncontrolled proliferation	16, 18, 19, 29
			miR21: down-regulation of tumor suppressors	16, 18,19
B-cell Lymphoma	Hepatitis C	E2	Bind to CD81 that activates CD19-CD21 and proliferation	19,20
Marginal zone lymphoma		Unknown	miR26b: activates BCL-2 (antiapoptotic, reduces caspases activity)	
		NS3 y E7	IL2, IL-10 increases somatic hypermutation	20, 21, 65
MALT			Translocation 14-18 overexpression of BLC-2	
No Hodgkin lymphoma	Herpes 8	vCyc	Integrates the host genome and generates cell expansion	17, 23
Leukemia/T-cell lymphoma	HTLV-1	Tax	Recruit transcription factors, activate the NF-kB and AKT pathways; it results in proliferation. Accelerates the cell cycle, causes structural DNA damage, inhibits repair and facilitates overduplication of centrosomes	17, 24, 65
		HBZ	Activates the alternative NF-kB pathway, which determines cell proliferation; Promotes the transcription of factor E2F1 and proliferation, increases the viral load and the action of telomerases.	24
EPITELIAL				
Nasopharyngeal carcinoma	Epstein Barr	EBNA 1	Protooncogene translocation c-MYC (8-14), proliferation.	15, 30
Gastric carcinoma			DNA metilation and suppression of p16	15, 27
Lymphoepithelial carcinoma		LMP1	Activate p13K y BCR. Deregulation of apoptotic pathways: JAK y JNK	26, 30
			Deregulation of proliferation: MAP-Kinasa	15, 30
Hepatocellular carcinoma	Hepatitis B and C	Pre-S2 deletion mutant proteins	TGF-β, IL1-β y TNF-α active JNK that increases the rate of cell proliferation	31, 34
		STAT3 y NF-kB	Antiapoptotic and regulate tumor angiogenesis	32
		Viral protein HBx	Activates mitogenic signals, generates chromosomal instability, increased metalloproteinase matrix production, which facilitates cell migration	4, 32, 33
		Viral protein HBVs	Mitochondria: inhibits JTB by increasing the life of the cell and preventing apoptosis	41
		Unknown	miR602: inactivation RASSF1A	19
	Hepatitis C	FNDC3B	Codifies a product that facilitates cell motility and metastasis	36
Merkel carcinoma	Merkel	T (large) antigen	Bind pRB, active proliferation	5, 38
		T (small) antigen	It binds and reprograms PP2A, promotes cell cycle and transformation.	
Cervical	HPV	E6	Degrade p53, with deregulation	39, 41, 43
Skin: Baso- and Squamous-cell carcinoma		E7	Degrade pRB	39, 43
Oropharyngeal Carcinoma			Suppression of miR203 that activates TP63 by promoting proliferation	19
Squamous neoplasm of the ocular surface			Induces telomerase activity, increasing the life of keratinocytes	9
		Unknown	miR100: inhibits PLK1 gene, promotes early carcinogenesis	19, 43

Neoplasm	Virus	Oncogenic viral particle	Cellular target/ Cellular effects	Reference
MESENQUIMAL				
Leiomyo-sarcoma	Epstein Barr	EBNA 1	Protooncogene translocation c-MYC (8-14), proliferation.	5, 19, 30, 62
Follicular dendritic				
Mesenchymal neoplasm				
Kaposi Sarcoma	Herpes 8 - Kaposi	LANA-1	Inhibit p53 y pRB	47, 49
		Prox-1	Causes lymphoendothelial differentiation.	
		vFLIP,	Induces endothelium-mesenchymal transition. Responsible for fusiform morphology.	
		kaposins A, B, C y ORF K1	Tumorogenesis promoter	
		miR-K12-1	Arrest p53	19
		miR-K12-3 y miR-K12-7	Active secretion of IL6 and IL10, which promote cell growth, angiogenesis and suppression of T cells.	19

Lymphoepithelial carcinoma: It is a poorly differentiated carcinoma with dense lymphocytic infiltrate[26]. It has been described in locations such as stomach, esophagus, tonsils, salivary glands, parotid[29], thymus, lungs and intrahepatic biliary epithelium[30]. The poor differentiation of these epithelial cells and an inflammatory environment are the oncogenic characteristics of the Epstein Barr virus, characterized by type II latency that interrupts multiple cellular processes and signaling pathways mentioned above.

