UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

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DOCTORADO EN CIENCIAS VETERINARIAS

EVALUACIÓN DE UNA VACUNA SINTETICA CONSTITUIDA POR TRES PEPTIDOS (KETc-1 KETc-12 Y GK-1), CONTRA LA CISTICERCOSIS PORCINA POR *Taenia solium* EN CONDICIONES NATURALES DE TRANSMISIÓN.

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MIRNA AUREA HUERTA OREA

Tutores:

ALINE S. DE ALUJA
EDDA SCIUTTO CONDE
GLADIS FRAGOSO GONZALEZ

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Por

MIRNA AUREA HUERTA OREA

ASESORES:

DRA. ALINE S. DE ALUJA

DRA. EDDA SCIUTTO CONDE

DRA. GLADIS FRAGOSO GONZALEZ

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DEDICATORIAS

A mis padres por que gracias a ellos y a su gran amor me dieron la vida, así como sus sueños, sus lagrimas y su alma Ing. Quim. Pedro Huerta Ruiz + Sra. Margarita Orea Vda. De Huerta.

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A mis familiares, amigos y compañeros de trabajo.

La vida es un constante movimiento
caracterizado por múltiples actividades
Como son: sorpresa, alegría, infortunio
Más día a día nos hacemos la pregunta.
¿Qué es la vida?, ¿ Cuál es el objetivo de esta?
¿Qué camino es el adecuado? y si lo elegimos
¿Qué tan buena es la elección que se ha escogido?
Lo importante es intentarlo día a día luchando
No importan tantos tropiezos y caídas, hay que levantarse
Y lograr esas metas qué nos trazamos.
Ya qué la vida es lucha.

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Resumen.

La cisticercosis por *Taenia solium* causa graves problemas de salud humana y pérdidas cuantiosas en la porcicultura en países en vías de desarrollo. En este estudio evaluamos la capacidad protectora de una vacuna sintética exponiendo los cerdos a condiciones naturales de transmisión, en comunidades rurales de alta prevalencia de cisticercosis porcina. La vacuna esta compuesta de tres péptidos protectores denominados KETc1, KETc12, y GK-1 de 12, 8, y 18 aminoácidos respectivamente, identificados en una librería de cDNA de *Taenia crassiceps* y en *T. solium*. Los cerdos rústicos utilizados nacieron y se mantuvieron en corrales en condiciones controladas donde se inmunizaron con los tres péptidos y saponina como adyuvante a los 60 y 90 días de edad. Al grupo control solo se le aplicó adyuvante (saponina). El experimento fue realizado en forma ciega. Después de la vacunación los cerdos se distribuyeron por pares, un inmunizado y un control en casas de habitantes de comunidades con alta prevalencia de cisticercosis porcina (14%). A todos los cerdos se les tomó muestra de sangre durante la experimentación. Se sacrificaron entre los 10 y 12 meses de edad y se inspeccionaron minuciosamente en busqueda de cisticercos. La mitad de cada canal fue inspeccionada para determinar el número y el estado histológico de los cisticercos. De un total de 120 cerdos controles, 18 se encontraron infectados en músculo esquelético con un total de 66563 cisticercos y se observó microscópicamente que el 3.9% de los cisticercos recuperados presentaban daño (caseosos y calcificados). En los 120 cerdos vacunados, 9 estuvieron infectados con un total de 1369 cisticercos, de estos 41% de los cisticercos obtenidos resultaron caseosos y/o calcificados. El daño inflamatorio que mostró la observación histológica en los inmunizados fue de 84.1 % y en los controles 19.3%. Se observó un incremento significativo de los niveles de anticuerpos después de la vacunación. La vacunación redujo el porcentaje de cerdos infectados, el número de cisticercos instalados y su viabilidad.

Abstract

Taenia solium cysticercosis seriously affects human health when localised in the central nervous system (CNS) and causes great economic loss in pig husbandry in rural areas of endemic countries. Increasing the resistance to the parasite in the obligatory host pig may help in curbing transmission. Three synthetic peptides based on protein sequences of the murine parasite Taenia crassiceps, which had previously been shown to induce protection in mice against homologous challenge, were tested as a vaccine against T. solium cysticercosis in pigs. Vaccinated and unvaccinated piglets (240 in all) were distributed in pairs among the peasants' households of two rural villages in Mexico in which 14% of the native pigs were cysticercotic. Ten to twelve months later, the effect of vaccination was evaluated at necropsy. Vaccination decreased the total number of T. solium cysticerci (98.7%) and reduced the prevalence (52.6%). The natural challenge conditions used in this field trial strengthen the likelihood of successful transmission control to both pig and human through a large-scale pig vaccination program. We believe this is a major contribution in anticysticercosis vaccine development as these rather simple yet protective peptides are potentially more cost-effective to produce and less variable in results than antigens that are more complex.

Key words: cysticercosis, synthetic peptides, vaccines

Abbreviate article title: T. solium cysticercosis vaccination

I. INTRODUCCIÓN

La cisticercosis causada por *Taenia solium* es una parasitosis frecuente en humanos y en cerdos de países que se encuentran en vías de desarrollo, donde prevalecen condiciones que favorecen su transmisión, como son la falta de higiene y fecalismo al aire libre, aunado a la crianza rústica de cerdos y consumo de carne sin la inspección sanitaria adecuada (Gemmell *et al.,* 1982, Aluja *et al.,* 2000). Dentro de las zonas más afectadas se encuentran América Latina, Asia y África (Sotelo *et al.,* 1996) en donde esta parasitosis causa serios problemas tanto de salud como económicos (Larralde *et al.,* 1992). Aún cuando la cisticercosis ha sido prácticamente erradicada en los países desarrollados, esta enfermedad ha comenzado a reaparecer, reportándose en países como Estados Unidos, debido a la afluencia de inmigrantes que provienen de zonas endémicas (Richard *et al.,* 1991; Rosenfeld *et al.*, 1996). Más aún, publicaciones recientes indican que algunos países de Europa no se encuentran totalmente libres de esta parasitosis (Tamburrini et *al.,* 1995).

En México es un padecimiento frecuente, donde las zonas de mayor seroprevalencia en seres humanos se encuentran en las regiones centro occidental y en el sureste del país (Larralde, 1992).

La Organización Mundial de la Salud (OMS) ha reportado un 1.9 % de defunciones por cisticercosis cerebral y un 3.5 % de cisticercosis en necropsias a nivel mundial (OMS, 1979). En la Figura 1 se ilustra la seroprevalencia en los distintos estados del territorio mexicano.

Tabla 1. **Seroprevalencia de anticuerpos contra el cisticerco de *Taenia solium*, reportados por entidad federativa en población abierta de 1 a 98 años (tomado de Larralde *et al*, 1992).**

Entidad	Población Muestral	Seropositividad No. de Población	Seropositividad Porcentaje
Baja California Sur	1 739	1	0.06
Sonora	2 251	5	0.22
Baja California Norte	1 605	5	0.31
Tabasco	2 958	10	0.34
San Luis Potosí	2 125	10	0.47
Tlaxcala	1 434	7	0.49
Nuevo León	3 174	16	0.50
Tamaulipas	1 937	10	0.52
Veracruz	2 257	12	0.53
Coahuila	1 997	12	0.60
Oaxaca	1 709	12	0.70
Sinaloa	2 292	17	0.74
Chihuahua	2 194	17	0.77
Querétaro	1 642	13	0.79
Campeche	1 541	13	0.84
Morelos	1 254	13	1.04
Chiapas	1 912	20	1.05
Hidalgo	2 042	23	1.13
Estado de México	2 837	34	1.20
Yucatán	1 775	23	1.30
Colima	1 703	23	1.35
Puebla	2 814	38	1.35
Michoacán	2 036	29	1.42
Quintana Roo	1 515	22	1.45
Aguascalientes	1 518	24	1.58
Durango	1 963	31	1.58
Nayarit	1 474	30	2.04
Jalisco	3 563	75	2.10
Guanajuato	2 970	66	2.22
Zacatecas	2 162	58	2.73
Distrito Federal	2 644	78	2.95
Guerrero	1 717	51	2.97
Total	66 754	799	1.20

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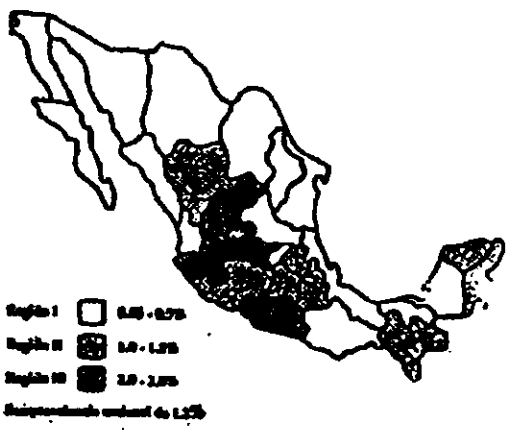
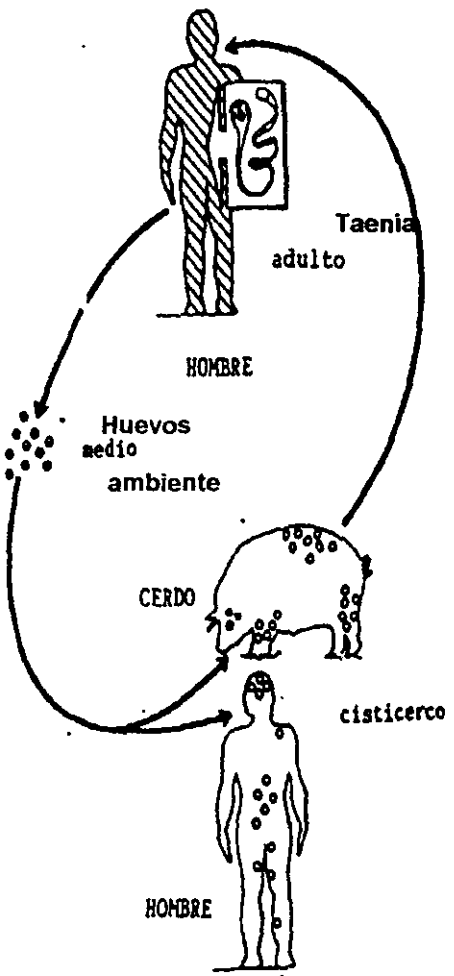


Figura1. Población de 1 a 98 años según Seroprevalencia de anticuerpos por entidad Federativa, México 1987-1988. Distribución De Seroprevalencia en la República Mexicana (Tomado de Larralde et al ., 1992).