Hepatocellular carcinoma (HCC): This carcinoma has low development associated with fibrosis. Epidemiologically it has been related in 80% of the cases with the chronic infection by the Hepatitis B (HBV) and the Hepatitis C virus (HCV), turning the infection into one of the most important risk factors. In the natural process of viral hepatitis, cirrhosis is not reached until 20 years after infection and oncogenesis takes at least 10 more years[31]. These viruses are widely distributed throughout the world and have a range of oncogenic mechanisms[32] as follows:

a. The inflammatory microenvironment that promotes the activation of the transforming growth factor beta (TGF-β) stimulates mitogenic factors such as JNK (of the MAPK family), producing rapid cell division with decreased genetic repair. Other endogenous inflammatory products such as IL- 1β, TNF-α and IL-6 (which generate resistance to apoptosis), are capable of activating these pathways. TGF-β maintains a relationship with pSmad3C under normal conditions, but when the latter is persistently phosphorylated, a mitogenic effect occurs and inhibits the apoptosis of transformed cells, a critical step for the progression of malignancy[31].
b. The induction of oxidative damage in lipids, mitogenic proteins such as MAPL, P13K, p53 and β-catenin, and in DNA. These reactive oxygen species induce intracellular calcium signaling, which increases their mitochondrial concentrations by activating STAT3 as antiapoptótico[32].
c. The insertion of viral DNA into the genome of the host in early stages of infection, which in some cases results in major genetic alterations, such as genomic instability, deletions, amplifications and chromosomal translocations. Multiple studies have described common integration sites, generally close to segments that control proliferation, survival, differentiation and immortalization, with advantages for tumorigenesis such as production of mutated HBx and mutated PreS2[32].
d. The products of the virus like Hbx and PreS / S. Hbx is a viral protein that activates aforementioned mitogenic factors. It also binds directly to p53 inhibiting apoptosis; it has been identified as a paracrine activator of stellate cell activation, promotes cell migration by increasing intracellular metalloproteinase and angiogenesis when activating HIF1 that activates VEGF and ANG2[32]. PreS / S is a viral antigen retained in the endoplasmic reticulum of the hepatocyte that generates oxidative stress, this retention activates cyclin A and therefore proliferation, as well as the over-duplication of centrosomes. It also activates hTERT that increases the activity of telomerase, generating telomere instability and even polyploidy and inhibits JTB by increasing cell motility, facilitating metastasis[32,33]. PreS2 also interacts with c-Jun and hyperphosphorylates pRB[34].
e. The aberrant methylation of promoter areas (CpG islands), which inactivate tumor suppressors in early stages of the disease. The enzyme DNMT is in charge of maintaining methylation patterns of the cell, with an up-regulation of this enzyme by Hbx. It has a preference for methylation near tumor suppressors such as RASSF1A, p16, p21, as well as adhesion controllers cellular such as E-cadherin, which, when inhibited, facilitates tumor metastasis[32].

The control of the microRNAs: Multiple interaction between the virus and these non-coding particles[22] have been established. The following are highlighted: miR602 attacks RASSF1A; miR143 usually considered a tumor suppressor, which contributes to metastasis by over expression, inhibiting the expression of FNDC3b of the fibronectin 3 gene, whose product regulates cell motility[19]. HBV and HCV share multiple mechanisms of transformation and tumorigenesis, with small diffe-

rences; one of them is the inhibition of the tumor suppressor of promyelocytic leukemia (PML)[35,36]. This possesses nuclear domains (PML-NBs), involved in the regulation of apoptosis, cellular senescence and antiviral response. It has been shown that HCV nuclear proteins interfere with multiple regulatory host particles including p53 by attacking their coactivator PML.

Merkel's carcinoma: This is one of the most aggressive and lethal types of skin cancer in up to 30% of patients. It occurs mostly in immunocompromised patients[37]. In 2008 its relationship with the virus of the polyomaviridae family was found. The Merkel cell carcinoma virus (MCV) has two oncoproteins; the long T (LT), which binds to pRB stimulating proliferation and the small T protein (Ts) which binds and reprograms the PP2A protein, to induce cell cycle progression and transformation. It also activates cell translocation factors such as 4E-BP1 that favor integration and genetic instability[37,38].

Oropharyngeal squamous cell carcinoma: This is a unique clinical entity, characterized by the anatomical location in the junction of soft and hard palate to the hyoid bone, including the base of the tongue It is associated with the human papilloma virus (HPV). It affects a heterogeneous population with determined risk factors and it is related to high-risk serotypes (HPV 16 and 18)[39] such as cervical carcinoma[40]. The virus has a common pathogenic pathway characterized by an advance in malignancy in which each time there is amount of virus present. Dysplastic changes are found in early stages, followed by carcinomas in situ, to culminate in infiltration of squamous cell carcinoma. It is noteworthy that oropharyngeal cancers are reported with viral changes for HPV 6 and 11, usually considered low risk. It is postulated that their behavior is not associated with benignity in this location[39].

The HPV has certain viral proteins with different functions during the infection and progression of the disease. E1 is essential for cellular regulation and thus, it supports viral replication. E2 is inactivated to increase the number of viral and epithelial cells with the integrated viral genome. The viral oncoproteins E6 and E7 cause genomic instability by coupling to the host genome and inactivate and/or reduce the half-life of p53 and pRB respectively. This translates into a lack of control during the cell cycle and the activation of cell signaling that causes uncontrolled cellular differentiation and proliferation by increasing the activity of p16[41,42]. The oncoproteins in turn have effects on the miRNA. E7 suppresses the activity of miR203 in epithelial cells, promoting proliferation and it is believed to facilitate viral replication, forcing the cell into the S phase, where the viral DNA is easily amplified[39]. E6 inhibits miR219 which overstimulates the LAMB3 gene involved in cell migration and tumorigenesis[17]. Pedroza et. al, relates other microRNAs related to early carcinogenesis, such as miR100, which promotes the activation of the PLK1 gene that promotes early carcinogenesis, as well as miR10a, miR196a and miR132 that are upregulated and contribute to cell transformation through the HOX and miR886-5p overexpression. This inhibits the process of apoptosis through the BAX gene[43].

The HPV has also been associated with squamous neoplasms of the ocular surface (pterygium, papilloma, carcinoma in situ, conjunctival intraepithelial neoplasia), which comprise the same phases of pre-malignancy and malignancy[44] and an oncogenic pathway similar to that previously described. However, the clinical evidence reported is scarce and is basically explained by two mechanisms: suboptimal study designs and techniques to identify HPV in biological specimens[45].

The role of HPV in keratinocytic carcinomas such as basal cell carcinoma and squamous cell carcinoma of the skin is not fully understood. Multiple clinical, epidemiological and experimental studies suggest a carcinogenic role of type B HPV in the development of these malignancies. This includes the viral characteristics (presence of E6 and E7 that degrade tumor suppressors and activate telomerase) and other factors such as exposure to UV radiation and the response of immunocompromised patients. That is, a patient with a deficient immune system[46] has more viral copies that cause damage to the DNA and prevent its repair, blocking in turn apoptotic pathways, these added factors culminate in the development of cancer[9].

Tumors of Mesenchymal Origin

There are multiple presentations of this type of malignancy, such as leiomyosarcoma, follicular dendritic cell sarcoma and myopericytoma[30] that have been related to the Epstein Barr virus and the nuclear antigen EBNA-1, which mediates oncogenesis, mainly by the translocation of the c-MYC protooncogene and the subsequent proliferation.

Herpes virus 8 or Kaposi's sarcoma virus induces transformation of mesenchymal cells, through different routes. LANA-1 binds to tumor suppressor p53 and inhibits the ability to induce apoptosis, as well as the pRB controller to modulate the cell cycle transition from G1 to S, where it allows replication and viral latency[47,48]. Prox-1 and vFLIP induce the lymphoendothelial and endothelial-mesenchymal transition resulting in the fusiform morphology of the cells. Kaposins A, B and C, and the K1 ORF are tumor promoters[49]. Lin and Flemington, describe the viral microRNA K12-1, which allows the increase of the cell cycle speed when arresting p53, K12-3 and K12-7. This selectively activate the secretion of IL6 and IL10 promoting cell growth, the angiogenesis and the suppression of T cells[19].