2.1 Antecedentes Generales.

El ciclo biológico de este parásito incluye una fase larvaria (cisticerco) que afecta tanto a humanos (huésped definitivo) como a cerdos (huésped intermediario). La ingestión de huevos, expulsados al medio ambiente en las heces de pacientes teniósicos, produce la cisticercosis en humanos y cerdos. En seres humanos el parásito se aloja preferentemente en el sistema nervioso central (Lombardo. 1982), pero también se ha informado de su presencia en el tejido subcutáneo, en menor grado en el tejido muscular y en el ojo (Echeverría, 1952; Ochoterena, 1935) mientras que, en el cerdo el parásito se desarrolla tanto en todas las masas musculares como en el sistema nervioso central. Cuando el hombre ingiere carne de cerdo mal cocida que contiene cisticercos vivos, estos al llegar al intestino delgado, evaginan y el escólex se fija en la mucosa por medio de ventosas y ganchos. Aproximadamente después de dos meses de desarrollo, el parásito alcanza la forma adulta *(Taenia solium)* y comienza a producir millones de huevos completándose así su ciclo de vida (Figura 2). Debido a que los huevos se eliminan en las heces diariamente, la transmisión se favorece en zonas donde prevalecen bajos estándares de sanidad, de higiene personal, de control ambiental y donde el fecalismo al ras del suelo es la regla y los cerdos deambulan por los pueblos teniendo acceso a la materia fecal humana, que contiene segmentos o huevos de *T. solium* (Aluja 1985).

Figura 2. Ciclo de vida de *Taenia solium*.



Cuando el hombre come carne de cerdo infectada con cisticercos, éstos llegan al intestino delgado y se desarrollan a la forma adulta (tenia). Por reproducción sexual, la tenia produce millones de huevos que son expulsados al medio junto con las heces humanas. La disposición inadecuada de las heces causa la diseminación de los huevos al medio, los cuales pueden ser ingeridos por el hombre ó por el cerdo. La ingestión de los huevos en ambos, causa el desarrollo de cisticerco en diferentes tejidos. En humanos se establece principalmente en el cerebro y ojos. En cerdo, el parásito se instala tanto en músculo como en cerebro. El ciclo de vida del parásito se cierra cuando el hombre come carne de cerdo infectada con cisticercos, los cuales se transforman en la tenia adulta en el tracto intestinal.

El estadio larvario de este gusano puede afectar prácticamente todos los órganos y tejidos del cuerpo humano. Sin embargo se ha declarado que la cisticercosis es más frecuente en tejidos subcutáneos e intramusculares en Asia y África, mientras que en el continente Americano su localización más frecuente es en el sistema nervioso central . Los metacéstodos ocasionan lesiones parenquimatosas y/o subaracnoideas dependiendo de su localización. También se pueden encontrar en el ojo (Flisser *et al.* , 1982). Los síntomas que producen varían de acuerdo al número de parásitos en el tejido afectado y de su localización en tejidos nerviosos. Las alteraciones histológicas son muy variables en tipo, severidad, y localización topográfica. Entre los síntomas en pacientes humanos, muy frecuentemente asociados a neurocisticercosis, figuran la epilepsia, cefaleas intensas, mareos y convulsiones. Así, en zonas endémicas, un adulto que presente epilepsia tiene muy altas probabilidades de padecer neurocisticercosis. A pesar de la potencial gravedad de la localización en el sistema nervioso, llama la atención que aproximadamente el 50% de los pacientes neurocisticercosos no presentan síntomas ostensibles y la neurocisticercosis es un hallazgo de autopsia o casual (Rabiela, 1972). En el caso de la cisticercosis subcutánea u ocular, el parásito es más fácilmente identificable. El tiempo entre la infección inicial y la aparición de los síntomas es también muy variable y puede ser de algunos meses o hasta de años (Aluja *et al.* , 1986).

El cerdo, el principal hospedero intermediario de este parásito, desempeña un papel muy importante en la transmisión de la teniosis-cisticercosis por *T. solium.* La porcicultura rústica constituye un factor de suma importancia en la transmisión ya que la crianza de cerdos, se practica en zonas donde el fecalismo al ras del suelo es la regla. Algunos hogares disponen de letrinas pero, éstas con frecuencia están construidas de tal forma que los

cerdos tienen acceso a heces humanas lo que permite la persistencia del ciclo de éste parásito. Por el contrario, en la porcicultura altamente técnificada, en donde los cerdos son criados con alimento comercial y no tienen contacto con materia fecal humana, no existe la posibilidad de infección. Otro factor de suma importancia es que la inspección sanitaria es deficiente. Sin un estricto control sanitario de la carne, no se podrá evitar el consumo de carne de cerdo parasitada con cisticercos de *T. solium* y por ende no se podría controlar la transmisión de esta zoonosis.

Dentro de los principales factores responsables de un ineficiente control del consumo de cerdos criados rústicamente, figuran la matanza doméstica y el consumo directo de carne sin previa inspección. En particular algunos de los rastros municipales de Puebla y de pequeñas ciudades la inspección sanitaria, o bien no se lleva a cabo o se efectúa en forma superficial y en ocasiones corrupta. A los rastros "Tipo Inspección Federal" (TIF) de la SAGAR y en aquellos municipales en los que la inspección sanitaria se efectúa correctamente, los cerdos con cisticercosis no llegan, ya que los dueños e introductores saben que se los van a decomisar.

Debido a la falta de control de la teniosis-cisticercosis, el número de personas y cerdos afectados no disminuye y las derogaciones del gobierno para diagnosticar y darles tratamiento a seres humanos son considerables. El tratamiento en seres humanos que padezcan teniosis se puede hacer en forma masiva o sólo a personas infectadas, a través de la identificación y tratamiento de teniósicos (Sarti *et al.* , 1994). Sin embargo existen serias limitaciones para aplicar este tipo de estrategias considerando la dificultad para el diagnóstico de teniósicos. La reducción del riesgo de contraer teniosis, mediante campañas

educativas y sanitarias recomendada por (Sarti *et al.* , 1994) no se han llevada a cabo a gran escala hasta la fecha.

Se han estimado pérdidas a la porcicultura por cisticercosis del orden de USS $ 43,310,524.00 (Acevedo, 1982).

Dada la importancia de la cisticercosis en la salud humana y en la porcicultura en México, ésta ha sido objeto de numerosos estudios relacionados con aspectos clínicos y patológicos, así como con el diagnóstico, tratamiento y prevención. El tratamiento de cerdos con Albendazol (Escobedo *et al.*, 1989) ó con Praziquantel (Flisser *et al.*, 1990), es muy efectivo, pero poco práctico para el criador, ya que después de la administración del medicamento habría que esperar varias semanas antes de poder ofrecer la carne al consumidor lo que aqumenta el costo de las canales.

Keilbach *et al.*, 1989; Sarti *et al.*, 1998; Flisser *et al.*, 1990, han publicado sus experiencias en comunidades rurales. Flisser *et al.*, 1990; Sarti *et al.*, 1998 han comparado diferentes estrategias de control en 3 comunidades rurales detectadas. Los resultados claramente indican que la educación es el principal factor para crear conciencia en la población del peligro que la falta de higiene, en general, y la ingestión de carne infectada con el metacestodo, en particular, representa riesgo para su salud.

Debe tenerse claro que una campaña para controlar la teniosis-cisticercosis en un país debe comprender:

1. Educación de la población que incluye la higiene personal, higiene para preparar los alimentos e instalaciones obligatorias de letrinas en todos los hogares.
2. Inspección sanitaria de toda la carne destinada para el consumo humano.

3. Combate de la corrupción en la compra y venta de animales y sus derivados.

Entre las alternativas para controlar esta zoonosis no estrictamente relacionadas con la conducta humana figuran :

1. La irradiación de la carne con dosis bajas (0.3 kGy), según la Nom-033-SSA1-1993, es una tecnología ya bien establecida y permitida en muchos países, México entre ellos. Es otro método recomendable que tiene la ventaja de inactivar varios agentes patógenos al mismo tiempo (Aluja *et al.*, 1995; Flores *et al.*, 1996), lo que beneficiaría en forma importante a la salud de la población en general. Pero de un difícil aplicación en el medio rural.
2. La vacunación contra la cisticercosis porcina con resultados promisorios, ya publicados (cuadro 1).
3. Modificación genética de hospederos susceptibles para aumentar su resistencia innata mediante transferencia de genes de resistencia (Fragoso, *et al.*, 1998).

La alternativa para interrumpir el ciclo de vida del parásito por vacunación se ha considerado como posibilidad realista de interferir con la transmisión del parásito, disminuyendo la prevalencia de cisticercosis porcina. Esta medida permitiria interrumpir el ciclo de la parasitosis y reducir la enfermedad humana.