In this review, seven viruses are reported as causal agents of cancer in humans, but the literature proposes some others that have viral characteristics compatible with the oncogenic mechanisms described above, along with epidemiological and experimental associations that sustain them[5,50,51]. Among them, the Cytomegalovirus of the herpesviridae family are found as potential causal agents of CNS malignancies (gliomas and multiform glioblastoma)[52], colon and prostate carcinoma[53,54]. JC and BK viruses of the polyomaviridae family are causal agents of tumors of the central nervous system[1,54] and colorectal carcinoma[50]. The postulated mechanisms for tumorigenesis are explained in Table 4. There has been descri-

Table 4. Viruses and Virus y possible oncogenic mechanism without enough evidence.

Neoplasm	Virus	Oncogenic viral particle	Cellular target/ Cellular effects	Reference
Glioma	Cytomega-lovirus	Prolonged infection of monocytes and macrophages.	TAMs, releasers of IL6, IL10 TGF-β, that activate proliferation by means of STAST 3	51
		Gen US3	Retention of MHC I in the endoplasmic reticulum	
		US28	Promotes cellular migration by RANTES and MCOP-1	51, 54
Colon Carcinoma		UL 3	It binds to caspase-8 and inhibits Fas-mediated apoptosis.	51
		UL37	Inhibits proapototic pathways, Bax and Bak.	51, 54
Prostate Carcinoma		Gen UL83	Produces ppg65 that blocks the presentation of antigens	51
		IE1	Mutagenics, break into DNA repair interfering with RM and ATR.	
			Delete p53 and pRb	
		IE2	Disable p53	
		mirR-UL112	Suppress the expression of MHC I	
Glioblastoma multiforme		RNABeta 2.7	Mitochondria, prevents apoptosis.	
		UL36	Inhibits Fas-mediated apoptosis	54
		UL123/124	Inhibits apoptosis by activating PI3 kinase.	
		Unknown	Stimulates hTERT increases the activity of telomerase	
		Unknown	Induces CD40 expression by increasing proliferation cell signaling	
Oligo-dendorglioma, astrocytoma, ependymoma, medullo-blastoma.	Polyoma-virus (JC y BK)	TAG	Inhibits Rb, releasing E2F. Inhibits p53	54
		tAG	Deregulates WTN of β-catenin and stimulates gene expression.	
			Activates PP2A, affects the cytoskeleton and promotes migration.	

be another virus linked to the development of breast cancer like bovine leukemia virus (BLV), mouse mammary tumor-like virus (MMTV) both retroviruses[55] their oncogenic mechanism still no clear, there is substantial information, but cannot be regarded as conclusive[56] more over in recent studies has been demonstrated the interaction of different types of virus, Drop et. al.,[57] describes confection between 22-34%, involucre tow or more of the following viruses: BKV, HPV, CMV, HSV and EBV, this combinations can induce transformation or ether exhibit a more rapid developing; so that associated with esophagus, prostate, bladder, breast, lung, colon and even central nervous system[58], not even this association are no documented properly.

Discusion

Pathologies of viral origin begin at the time of infection, although this is only one of the steps for the development of malignancy. The process of cellular transformation begins with the deregulation of cell signaling induced by the virus, stimulating oncoproteins that tend to have self-sufficiency in replication and adaptation, insensitivity to inhibitory and apoptotic signals, as well as to angiogenesis, tissue invasion and metastasis[59,60]. These provide proliferative advantages even under conditions of nutrients and poor oxygen[61].

The Epstein Barr virus is the oncogenic prototype, one of the most ubiquitous and successful known viruses. It has developed strategies to infect multiple cell types, evade the immune system, develop viral latency and contribute to the development of malignancies in the three types of tissue mentioned in this review (lymphoid, epithelial and mesenchymal), to the point that 1% of human cancer types are related to these agents[7,30,62].

Viral infections are one of the risk factors for the development of cancer. Research and understanding of the pathogenic mechanisms will allow the development of strategies for the prevention of these malignancies. The development of effective vaccination strategies, as reported by Stanley in a recent review about hepatitis B virus infection, indicating that there are approximately 250 million people infected and about 887,000 deaths in 2015. In addition, it appears that up to 50% of children infected at an early age develop cirrhosis or carcinoma hepatocellular. The implementation of the universal vaccination program in 1992, resulted in a decrease in the rate of carriers of the virus. Regarding the human papillomavirus, approximately 290 million women are estimated to be infected. In an optimal scenario, in countries with more than 50% vaccine coverage, the v2VPH v4 HPV vaccine could reduce the incidence of cervical cancer by 70%, and the v9VPH could reduce it by 90%. The infections by HPV16 and 18 were decreased by 68%, with a reduction of cross infection by strains 31, 45 and 33[63]. In the near future, specific treatments should be implemented, perhaps through vectors of genetic therapies or by the use of oncolytic therapy agents[64,65].

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REPORTE DE CASO

Leclercia adecarboxylata, a rare cause of soft tissue infections in immunocompromised patients, case report and review of the literature

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Abstract

Leclercia adecarboxylata is a member of the *Enterobacteriaceae* family that has been isolated from several environmental and animal specimens, however it rarely causes diseases in human beings. It has natural resistance to several antibiotics, and has shown the ability to harbor and produce enzymes capable of hydrolyzing most of the antibiotics used in daily clinical practice, making its treatment a challenge when a strain with such characteristics causes disease. Here we report the first known case of infection by *Leclercia adecarboxylata* after a trauma with plant material, in a 69-year-old male patient, with poorly controlled Diabetes Mellitus type 2.

---Resumen---

Introduction

Leclercia adecarboxylata is a member of the *Enterobacteriaceae* family, it is phenotypically similar to *Escherichia coli*, differentiated in 1986 by Tamura et al[1,2]. It is thought to be present widely in nature and it is a rare cause of disease in humans, with an antimicrobial susceptibility ranging from natural resistance to multiple antibiotics[3], to multidrug resistance due to the production of several enzymes[4-6]. We report the first known case of infection by this microorganism related to trauma with plant material, a review of reported cases around the world with interesting clinical manifestations, natural antimicrobial susceptibility and acquired resistance patterns are also presented.

Case Report

A 69-year-old male patient, with medical history of Diabetes Mellitus type 2, presented to the emergency department of a hospital in Bogotá, Colombia, after losing his postural tone and hitting his head to the ground, with no consciousness loss, motor or sensitive alterations, vomiting or other relevant symptoms afterwards. At initial examination, the patient had a heart rate of 71 beats per minute, a respiratory rate of 19 breaths per minute, blood pressure of 169/97 mmHg, temperature of 36°C, blood glucose of 350 mg/dL, a hematoma on the right frontal zone of the head and a 4*4 cm erythematous, indurated, painful lesion with abundant purulent secretion, that limited the metatarsophalangeal flexion of the third, fourth and fifth fingers of the right hand, with no other relevant findings.

The patient was then asked about the injury on his right hand, and he referred that five days before consultation, he was at his country house (Carmen de Apicalá, Tolima, Colombia) carrying some wooden logs, when he stumbled and fell, hurting his right hand with the logs he was carrying, with subsequent abundant bleeding and edema; the patient grabbed his medic kit and cleaned the lesion with hydrogen peroxide (H2O2), he checked for foreign objects and applied

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Figure 1.

a bandage on his hand. Three days before consultation, the patient noticed edema, pain, fetid smell and movement limitation of his right hand. After removing the bandages, he found blood stains and purulent secretion, he drained the lesion and massaged his hand with an ointment made of coke and marihuana leaves; on the day of consultation, although it was not the reason for his attendance to the hospital, the lesion had progressed, as described before.