En la búsqueda de este objetivo resulta prometedor la existencia de una vacuna efectiva desarrollada contra la cisticercosis ovina por (Richards *et al.*, 1991). Estos resultados nos alientan en las posibilidades de desarrollar una vacuna eficiente en contra de la cisticercosis porcina, considerando las similitudes entre *T. solium* y *Taenia ovis.* Al respecto, numerosos estudios han sido realizados a fin de proponer la vacunación como una estrategia para poder

interrumpir el ciclo de la transmisión como se ilustra en el cuadro 1. (Kumar *et al.*, 1987., Pathak y Gaur 1990; Molinari *et al.*, 1993; Sciutto *et al.*, 1990, 1995., Nacimiento *et al.*, 1995., Manoutcharian *et al.*, 1996., Molinari *et al.*, 1997., Rosas *et al.*, 1998, Huerta *et al.*, 2000).

Cuadro 1. Evaluación de diferentes inmunógenos en contra de la cisticercosis porcina causada por *T. solium*.

AUTOR	TIPO DE VACUNA	No. ESPECIE ANIMAL	No. INMUNIZACIONES	DOSIS DE ANTÍGENO	TOTAL DE CISTICERCOS VACUNADO/CONTROL	EFICIENCIA DE PROTECCIÓN (%)
Kumar *et al.*, 1987	1° y 2° de fraccionamiento de antígenos de escólex de *Cysticercus cellulosae*	S/D	2	1.0 ml de cada antígeno/cerdo	126 /416	77
Pathak y Gaur 1990	Antígenos de secreción /excreción de cultivo de oncosferas.	S/D	1	5.0 ml de antígenos	200 /515	95
Molinari *et al.*, 1993	Extracto de cisticerco de *T.solium*	S/D	1	250 µg/cerdo	2/216	86
Sciutto *et al.*, 1990, 1995	Antígenos de *T. solium*			Ratones y en cerdo		
Manoutcharian *et al.*, 1995	Antígenos purificados de 56, 66 y 74 Kda de cisticerco de *T. crassiceps*	S/D	3	60 µg/Kg de peso/cerdo	3/7	96
Nascimiento *et al.*, 1999	Antígenos de excolex de cisticerco de *T. solium*	S/D	3	300 µg /cerdo	448 /977	71.43
Huerta *et al.*, 2000	Péptidos sintético (GK1 KETc12, KETc 1)	240 cerdos	2	250µg/cerdo	1369 / 66563	98.7

S/D sin datos

Un primer obstáculo para la evaluación de inmunógenos de interés para el desarrollo de una vacuna fue las dificultades económicas y experimentales que se presentan en la evaluación con cerdos.

2.2 Antecedentes específicos.

Antecedentes de la vacuna.

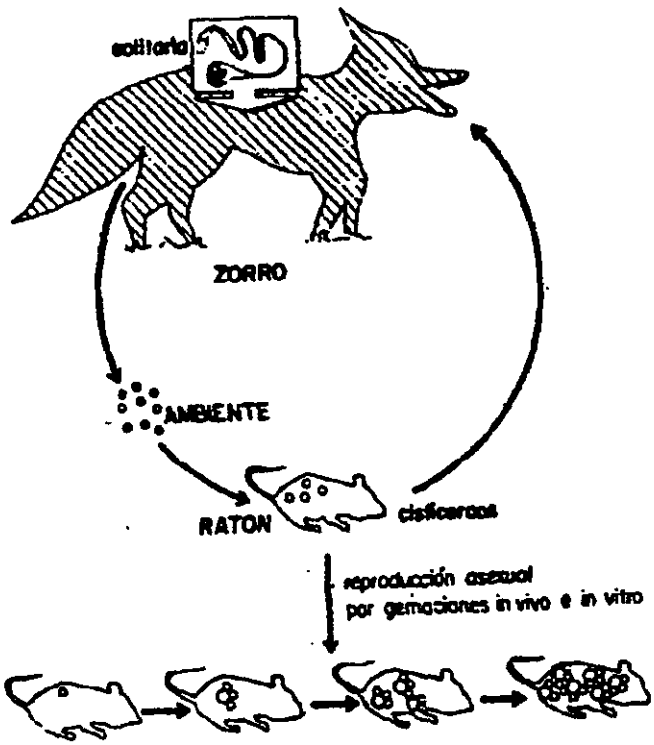
La dificultad de conseguir inmunógenos a partir de *Taenia solium* y poder experimentar en el desarrollo de vacunas y con el fin de mejorar este punto se comenzó a estudiar la cisticercosis murina causada por otro céstodo, *Taenia crassiceps* (Freeman, 1962). Este parásito se reproduce rápidamente por gemación polar múltiple en la cavidad peritoneal de ratones, ofreciendo así una importante fuente de antígenos obtenidos en condiciones experimentales controladas (Figura 3) (Dorais *et al.*, 1962, Freeman, 1962, Larralde *et al.*, 1992). Ambos cestodos presenta muchas similitudes:

1. La cisticercosis por *Taenia crassiceps* afecta a roedores y zorros. Los hospederos intermediarios son pequeños roedores de Europa y Norteamérica, los cuales adquieren la infección al ingerir los huevos de tenia presentes en las heces de hospederos definitivos (zorros, lobos y perros).
2. Los cisticercos como las tenias de ambas especies presentan una estructura macroscópica similar, con la diferencia de que los cisticercos de *T. crassiceps* son más pequeños y presentan la característica única de dividirse por gemación. Esta característica permite mantenerlos en el laboratorio, infectando ratones con sólo algunos cisticercos, pudiéndose recuperar entre 3 a 4 meses, una gran cantidad de larvas

que facilitan obtener hasta gramos de antígeno a partir de cada ratón infectado (Sciutto *et al.*, 1992).

3. La extensa similitud antigénica que existe entre ambos metacéstodos (Larralde *et al.*, 1990) ha permitido utilizar los antígenos de *T. crassiceps* con fines diagnósticos. Así, los antígenos de *T. solium* se pueden reemplazar por los de *T. crassiceps* en el inmunodiagnóstico de la cisticercosis humana (Larralde *et al.*, 1989; Ramos-Kurí *et al.*, 1992) sin mayor detrimento de sensibilidad ni especificidad (figura 3).

Figura 3. Ciclo de vida de *Taenia crassiceps.*



El cisticerco se desarrolla en roedores, los cuales adquieren la infección por ingestión de huevos dispersos en el medio ambiente, provenientes de heces de cánidos infectados por la tenia adulta. En el roedor las larvas eclosionan de los huevos originando los cisticercos. Cuando un roedor infectado es devorado por algún cánido, los cisticercos se instalan en el intestino de éste y se desarrollan en forma adulta. Al alcanzar la madurez sexual, la tenia produce huevos infectivos, cerrando de este modo el ciclo de vida del parásito. En la cisticercosis experimental murina, los cisticercos son inyectados en la cavidad abdominal del ratón donde se reproducen por gemación, produciendo a lo largo de varios meses una cantidad considerable de parásitos.

Cuadro 2.

Inmunización con antígenos de cisticercos *Taenia crassiceps* contra la cisticercosis por *T. solium* en cerdos y por *T. crassiceps* en ratones.

	Ratones		Cerdos	
Inmunógeno	**control**	**vacunado**	**control**	**vacunado**
T. crassiceps **(extracto total)**	**25.0±1.9[a]**	**0[a]**	**519.0 ± 40.2[a]**	**216.8 ± 53.6[a]**
T.solium **(extracto total)**	**25.0±1.0[a]**	**1 ± 0[a]**	**S/D**	**S/D**
T.crassiceps **(fluido vesicular)**	**30.0±6.7[a]**	**14.3±1.7.2[a]**	**5.0±0.85[a]**	**2.5±11.19[a]**
T.crassceps **(fracciones antigenicas de 56,66 y 74 kD)**	**30.0±6.7[a]**	**9.7±6.6[a]**	**5 ±0.05[a]**	**0.16±0.17[a]**
KETc1	**28.2 ± 18.38**	**6.6±4.7[a]**		
KETc7	**28.2 ± 18.38**	**7.6 ± 7.57[a]**		
Ketc11	**28.2 ± 18.38**	**40.6 ±22.2[a]**		
KETc12	**28.2 ± 18.38**	**4.4 ± 5.30[a]**		

[a] Los valores representan el promedio ± 0.5 número de cisticercos recuperados en cada grupo experimental.
Modificado de Manoutcharian et al., 1995.
SD sin datos.

En trabajos previos, se ha demostrado que los antígenos totales de *T. crassiceps* protegen en forma parcial a los cerdos en contra de la cisticercosis porcina (Valdez *et al.*, 1994). Sin embargo, los efectos de la vacunación con extractos totales no resultan la alternativa más adecuada, ya que se ha observado que la capacidad de inducir protección, utilizando antígenos totales, depende críticamente de la dosis utilizada y puede a altas dosis inducir incluso facilitación de la parasitosis (Sciutto *et al.*, 1995). En las mejores condiciones, los antígenos totales de *T. crassiceps* son capaces de reducir a la mitad la carga parasitaria (Sciutto, *et al.*, 1995). Estos resultados indican que la cisticercosis murina es un modelo adecuado para identificar, evaluar y seleccionar antígenos

de interés en vacunación (Sciutto *et al.*,1990, Valdez *et al.*, 1994; Manoutcharian *et al.*, 1995, 1996). Considerando estos hallazgos, se identificaron los antígenos protectores presentes en un extracto antigénico total de cisticercos de *T. crassiceps* (Valdez *et al.*, 1994). Se obtuvieron fracciones antigenicas que fueron separadas electroforéticamente obteniéndose 12 fracciones antigénicas (8 kDa-220 kDa) las cuales fueron utilizadas individualmente para evaluar su capacidad protectora en ratones. De estas 12 fracciones antigénicas sólo ocho tuvieron un efecto significativo en la reducción de la carga parasitaria de los animales inmunizados con respecto a los controles (Manoutcharian *et al.*, 1996). De ellas se selecionaron las tres fracciones antigénicas (56, 66 y 74 kDa) que indujeron los mayores niveles de protección a bajas y altas dosis y fueron reevaluadas en su capacidad protectora (Manoutcharian *et al.*, 1995). Los resultados mostraron que la inmunización con estas tres fracciones antigénicas redujo de manera importante la carga parasitaria en ratones (Cuadro 3, Valdez *et al.*, 1994). Al mismo tiempo dichos antígenos fueron capaces de ser reconocidos por sueros de cerdos infectados con cisticercos de T. *solíum (Valdez et al.*, 1994). Estas fracciones también fueron utilizadas como inmunógenos en el cerdo, obteniéndose altos niveles de protección en los animales vacunados, y confirmando así su capacidad protectora (Cuadro 2, Manoutcharian *et al.*, 1995).