On the emergency room, they started intravenous Oxacillin, 2gr every four hours, in continuous infusion, received Tetanus prophylaxis (single IM dose) and requested an X-ray from his right hand, which had no abnormalities, a Computed Axial Tomography of the head, both with no abnormalities, and blood analysis (Table 1).

The patient was assessed by the orthopedist, who considered he was a candidate for surgical drainage and debridement, he suspended the infusion of oxacillin and started intravenous Ampicillin/Sulbactam, 1.5gr every 6 hours, and ordered his hospitalization. During the surgical procedure, an abscess of 8cc of purulent secretion was drained, a small foreign object (apparently vegetal) was extracted, samples for culture were taken and the wound was washed thoroughly with 1000cc of 0.9% saline solution and H2O2, finally the wound was sutured and covered with a nitrofurazone gauze and a bandage.

The patient had fluctuant glycemia, thus he was assessed by the internist, who started basal insulin therapy, with an adequate response. Five days after the surgical intervention, *Leclercia adecarboxylata* was identified using the VITEK® 2 system healthcare with a probability of 96% showing a natural antimicrobial resistance pattern (Table 2). We tested the isolated strain 4 times in our laboratory for certainty, all of which resulted positive for the same microorganism. The patient completed 10 days with intravenous Ampicillin/Sulbactam 1.5 grams IV every 6 hours, he had no need for a second surgical intervention, achieving clinical recovery and recovering full functionality of his hand.

The strain was sent for extra institutional confirmation to bioMérieux in Bogotá, Colombia, where they used a Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometer (MALDI TOFVITEK® MS) to confirm the results yielding a trust percentage 99.6% and thus confirming the identification of L. adecarboxylata.

Discussion

Leclercia adecarboxylata, described by Leclerc in 1962 and first designated as "Enteric group 41" or "*Escherichia adecarboxylata*", is a Gram-negative, oxidase-negative, indole-positive, facultative-anaerobic, motile bacillus, member of the *Enterobacteriaceae* family, that is phenotypically similar to *Escherichia coli*[1,3,7]; it was differentiated in 1986 by Tamura et al. through DNA hybridization, biochemical and computer iden-

Table 1. Blood Analysis

	Hospitalization Day	Completion day of antibiotic therapy	Reference Values
Blood Glucose	219 mg/dL	254 mg/dL	70 – 115 mg/dL
Serum urea nitrogen	17.7 mg/dL	–	6 – 20 mg/dL
Serum creatinine	0.92 mg/dL	–	0.7 – 1.2 mg/dL
Serum sodium	134 meq/L	–	136 – 145 meq/L
Serum potassium	3.68 meq/L	–	3.5 – 5.1 meq/L
Serum chloride	101.6 meq/L	–	98 – 107 meq/L
White Blood Cells	7000 mm^3	7900 mm^3	4500 - 11000 mm^3
Neutrophils	3730/uL	5050/uL	1900 – 8000/uL
Lymphocytes	2400/uL	2100/uL	900 – 5200/uL
Hematocrit	37.7 %	43.8 %	41 – 50 %
Hemoglobin	13.1 g/dL	15.4 g/dL	13.5 – 16.5 g/dL
Platelets	220000/uL	259000/uL	150000 – 450000 /uL

Table 2. Leclercia adecarboxylata antibiogram

Antibiotic	MIC	Interpretation
Amikacin	≤ 2	S
Ampicillin/Sulbactam	≤ 2	S
Cefepime	≤ 1	S
Cefoxitin	8	S
Ceftazidime	≤ 1	S
Ceftriaxone	≤ 1	S
Ciprofloxacin	≤ 0.25	S
Ertapenem	≤ 0.5	S
Gentamicin	≤ 1	S
Imipenem	≤ 0.25	S
Meropenem	≤ 0.25	S
Piperacillin/Tazobactam	≤ 4	S

MIC: Minimal inhibitory concentration; S: Sensitive

tification tests, resulting in a new genus[1,2]. *L. adecarboxylata* is thought to be distributed widely in nature, present in food, water sources, egg shells, milk, industrial fat and oil reservoirs and as a commensal of the gut flora of some animals[1,3,7-9]; it has also been recovered, uncommonly, from human samples like blood, bone[10], cardiac valve, wounds, bronchial wash[11], peritoneal fluid, urine, gall bladder, synovial fluid, abscesses and epididymo-orchitis, from immunocompromised and immunocompetent patients, being the former the most relevant and frequent scenario, as the case of our patient, and the later generally but not always associated to polymicrobial infections, suggesting the dependence of this bacteria on other microorganisms to infect immunocompetent hosts[1,7].