Cuadro 3.

Identificación de la capacidad protectora en contra de la cisticercosis murina en la inmunización con antigenos aislados electroforéticamente utilizados individualmente o combinados empleando adyuvante completo de Freund.

Grupos	Dosis (µg)	*Número de cisticercos (n)	+ Protección eficiencia PE (%)	Número de ratones
Total de antigenos+ Separación de antigenos (kDa)	100	14.3 ± 7.2 &	62.0	16
56	5	27 ± 11.21 &	27.8	14
66	5	17.9 ± 6.9 ++	53.0	13
74	5	16.6 ± 10.7 ++	56.4	13
56 + 66	10	12.6 ± 7.0 ++	66.9	15
56 + 74	10	14.2 ± 6.7 ++	62.7	15
66 + 74	10	12.6 ± 7.7 ++	66.9	14
56 + 66 + 74	15	9.7 ± 6.6 ++	74.5	11
Controles	S/D	38.1 ± 20.3 &	S/D	9

- *Promedio y desviación estándar individual de parasitos recuperadaos 30 días después de infección en ratones inmunizados y controles.
- PE – (n ratones control) – (n ratones inmunizados) / (n ratones control).
- + Antigenos totales incluidos de bajo y alto peso molecular en un 7% de geles.
- ++ Protección significativa comparada con el control, considerando un nivel de 99%.
- & datos considerados con un nivel de significancia diferente al 99%.

Modificado de Valdez *et al.*, 1994.

A pesar que este método permitió identificar antígenos de interés y con el propósito de disponer de cantidades adecuadas de estas fracciones para vacunaciones en forma masiva, este tipo de obtención de antígenos no era él

adecuado, por su alto costo, reproducibilidad y tiempo. Por lo tanto, se decidió producirlos por medio de ADN recombinante. Para ello, se construyó una biblioteca de ADNc en el vector UniZapXR utilizando ARNm de cisticercos de *T. crassiceps.* Se obtuvieron un total de 180,000 clonas individuales. A partir de esta biblioteca se seleccionaron trece clonas recombinantes, las cuales fueron identificadas por medio de inmunodetección, utilizando anticuerpos policlonales específicos producidos en conejo, en contra de las fracciones antigénicas protectoras de 56, 74 y 66 kDa. De las clonas identificadas se seleccionaron aquellas que también fueron reconocidas por sueros de cerdos infectados con *T. solium,* ya que de esta manera se pudo asegurar que las clonas codificaran a antígenos de *T. crassíceps* homólogos a los de *T. solium.* Las clonas se denominaron KETc-1, KETc-4, KETc-7, KETc-11 y KETc-I2. (Manoutcharian *et al.*, 1995 - 1996).

Por medio de técnicas de inmunoelectrotransferencia (IB), se observó que los anticuerpos contra los antígenos codificados por cada una de estas clonas, fueron

capaces de reconocer antígenos específicos presentes en el fluído vesicular de cisticercos de *T. crassiceps.* Estos anticuerpos purificados utilizando los propios antígenos transferidos, reconocieron específicamente a los antígenos nativos de 56 y 74 kDa. Esto nos confirmó que los 5 antígenos recombinantes representan al menos parte de los antígenos incluidos en las fracciones antigénicas protectoras.

Posteriormente, se evaluó la eficacia de las proteínas recombinantes en la prevención de la cisticercosis, procediendo a la vacunación de ratones de la cepa susceptible BALB/cAnN (*Manoutcharian et al., 1995; 1996).* En los ratones vacunados con lisados de las clonas KETcl, KETc4, KETc11, y KETcl2 se observaron eficientes niveles de protección contra la cisticercosis murina causada por *T. crassiceps* (Cuadro 4, Manoutcharian *et al.,* 1996).

Cuadro 4.

Evaluación de la inmunización de cisticercosis por *T. crassiceps* en ratones machos y hembras BALB/cAnN *.

Grupo	Promedio de parasitos recuperados ± desviación estándar (% protección)	
	Hembras	Machos
Control	61.3 ± 29.7	28.2 ± 18.4
Inmunizados con antigenos de lisados crudo de:		
KETc1	28.5 ± 17.5 + (53.5%)	6.6 ± 4.7 + (76%)
KETc4	22.0 ± 15.9 + (64.1%)	N/D
KETc7	42.5 ± 6.4 (30.5%)	7.6 ± 7.6 + (73%)
KETc11	46.0 ± 7.2 + (24.9%)	40.6% ± 22.2 + (44%)
KETc12	26.3 ± 7.2 + (57.1%)	4.4 ± 5.3 + (84.4%)

Promedio y D.S. del número de cisticercos recuperados en ratones inmunizados con los diferentes extractos crudos, de las clonas recombinantes. Los controles solo fueron inmunizados con lizados crudos combinado aisladamente al fago λ
Fueron usados grupos de 5 machos y 8 hembras. N/D.- no determinado.
+ Significancia estadística con respecto al grupo control de ratones de P < 0.05.
(Modificado de Manoutcharian *et al.*, 1996).

Con el propósito de identificar las regiones antigénicas protectoras en estos antígenos recombinantes, se comenzó a analizar el antígeno KETc-7. Con base en su secuencia, que consta de 100 aminoácidos, se realizó una predicción

teórica de las regiones de mayor antigenicidad de éste polipéptido (Gevorkian *et al.*, 1996). Por medio de esta predicción se seleccionaron tres regiones que se denominaron GK-I, GK-2, y GK-3, las que fueron sintetizadas químicamente. Se confirmó su antigenicidad utilizando un panel de sueros de individuos infectados (humanos y cerdos) con *T. solium* y con sueros de ratones parasitados con *T. crassiceps* (Gevorkian *et al.*, 1996). Los tres péptidos fueron reconocidos por individuos cisticercosos y no así por no infectados (Gevorkian *et al.*, 1996).

Respecto a la clona KETc1 y KETc12 se identificó que su secuencia codificaba para dos peptidos de 12 y 8 aminoacidos, respectivamente. Estos peptidos se identificaron químicamente y su reactividad fue confirmada utilizando la misma estrategia

Teniendo como evidencia que los tres péptidos Gk1, KETc-1 y KETc-12 fueron ampliamente reconocidos por individuos infectados así como su capacidad protectora contra la cisticercosis murina en el presente trabajo nos propusimos evaluar la capacidad protectora en contra de la cisticercosis porcina, así como la respuesta inmune humoral asociada a protección.

2.3 Justificación

La prevención de esta enfermedad se justifica considerando las consecuencias que implica en la salud humana, tales como:

1.- El reporte de 50,000 muertes al año en el mundo por neurocisticercosis en humanos (International Task Force for Disease Erradicatión 1992).

2.- Los altos costos que se implica el diagnóstico y tratamiento de la neurocisticercosis. Se calcula un costo mensual aproximado de 217 dólares por paciente (Velasco et al., 1982). La enfermedad comúnmente disminuye la capacidad productiva del individuo afectado.

3.- Estas cifras adquieren mayor relevancia si consideramos el número de personas infectadas por la neurocisticercosis, que en México se estima en 500,000 individuos (comunicación personal, Julio Sotelo, 1996).

En particular en el Estado de Puebla existen las condiciones socioeconómicas y culturales que favorecen la existencia de zonas de alta prevalencia. Así se practican frecuentemente la crianza de traspatio de cerdos, el fecalismo al ras del suelo y existe una inadecuada inspección sanitaria de la carne, lo cual favorece la existencia de esta parasitosis. En el Estado de Puebla se reporta un incremento de la prevalencia de neurocisticercosis de 0.9 a 2.8 del periodo de 1979 a 1988 (Vol. Epi. IMSS., 1988). Un estudio seroepidemiológico en el rastro de la Ciudad de Puebla, reporta 6.1% en 1992, contra del 0.97% resultado de Inspección sanitaria de los canales de cerdo el mismo año. (Huerta *et al.*, Congreso N. IMSS 1992). Aunque estas modificaciones fueron consecuencia de la optimización en el diagnóstico así como en la inspección sanitaria Puebla presenta comunidades endémicas de cisticercosis porcina

Planteamiento del problema

¿Es capaz la vacuna constituida por tres péptidos sintéticos GK1, KETc1, KETc12 de proteger a los cerdos contra la cisticercosis por *T. solium*?

Objetivos:

General.

1. Evaluar la eficacia de la vacuna constituida por tres péptidos GK1, KETc1 y KETc12 contra la cisticercosis porcina en condiciones naturales de transmisión.

Específicos.

1. Identificación de comunidades rurales del Estado de Puebla, con alta prevalencia de cisticercosis porcina (>10 %).

2. Determinar la edad óptima de cerdos mestizos en los que se induce inmunidad por vacunación en condiciones experimentales de infección.

3. Evaluar la capacidad de transferir inmunidad a través de calostro de madres a hijos en cerdas gestantes, vacunadas y no vacunadas.

4. Diseñar y aplicar un protocolo de vacunación de los cerdos de la comunidad, para evaluar la vacuna sintética.

4. Estudiar el estado histopatológico de los cisticercos, localizados en los diferentes tejidos de los cerdos vacunados y no vacunados, tanto de los infectados experimentalmente como de los desafiados en forma natural.

Hipótesis

Ho: La aplicación de una vacuna contra cisticercosis porcina constituida por 3 péptidos sintéticos (GK1, KETc1 y KETc12) aplicada en cerdos en una comunidad rural expuestos al desafio natural, no reduce la prevalencia ni la carga parasitaria promedio del cerdo.

Hi: La aplicación de una vacuna contra la cisticercosis porcina constituida por 3 péptidos sintéticos (GK1, KETc 1 y KETc12) aplicada en cerdos en una comunidad rural expuestos al desafio natural reduce la prevalencia y carga parasitaria del cerdo.

III. Materiales y métodos.

Se trata de una cohorte experimental prospectiva cuya población de estudio son los cerdos de traspatio de las comunidad del Estado de Puebla.

1. Identificación de la comunidad.

Se realizó un estudio en los cerdos en pie, para lo cual se detecto cisticercos en lengua y tomo una muestra de sangre periferica para conocer los niveles de anticuerpos anticisticerco. La detección de cisticercosis por inspección de lengua es un método de sensibilidad del 60 al 70 % (Aluja et al., 1982) aunque de muy alta especificidad de más de 90%. Con base en esta inspección se seleccionó el área en donde se observara un prevalencia de cisticercosis porcina al menos igual o mayor de un 10 %.

2. Determinación de la edad optima de inmunización.

Se realizó una prueba piloto en la que se identificó en cerdos rústicos la edad optima para vacunación. Para este fin se adquirieron cerdos mestizos sanos de 40 y 70, días de nacidos, mismos que fueron vacunados unos, con los antígenos totales de *T. crassiceps* a una dosis de 40 ug/kg de peso, mezclado con saponina 100/μg/cerdos (adyuvante) en 1.5 ml de solución salina normal, inoculando por vía subcutánea en la base de la oreja y otros con placebo utilizando saponina 100/μg/cerdos en 1.5 ml de solución salina normal, de cada cerdo se llevó un registro individual.

Los cerdos fueron sangrados antes y después de la vacunación, desafiados 30 días después de la vacunación con 100,000 huevos de *Taenia solium*. Sesenta días después fueron sacrificados, se cuantificó el número de cisticercos observados en la mitad de la canal y se identificó la fase histopatológica del cisticerco. Este experimento permitió identificar si los antígenos totales desencadenaron una respuesta inmune efectiva capaz de controlar la infección. Con los sueros de los animales tratados y no tratados antes y después del desafío se detectaron Ac totales inducidos por vacunación

cuantificándolos por ELISA siguiendo el procedimiento previamente reportado (Larralde *et al.*, 1989). La capacidad protectora de los anticuerpos individuales fue estudiada utilizando un ensayo de transferencia pasiva de anticuerpos.

3) Evaluación de la capacidad de transferir inmunidad a través de calostro de madres a hijos.

En este estudio experimental prospectivo la muestra fueron dos cerdas híbridas gestantes. Cerda A de 80 Kg, se vacunó con 500 μg de antígenos totales de *T. crassiceps* + 100 μg saponina como adyuvante en 1.5 ml de solución salina y cerda B de 90 Kg se le administraron 100 μg saponina como adyuvante en 1.5 ml de solución salina como (control) por vía subcutánea en la base de la oreja durante el ultimo tercio de la gestación. Con la finalidad de determinar la presencia de inmunoglobulinas, se colectó calostro a las 4, 8, 12, 24, 48 horas tanto en la cerdas control como en la cerda vacunada.

Por otro lado los seis lechones provenientes de la cerda vacunada y cinco de la cerda control se identificarón con registro individual y se procedió a medir el nivel y tipo de anticuerpo presentes en sangre durante la lactación, el destete y la engorda de acuerdo al método de Larralde et al., 1989. Se identificó el tiempo en que los lechones presentaron niveles máximos de anticuerpos. Los lechones se desafiaron con 50 000 huevos de *Taenia solium* , 30 días después del nacimiento con la finalidad de observar la capacidad protectora de la inmunidad materna transferida.

En los sueros de animales tratados se detectaron Ac inducidos por vacunación, cuantificándolos por ELISA, siguiendo el procedimiento previamente reportado (Larralde et al., 1989). Tres días después de la vacunación se realizó el desafío de los lechones con 50,000 huevos viables de *T. solium* en alimento humedecido, de forma individual y se verificó que toda la papilla fuese ingerida por el lechón. Se tomaron muestras sanguíneas a los 30, 50 y 100 días de edad.

Alimentación. Las cerdas recibieron una dieta comercial a base de soya sorgo y maíz. Para la etapa de lactancia se aumentó la cantidad y calidad de proteína de la dieta (alimento comercial). Después del destete que se efectuó, a los 28 días, los lechones recibieron un alimento de iniciación y posteriormente de crecimiento. El alimento fue especialmente elaborado para el proyecto sin desparasitantes que normalmente incluyen los alimentos comerciales y que, en este caso, podrian interferir en los resultados del estudio.

Sacrificio y toma de muestras. A los 100 días de edad se realizó el sacrificio, se colecto cisticercos de: lengua, esófago, faringe, tráquea, aparato respiratorio, digestivo, urinario y por último genital. Se cortó la mitad de la canal para luego contar la carga parasitaria de esta; se tomaron muestras para estudio histológico de la región retroauricular que fue donde se vacunó; se tomaron muestras de los ganglios linfáticos cervicale, se fijaron en formol al 10% y se mantuvo en refrigeración. A la mitad de cada canal se le tomaron además muestras de tejido que midieron aproximadamente 0.5 cm de tejido y se colocaron en formol al 10%. Se realizaron cortes muy finos para permitir contar los parásitos; cada recipiente se identificó con el numero de cerdo y órgano del cual procedía la muestra. Los tejidos que se estudiaron fueron de: lengua, músculo masetero, corazón, hígado, músculos abdominales, espaldilla, pierna, psoas, diafragma y cerebro. En las observaciones se mencionaron las características del cisticerco: hialino, vesicular o caseoso.

El análisis estadístico se realizó para los datos de cerdas y de los lechones utilizando la prueba de *t* de Student. En el análisis de los datos del suero de los lechones se utilizó un análisis de varianza (ANOVA). Se utilizó el programa Instat, Statistica para Windows, 1993.

4. Diseño de un protocolo de vacunación. Con base en la prevalencia

identificada en las comunidades de cisticercosis porcina (14%), al explorar lengua se calculó, el número de cerdos que deberían vacunarse para evaluar el efecto de la vacuna ante el desafio natural, se utilizó un muestreo aleatorio simple ciego, en grupos apareados, aretando a cada animal para su identificación. Se estimaron 240 cerdos del grupo 120 y grupo inmunizado 120.

Un conjunto de cerdas fueron vacunadas en el ultimo tercio de la gestación con los 3 pétidos sintéticos (500 μg de cada péptido / cerda) más adyuvante en 1.5 ml de solución salina normal y otro conjunto con solo adyuvante (saponina) en 1.5 ml de solución salina normal, en la base de la oreja, por vía subcutanea. Se procedió a vacunar a los lechones a los 60 días considerando el estudio anterior realizado, las camadas que provenian de cerdas vacunadas se les aplicó vacuna y aquellos que provenian de cerdas control solo adyuvante (saponina).

A los lechones se les aplico una primera dosis a los 60 y una segunda dosis de 90 días edad. Cada lechón fue sangrado antes y después de la inmunización. A los 120 lechones de madres vacunada se les administró una dosis de 250 ug/kg de tres péptidos sintéticos (GK1, KETc1, KETc12) +100 μg de saponina en en 1.5 ml de solución salina normal en la base de la oreja y por vía subcutánea, a los lechones 120 provenientes de cerdas placebo se les administró 100 μg de saponina en 1.5 ml de solución salina normal por la misma vía. Luego de la segunda dosis de vacuna, los lechones se distribuyeron por pares (vacunado + control) a los habitantes de las comunidades donde se encontró cisticercosis porcina. Después de la vacunación los cerdos fueron sangrados en diferentes tiempos y continuaron con su vida libre manteniendo el riesgo de infección en forma natural, sin modificar su manejo rústico. Los cerdos se donaron a los habitantes de Huatlatlauca y se les dio una compensación para que los cuidaran, cuando ya alcanzaron el tamaño y edad para ser vendidos se sacrificaron humanitariamente entre 8 y 12 meses de edad, se cuantificó el número de parásitos, tanto en los cerdos vacunados como en los que se recibieron placebo.

IV. Resultados.

1. Se identificaron comunidades del Estado de Puebla, en las que la prevalencia de cisticercosis fue de un promedio de un 14 %, en las comunidades de Huatlatlauca, Tepetzezintla y San Nicolas Tolentino, estas pertenecen a Huatlatlauca municipio de Tepexi de Rodriguez Puebla, comunidades en donde prevalecen los factores que favorecen el ciclo de esta parasitosis.

2. Se determinó la edad óptima de cerdos mestizos en los que se induce inmunidad por vacunación en condiciones experimentales de infección la edad óptima fue a los 60 días. Los resultados se incluyen en el artículo: Vaccination against *Taenia solium* cisticercosis in underfed rustic pigs of México: roles of age, genetic background and antibody response. Publicado en Veterinary Parasitology, (2000) 90, 209-219 (Anexo 1).

3. Se evaluó la capacidad de transferir inmunidad a través de calostro de madres a hijos en dos cerdas gestantes, una vacunadas y otra no vacunada (apéndice de resultados 3).

4. Los resultados del protocolo de vacunación de los cerdos de la comunidad, para evaluar la vacuna sintética, se muestran en el artículo: Synthetic peptide vaccine against *Taenia solium* pig cysticeosis: successful vaccination in a controlled field trial in rural México. Enviado para su publicación a la revista Vaccine. Se anexa copia y comprobante de recibido.

5. Estudio de la fase histopatológica de los cisticercos, localizados en los diferentes tejidos de los cerdos vacunados y no vacunados (apéndice de resultados 5).