Zapor et al.[7] reported the case of a 25-year-old soldier who was wounded in Afghanistan by an improvised explosive device, with a subsequent prolonged hospital stay and multiple surgical interventions; after being discharged the patient consulted again due to limited right hip range of motion, during surgical intervention they found a focal collection in the right buttock with the isolation of *L. adecarboxylata* by BD Phoenix Automated Microbiology System and confirmed by 16s ribosomal sequencing, due to in-vitro resistance of the strain to Amoxicillin/Clavulanic acid, Ampicillin/Sulbactam and Cefazolin, the patient was successfully treated with orally Ciprofloxacin, 750 mg twice daily, for 10 days. Keren et al.[12] reported the case of a 46-year-old male patient who suffered a laceration at the dorsum of his left foot with a surfboard fin while surfing, developing a soft-tissue infection, with the identification, using VITEK® 2 systems as in our case, of *L. adecarboxylata* and *Enterobacter cloacae*, successfully treated with oral Ciprofloxacin for 14 days. Sanchez Porto et al.[2] reported the case of a 56-year-old female with Metabolic Syndrome and Diabetes Mellitus type 2, with a burn wound located in the left pre-tibia extremity with an open ulcer and signs of infection, with the identification in three sets of blood culture and a swab culture of the exudate of *L. adecarboxylata* by Microscan Automatic identification System, confirmed by API 20E System (bio-Mérieux, Marcy l'Etoile, France), successfully treated with Amoxacillin/Clavulanic acid 1 gram IV every 8 hours for seven days, then orally 875/125 mg every 8 hours for 7 additional days. Corti et al.[13] reported the case of a 37-year-old male patient that was involved in a major motor vehicle accident with multiple injuries, including Gustillo-Anderson type II open fracture of the left distal femur, type IIIB open fracture of the left distal tibia with bone loss and mangled left foot with multiple fractures and extensive soft-tissue involvement, no farm contamination expected as he was rescued on the road, with the isolation from several surgical samples of the wounds of *L. adecarboxylata* and *Enterobacter amnigenus*, successfully treated with Piperacillin/Tazobactam 4.5 grams three times a day. To our knowledge, our patient is the first report of infection by *Leclercia adecarboxylata* after a trauma with plant material (logs), in an immunocompromised patient (Diabetes Mellitus type 2), not associated to other microorganisms, successfully treated with a course of 10 days of Ampicillin/Sulbactam 1.5 grams IV every 6 hours for 10 days.

L. adecarboxylata has natural resistance to fosfomycin[3]. Natural resistance of *L. adecarboxylata* to fosfomycin may represent an additional parameter for its reliable phenotypic separation from *E. coli*.[15,16] In the case of our patient, we isolated a strain with a usual susceptibility pattern, however there are reports in the literature of *L. adecarboxylata* strains expressing diverse acquired resistance mechanisms. Sun et al.[6] describe the production of plasmid-encoded NDM-1 from a *L. adecarboxylata* strain (P10164) isolated in 2012 from the sputum of a 43-year-old male with pneumonia in Chongqing City in China, which not only harbored the bla_{NDM-1} gene, but also