4.1 ARTICULOS PUBLICADOS

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Vaccination against *Taenia solium* cysticercosis in underfed rustic pigs of México: roles of age, genetic background and antibody response

M. Huerta[c], E. Sciutto[a,*], G. García[a], N. Villalobos[b], M. Hernández[a], G. Fragoso[a], J. Díaz[c], A. Díaz[e], R. Ramírez[d], S. Luna[d], J. García[d], E. Aguilar[d], S. Espinoza[d], G. Castilla[d], J.R. Bobadilla[a], R. Avila[d], M.V. José[a], C. Larralde[a], A.S. de Aluja[b]

[a] *Department of Immunology, AP 70228, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México (UNAM), Mexico D.F. 04510, Mexico*

[b] *Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México (UNAM), Mexico D.F. 04510, Mexico*

[c] *Facultad de Medicina, Benemérita Universidad Autónoma de Puebla, Puebla, Mexico*

[d] *Escuela de Medicina Veterinaria y Zootecnia, Benemérita Universidad Autónoma de Puebla, Puebla, Mexico*

[e] *Instituto Mexicano de Seguro Social, Puebla, Mexico*

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Vaccination against *Taenia solium* cysticercosis in underfed rustic pigs of México: roles of age, genetic background and antibody response

M. Huerta[c], E. Sciutto[a,*], G. García[a], N. Villalobos[b], M. Hernández[a], G. Fragoso[a], J. Díaz[c], A. Díaz[e], R. Ramírez[d], S. Luna[d], J. García[d], E. Aguilar[d], S. Espinoza[d], G. Castilla[d], J.R. Bobadilla[a], R. Avila[d], M.V. José[a], C. Larralde[a], A.S. de Aluja[b]

[a] *Department of Immunology, AP 70228, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México (UNAM), Mexico D.F. 04510, Mexico*

[b] *Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México (UNAM), Mexico D.F. 04510, Mexico*

[c] *Facultad de Medicina, Benemérita Universidad Autónoma de Puebla, Puebla, Mexico*

[d] *Escuela de Medicina Veterinaria y Zootecnia, Benemérita Universidad Autónoma de Puebla, Puebla, Mexico*

[e] *Instituto Mexicano de Seguro Social, Puebla, Mexico*

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Abstract

Vaccination of pigs of mixed genetic make-up, raised as rustically as done in rural Mexico, resulted in effective protection to experimental challenge against *Taenia solium* cysticercosis. Maximum protection was achieved if pigs were immunized at 70 days of age. There was large variation of viable parasite load within vaccinated pigs and controls, which is suggestive of significant genetic factors influencing susceptibility, besides immunization. Our results strengthen the advisability of pig vaccination for control of *T. solium* cysticercosis, since it lowers the number of viable cysticerci capable of transforming into tapeworms.

Keywords: Porcine cysticercosis; Immunization; Vaccination; Antibodies; *Taenia solium*

1. Introduction

Taenia solium cysticercosis is highly prevalent in humans and pigs in developing countries of Latin America, Asia and Africa and has serious health and economic consequences (Sotelo et al., 1996). The life cycle of this parasite includes a larval (cysticercus) phase

* Corresponding author. Tel.: +5-6-22-38-18; fax: +5-6-22-33-69.
E-mail address: edda@servidor.unam.mx (E. Sciutto)

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affecting both pigs and humans after ingestion of eggs present in feces of human tapeworm carriers.

Transmission is clearly related with rustic rearing of pigs in impoverished sectors of the rural population (Larralde et al., 1992). There is evidence that rustically reared pigs become infected during the initial days after birth (Aluja et al., 1998). Since the pig is an indispensable intermediate host, transmission could be reduced by lowering the prevalence of pig cysticecosis through effective vaccination. Development of an effective vaccine for pig cysticercosis is being pursued by a number of researchers (Molinari et al., 1993, 1997; Nascimento et al., 1995; Sciutto et al., 1995).

Experimentation leading to a vaccine against porcine cysticercosis is hampered by the high cost and slow data retrieval involved in testing pigs. Therefore, another cestode, *Taenia crassiceps*, which exhibits extensive antigen similarities with *T. solium* (Larralde et al., 1989, 1990) and whose metacestodes easily and rapidly develop and proliferate in the peritoneal cavity of mice (Fragoso et al., 1998), has been used as an experimental model to select and test promising antigens before testing in pigs (Valdez et al., 1994; Manoutcharian et al., 1996). This approach led us to find that immunization of well-nourished adult York-Landrace pigs with total *T. crassiceps* antigens reduced almost 50% the expected parasite load in pigs experimentally challenged with *T. solium* eggs (Sciutto et al., 1995; Manoutcharian et al., 1996). We set out to test the validity of such encouraging results under field conditions that approximate those prevailing in rural areas of Mexico. Thus, we measured the effect of the reported vaccination and challenging procedures but now applied to underfed rustic pigs of different ages, all taken from a mixture of different genetic backgrounds consistent with typical rural pig populations. Serological studies were also performed to test the cysticidal effect of the vaccinated pigs' antibodies as measured by the blocking of *T. crassiceps* cell transformation into cysts (García et al., 2000).

2. Material and methods

2.1. Pigs

We used 20 pigs chosen at a rural farm in Atlixco, in the state of Puebla, Mexico, which was shown to be free of cysticercosis. Pigs were transferred and kept in the farm at the Veterinary School of the Benemérita Universidad Autónoma de Puebla, which was also free of cysticercosis. To simulate the nutritional deficiencies to which free-roaming pigs in rural areas are exposed, pigs were fed with only 50% of the recommended amount of commercial food which resulted in seriously reduced body weight (40%).

2.2. Experimental strategy

Two groups of 10 pigs each were used for immunization trials: one group was treated at 40 days of age and the other group at 70 days of age. Five pigs of each group were immunized with total *T. crassiceps* antigens in adjuvant and the other five were injected with adjuvant alone and used as controls.

2.3. Immunogen preparation and immunization schedule

The immunogen consisted of total antigens recovered from *T. crassiceps* (ORF) cysticerci obtained as previously described (Sciutto et al., 1995). Briefly, murine *T. crassiceps* cysticerci were collected from the peritoneal cavity of mice and placed in cold 0.01 M phosphate buffered saline, pH 7.2 (PBS). The metacestodes were repeatedly washed in ice cold PBS and then homogenized in a Polytron (Brinkmann Instruments); protein concentration was determined by the method of Lowry and adjusted to 9 mg/ml in PBS, and the samples were stored at −70°C until used. Pigs were immunized subcutaneously with one dose of 0.4 mg/kg in 2.5 ml of saline with 250 μg of saponin as adjuvant or with adjuvant alone (controls) at the base of the ear.

2.4. Experimental infection and assessment of parasite burden

A specimen of *T. solium* was obtained from a fully informed and consenting human carrier in a village (San Nicolás Tolentino) in the state of Puebla, Mexico, immediately after treatment with niclosamide (Yomesan, Bayer). The tapeworm was collected in saline. Under controlled laboratory conditions, the tapeworm was cut up with scissors to free eggs from gravid proglottids. Eggs were washed repeatedly with saline solution and kept at 4°C until used. Thirty days after collection, egg viability was confirmed by microscopic examination using the technique described by Aluja et al. (1998). Thirty days after immunization each pig was orally infected with 10^5 viable eggs of *T. solium*. Before immunization and at five different times thereafter, pigs were bled for antibody evaluation. Eighty days after infection, pigs were killed and cysticerci were counted after thoroughly dissecting skeletal muscles, tongue, heart and liver of all the animals. Samples of tissues containing metacestodes were fixed in 10% phosphate buffered formaline (pH 7.2) and processed for histological examination.

2.5. Histological examinations

Tissues containing metacestodes were sectioned (5 μ) and stained with hematoxylin-eosin as previously described (Aluja et al., 1998). Based on the microscopic appearance, cysticerci were classified according to the inflammatory reaction and to the degree of destruction in grades of 1–6 (Aluja and Vargas, 1988). A total of 693 cysticerci were analyzed in a blind evaluation from immunized and non-immunized pigs.

2.6. Distribution of cysticerci in experimentally infected pigs

All muscles, tongues, hearts and livers of the pigs were cut in slices of approximately 1 cm each and all metacestodes present in them were counted. A beta probability density function was used for the frequency distribution in order to fit the data. The STATGRAPHICS program was used for fitting procedures and for obtaining estimates of the two parameters (β=35.73 and α=1.84) for the beta density.

2.7. Antibody detection by ELISA

Serum antibody levels were obtained by indirect ELISA using *T. solium* cyst fluid as antigen with minor modifications following a previously described procedure (Sciutto et al., 1998). After incubation with 100 μl per well of *T. solium* cyst fluid (1 μg/ml) in borate buffered saline (BBS), pH 9.6, for 60 min at 37°C, the wells were washed and incubated with 100 μl of pig sera diluted in PBS-Tween, 1:100, for 60 min at 37°C. Antibodies were detected with 100 μl of rabbit alkaline phosphatase anti-pig IgG (whole molecule) conjugate (Sigma), diluted to optimum concentration (1:1000) and followed by substrate (*para*-nitrophenylphosphate (Sigma, at 5 mg/ml). The color reaction was stopped using 50 μl/well of 2 N NaOH and absorbance values were measured at 405 nm in an ELISA reader (Human GMBH, Humareader, Model 2106). A serum sample was considered to be positive when its ELISA optical density (OD) reading exceeded the 99% confidence interval (mean value of control (non-immunized) +3 standard deviations).

2.8. Relevance of the antibody response

To evaluate the anticysticercal properties of the antibodies induced by immunization and by the challenging infection, we used an experimental model of cysticercosis based on the infection of 4–6 weeks-old female BALB/cAnN mice with *T. crassiceps* cysticercus cells Freeman, 1962. This technique has as been recently developed as a bioassay for this purpose (Toledo et al., 1997; García et al., 2000). Cysticercus cells used for infection were obtained from cysticerci after extensively washing the parasites with sterile PBS, following the technique previously described (Toledo et al., 1997). Briefly, about 500 cysts were transferred to 50 ml RPMI 1640, supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, penicillin (100 U/ml)/streptomycin (100 μg/ml), 0.01 mM non-essential amino acids, 0.2 mM L-glutamine, 1.6 μM βmercaptoethanol, 25 mM HEPES, and 2 g/l $NaHCO_3$. Cysts were cut into small pieces with a scalpel and placed in 50 ml culture flasks (Falcon Labware, New Jersey) with 5 ml of culture medium. After 7 days of in vitro culture at 27°C, the fragments were transferred to fresh medium, containing 1% trypsin-EDTA (Gibco BRL, New York) and maintained at room temperature under strong shaking for 15 min. The trypsin digest was washed three times after 10 min centrifugation of lysate at 800 g and the pellet was resuspended in the supplemented culture medium. Infection of mice by isolated cysticercus cells was performed by intraperitoneal injection of 0.5 ml of the cell suspension (10^6 cells per mouse). Mice were killed 30 days after infection and the number of cysts in the peritoneal cavity was determined (Fragoso et al., 1998).

To study the effect of antibodies upon *T. crassiceps* cysts regeneration from the cysticercus cells, Igs from both non-immunized (control) and immunized pigs were collected using the technique previously described (Sciutto et al., 1998) before the challenging infection (Table 2). In an additional experiment sera from four already infected control pigs were used (Table 3). Two of the four pigs developed few cysticerci after challenge (resistant), and two a high number of cysts (susceptible). Immunoglobulins concentration was calculated by Lowry technique (Lowry et al., 1951) and were adjusted to a concentration of 75 μg/ml in RPMI 1640 and incubated in 1 ml with 10^6 cysticercus cells during 30 min at room temperature under sterile conditions. After incubation, the cysticercus cells that had been

incubated with medium alone or with immunoglobulins in the same medium were injected into the peritoneal cavity of BALB/cAnN mice. After 30 days the mice were killed and the number of cysticerci recovered in their peritoneal cavities were counted.

2.9. Statistical analysis

The statistical significance of results was calculated with the non-parametric Mann–Whitney *U*-test, considering that parasite density is a discrete variable (i.e. 0, 1, 2, . . . ,*n* parasites). Both the statistical analyses were performed with the INSTAT program (GraphPad, San Diego, CA). Differences were considered significant when *P* values were less than 0.05.

3. Results

3.1. Effect of immunization against pig cysticercosis

To evaluate the effect of immunization against cysticercosis, the number of cysts per pig as well as the histological state of the cysticerci recovered were determined. A total of 693 slices from cysticerci collected from infected pigs were analyzed and the histological state of cysticerci classified according to their degree of damage from 1 to 6, following the classification proposed by Aluja and Vargas, 1988. It is shown in Fig. 1 that the percentage of viable cysticerci (grades 1, 2, 3) present in immunized pigs was significantly lower ($P<0.05$) than those in control pigs; likewise, the distribution of cysticercal viability classes in immunized pigs was shifted by several intervals towards severely damaged metacestodes (grades 4, 5 and 6). More importantly, in pigs immunized at 70 days of age, in comparison to that of controls, cysticerci were distributed mostly in the low viability classes (grades 4, 5, 6), although similar total numbers of *T. solium* cysticerci had entered and established in both immunized and control pigs (Table 1).

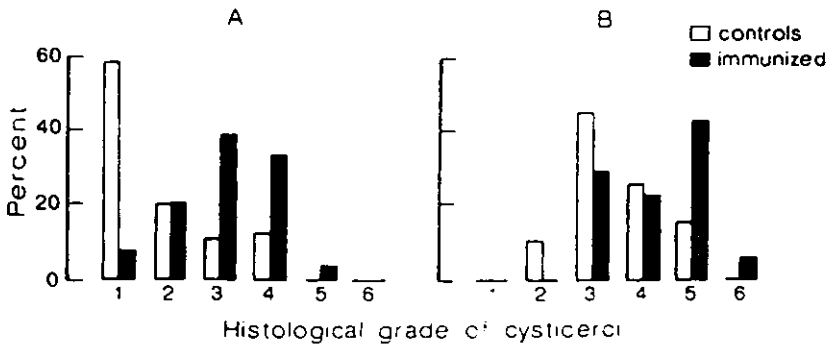

Fig. 1. Percentage distribution of cysticerci histologically classified according to the grade of damage in 693 parasites explored representative for non-vaccinated (controls) and pigs vaccinated at (A) 40 or (B) 70 days of age.

Table 1
Number of cysticerci recovered in immunized and controls pigs 80 days after challenge with *Taenia solium* eggs

	Total number of cysticerci/pig	Mean number of cysticerci/group ±S.D.
Pigs immunized at the age of 40 days		
Controls	175, 198, 251, 375, 858	371.4±252.9
Immunized	87, 172, 362, 487, 631	347.8±199.3
70 days		
Controls	172, 178, 294, 323, 812	355.8±235.9
Immunized	176, 183, 234, 355, 1021	393.8±320.0

3.2. *Relevance of the antibody response*

None of the pigs immunized at 40 days of age had detectable serum antibodies by ELISA at the time of infection challenge, whereas in sera from pigs immunized at 70 days of age, a clear increase in the antibody levels after immunization was detected (Fig. 2). However, after 80 days of infection, all immunized and non-immunized pigs of both ages had detectable and similar serum antibody levels. Cysticerci from pigs immunized at 70 days of

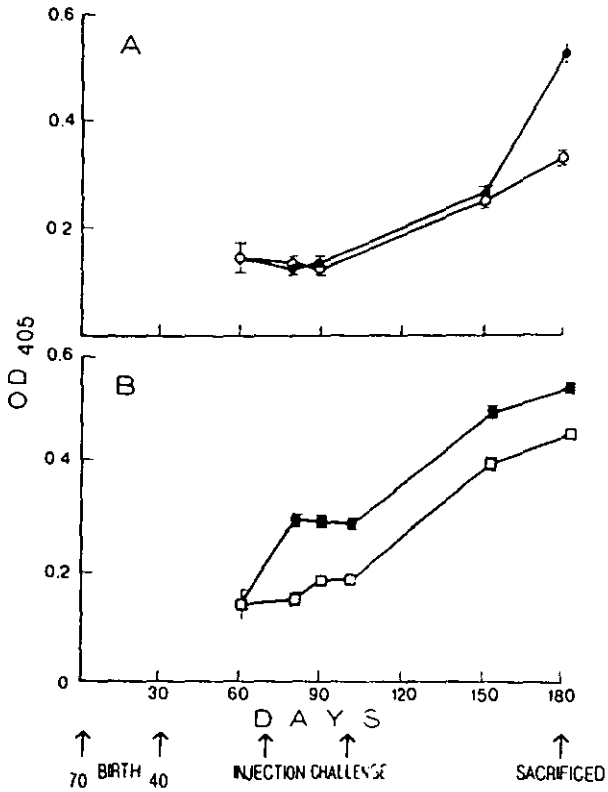

Fig. 2. Levels of antibodies determined by ELISA (O.D.) ±S.D. in sera from non-vaccinated (○, □) and vaccinated (●, ■) pigs before and after challenge with *T. solium* eggs. Schemes of events in the protocol used are signaled (A) 40 and (B) 70 correspond to the time of birth of pigs of each age group.

Table 2
Effect of pig anti-cysticercal antibodies in *Taenia crassiceps* cyst regeneration from isolated cysticercal cells

Immunoglobulins from pigs immunized at age of [a]	
40 days	
Non-immunized	69, 74, 89, 83, 79, 103, 88, 68, 79, 94[b]
(0.18±0.03)[c]	(82.6±10.5)[a,d]
Immunized	96, 101, 104, 70, 78, 70, 93, 82, 79, 94
(0.17±0.04)	(86.7±11.8)[a]
70 days	
Non-immunized	113, 99, 106, 86, 109, 125, 87, 98, 103, 99
(0.19±0.04)	(102.5±11.1)[a]
Immunized	46, 36, 69, 53, 42, 59, 61, 54, 48, 58
(0.32±0.05)	(52.6±9.3)[b]

[a] Immunoglobulins were purified from pig sera obtained 30 days after immunization with *T. crassiceps* total antigens (400 μg/kg).

[b] Number of cysticerci recovered after infection of mice with 10^6 *T. crassiceps* cysticercal cells incubated with purified immunoglobulins (70 μg/ 10^6 in 1 ml).

[c] Level of anti-cysticercal antibodies determined by ELISA (mean of the OD±S.D.). Data labeled with different letters are significantly different (non-parametric ANOVA test, $P<0.001$).

[d] Mean of the number of cysticerci recovered per group ±S.D.

age exhibited greater histological damage than those immunized at 40 days of age. The effect of purified antibodies from the older pigs was tested with *T. crassiceps* cyst regeneration in BALB/cAnN recipient mice and compared with the cysticidal effect of purified Igs from the younger immunized pigs. As shown in Table 2, there was a reduction in the number of cysts recovered from the peritoneal cavity of mice when cysticercal cells were incubated with Igs from pigs immunized at 70 days, compared with Igs from 70 days non-immunized pigs (52.6 versus 102.5, $P<0.001$), whereas no effect was observed in cells incubated with Igs

Table 3
Effect of antibodies induced by susceptible or resistant pigs on *Taenia crassiceps* cyst regeneration from isolated cysticercal cells

Immunoglobulins from pigs:	Experiment 1	Experiment 2
Non-infected	78, 112, 118, 89, 95, 92, 103, 83, 90, 104[a] (96.4±12.0)[a,b]	53, 101, 64, 78, 100, 50, 70, 80, 93, 93 (78.2±17.7)[a]
Infected		
Susceptible	114, 99, 100, 86, 93, 78, 64, 94, 89, 103	103, 59, 78, 93, 96, 118, 75, 88, 92, 86
(858, 812)[c]	(92±13.2)[a]	(88.8±15.3)[a]
Resistant	42, 56, 69, 65, 79, 45, 58, 69, 70, 82	38, 36, 48, 52, 46, 32, 48, 56, 43, 51
(175, 172)	(63.5±13.6)[b]	(45±7.2)[b]

[a] Number of cysts recovered after infection of mice with 10^6 *T. crassiceps* cysticercal cells incubated with purified immunoglobulins (75 μg).