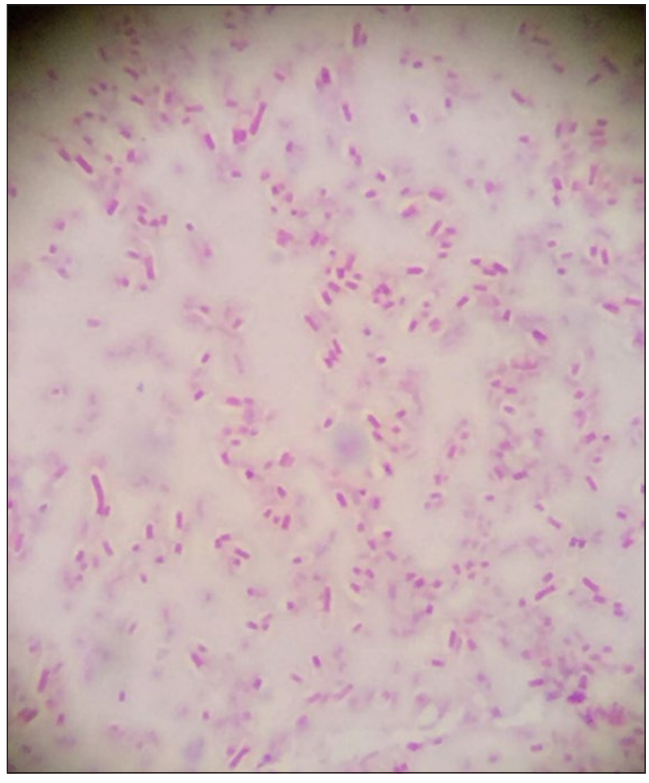

Figure 2. Gram Stain: 40x. Gram negative bacillus from tissue culture.

other genes conferring resistance, as $bla_{CTX\text{-}M\text{-}1}$ group, bla_{TEM} and $bla_{OXA\text{-}1}$ group genes and showed in-vitro resistance to all of the tested drugs. Papagiannitsis et al.[5] during a study of hand hygiene among the staff of the Na Homolce Hospital, Prague, Czech Republic, found a strain of *L. adecarboxylata*, identified by matrix-assisted laser desorption–ionization time of flight mass spectrometry and confirmed through 16s rRNA gene sequence, with a plasmid that contained the $bla_{VIM\text{-}1}$ gene, with the ability of conjugal transfer to the *E. coli* A15 strain. Shin et al.[14] isolated a strain of *L. adecarboxylata* from blood cultures of a patient with breast cancer with a peripherally inserted central catheter for chemotherapy administration, identifying through PCR assays the presence of $bla_{TEM\text{-}1}$, $bla_{CTX\text{-}M\text{-}3}$ and *intl1* with *dfrA12* and *aadA2* (resistance to trimethoprim-sulfamethoxazole and aminoglycosides, respectively) genes in such strain. Finally, Geffen et al.[4] isolated a strain of *L. adecarboxylata* in Laniado Medical Center, Israel, from rectal surveillance cultures of patients, identified using VITEK-2 system and confirmed by 16s rDNA aequencing, with a plasmid carrying the $bla_{KPC\text{-}2}$ gene.

Conclusion

Although *Leclercia adecarboxylata* is an uncommon infecting microorganism, it must not be forgotten in the clinical and epidemiological setting, as it is capable of carrying and expressing different mechanisms of resistance, reducing therapeutic options and threatening to alter the antimicrobial susceptibility of the flora in healthcare facilities and the community, as it has been isolated from the skin of healthy human beings, resulting in the spread of the microorganism and, if hand sanitation adherence is not adequate, its acquired resistance genes.

It is possible that infections caused by this microorganism have been under-reported due to its similarities with *Escherichia coli*, a situation that gains relevance in the setting of urinary tract colonization/infection, as the innate resistance of L. adecarboxylata to fosfomycin, threatens the effectivity of therapeutic/prophylactic regimens with this drug.

To our knowledge, this is the first reported case of the association between trauma with plant material (logs) and infection by *Leclercia adecarboxylata* in an immunosuppressed patient. It is important to take into account this way of inoculation, being a rather common event in the daily routine of thousands of people around the globe, that added to an underlying condition, immunosuppression or even an immunocompetent state, can result in a soft tissue infection as the case of our patient, a bone infection or even pneumonia or endocarditis.

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Ethical disclosures

Protection data of people and animals. The authors state that for this investigation have not been performed experiments on humans or animals.

Confidentiality of data. The authors declare that this article does not appear names or data of the patient.

Right to privacy and informed consent. The authors declare that this article does not appear patient data.

Conflict of interest

The authors declare no conflict of interest.

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