[b] Mean±S.D. of the number of cysticerci recovered.

[c] Number of cysticerci determined per pig after infection with 10^5 *T. solium* eggs. Genetically heterogeneous pigs were infected under the same conditions. Data labeled with different letters are significantly different from each other (non-parametric ANOVA test, $P<0.05$).

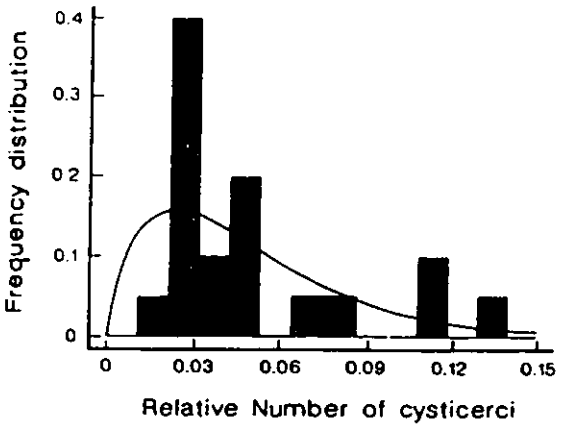

Fig. 3. Frequency distribution of metacestodes of *T. solium* in 20 experimentally infected pigs. The solid curve represents the best fit of the beta probability density function. The maximum likelihood estimates of the parameters are $\alpha=1.84$ and $\beta=35.73$. The number of cysticerci of each pig shown in Table 2, immunized and non-immunized pigs for 40 and 70 days, are normalized to 0 and 1. Then the relative number of cysticerci denoted by x is a random variable whose range goes from 0 to 1. The beta distribution is defined as: $F(x) = (\Gamma(\alpha+\beta)/(\Gamma(\alpha)\Gamma(\beta))(x^{\alpha-1})(1-x)^{\beta-1}$ where Γ is the gamma function, α and β are shape parameters.

from pigs immunized at 40 days, or with sera from control pigs (86.7 versus 82.6, $P>0.4$). Using the same *T. crassiceps* murine model we analyzed the relevance of antibodies elicited by four experimentally infected pigs with *T. solium* eggs. As shown in Table 1, two of them were naturally more resistant (with 175 and 172 cysts) and two were more susceptible (with 858 and 812 cysts). When parasite cells were treated with antibodies from the resistant pigs, a significant reduction in the number of cysticerci was detected in two independent experiments (96.4 versus 63.5; 78.2 versus 45, $P<0.05$, Table 3). No effect was detected when antibodies from susceptible pigs were evaluated (96.4 versus 92, $P>0.5$; 78.2 versus 88.8, $P>0.3$, Table 3).

3.3. Distribution of cysticerci in experimentally infected pigs

The frequency distribution of cysticerci in the 20 experimentally infected pigs is shown in Fig. 3. This distribution clearly follows an overdispersed distribution of the number of cysticerci among the pigs. The solid curve represents a beta density, which is the best fit to experimental data. As shown, most of the pigs harbored low number of cysts and only a few pigs exhibited a clearly higher number of parasites.

4. Discussion

In this study we observed that immunization of rustic pigs with heterologous total *T. crassiceps* antigens reduced the viability of *T. solium* cysticerci that developed from experimental challenge with *T. solium* eggs. Protection by vaccination was more pronounced in older pigs (70 days of age), an effect possibly mediated in part by antibodies. In a previous study we had found that immunization with *T. crassiceps* total cysticercus antigens

significantly reduced the expected parasite load by almost 50% of experimentally infected York-Landrace pigs, kept in ideal conditions and fed ad libitum with fully balanced commercial food (Sciutto et al., 1995). In this study, we found that the same immunization protocol applied to genetically hybrid insufficiently fed pigs induced a cysticidal effect clearly observed in pigs immunized at 70 days of age (Fig. 1) although the total number of parasites found, dead or alive, was similar in both the groups of pigs (Table 1). The damage to cysticerci induced by vaccination is particularly relevant for practical reasons, as it strengthens the feasibility to control transmission of the infection by pig vaccination, even if applied to rustically reared pigs. The failure to detect an antibody response after immunization at 40 days of age could indicate that younger animals are still in the process of developing their immune system and perhaps precludes the use of early vaccination.

These results point to three main issues that merit comment. The first is the optimal age at which pigs should be vaccinated to induce an effective immune response, a very important issue to consider in vaccination protocols, particularly when taking into account that pigs come in contact with the parasite as early as a few days after birth (Aluja et al., 1998). Additionally, the higher viability of cysticerci in pigs immunized at 40 days of age and challenged at 70 days than of those from pigs immunized at 70 days and challenged at 100 days of age (Fig. 1) is of interest, as it suggests that once cysticerci are established the immune system has difficulties to damage them. The pig's slow development of effective immunity against cysticerci, combined with the early exposure to the infective eggs, may warrant the development of vaccination of pregnant sows for passive transfer of antibodies via colostrum. The second comment relates to the evaluation of vaccine protocols in cysticercosis and the relevance of assessing the total number of cysticerci that establish as a result of challenge as well as the histological degree of parasite damage. Counting only the total number of cysticerci found in the challenged host may be misleading (García et al., 2000). Antibodies that cripple biological functions of the parasite, such as development to reproductive or pathogenic stages, make antibodies important protagonists in pathogenesis and transmission of taeniosis/cysticercosis: thus, not all infected pigs, and perhaps not all cysticerci within the same pig participant, are equally capable of conversion to tapeworms (García et al., 2000).

The third issue pertains to the relevance of antibodies in the cysticidal effects of vaccination. A new more sensitive system has been used here, one based on the use of *T. crassiceps* cysticercus cells as challenge infection in murine hosts that regenerate to complete cysticerci (Toledo et al., 1997). When *T. crassiceps* cysticercus cells were previously incubated with antibodies purified from sera of pigs immunized at 70 days of age, a clear reduction in the number of regenerated cysticerci was observed, whilst no effect was detected when sera from control or 40 days immunized pigs were used. Thus, it would appear that antibodies might be involved in the protection conferred by vaccination.

Another interesting finding that emerged from this study is the overdispersed distribution, a beta density, of parasite loads in challenged hybrid pigs. The gamma distribution (see e.g. Medley et al., 1993) and in particular the negative binomial distribution (see e.g. Anderson and May, 1985) have been used several times for describing the distribution of helminth parasites among hosts. The fitted beta distribution describes better the aggregation of parasites among pigs, which had the same degree of exposure than the negative binomial distribution. Indeed, the distribution of the number of parasites harbored by each of the

20 pigs that were experimentally and simultaneously challenged with the same inoculum of eggs, indicated that 80% of the pigs contained less than 400 parasites and 20% carried more than 600 cysticerci (Fig. 3). As all the pigs were equally challenged with a thoroughly mixed population of eggs derived from a single harvest, this huge variation in the pigs' parasite load indicates that in *T. solium* cysticercosis there are important genetic differences in susceptibility within the pig population. This is of special interest considering recent findings in which at least one gene seems to be related with resistance to murine cysticercosis (Fragoso et al., 1998). Thus, it is of great interest to explore the involvement of some homologous gene in the genetic differences in susceptibility of pigs reported herein. The important genetic differences found in pig susceptibility to *T. solium* cysticercosis stresses the need for further research on genetic factors involved in transmission.

The results observed in this vaccine trial in which we used rustic pigs are clearly encouraging and offer the possibility of diminishing transmission of *T. solium*.

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Synthetic peptide vaccine against Taenia solium pig cysticercosis: successful vaccination in a controlled field trial in rural Mexico

[c]M. Huerta, [b]A.S. de Aluja, [a]G. Fragoso, [a]A. Toledo, [b]N. Villalobos, [a]M. Hernández, [a]G. Gevorkian, [a]G. Acero, [d]A. Díaz, [e]I. Alvarez, [e]R. Avila, [a]C. Beltrán, [a]G. Garcia, [b]J.J. Martinez, [a]C. Larralde, [a]E. Sciutto*

[a]Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México.D.F., A.P. 70228. 04510; México. *Howard Hughes Medical Institute International Research Scholar. [b]Facultad de Medicina Veterinaria y Zootecnia, [c]Facultad de Medicina, Universidad Nacional Autónoma de México. México. D.F. 04510 México.

[d]Instituto Mexicano del Seguro Social, [e]Escuela de Medicina Veterinaria y Zootecnia, Benemérita Universidad Autónoma de Puebla, Puebla, México.

*Corresponding author. Tel.: (525) 6223818; fax: (525) 6223369; E-mail: edda@servidor.unam.mx.

Abstract

Taenia solium cysticercosis seriously affects human health when localised in the central nervous system (CNS) and causes great economic loss in pig husbandry in rural areas of endemic countries. Increasing the resistance to the parasite in the obligatory host pig may help in curbing transmission. Three synthetic peptides based on protein sequences of the murine parasite Taenia crassiceps, which had previously been shown to induce protection in mice against homologous challenge, were tested as a vaccine against T. solium cysticercosis in pigs. Vaccinated and unvaccinated piglets (240 in all) were distributed in pairs among the peasants' households of two rural villages in Mexico in which 14% of the native pigs were cysticercotic. Ten to twelve months later, the effect of vaccination was evaluated at necropsy. Vaccination decreased the total number of T. solium cysticerci (98.7%) and reduced the prevalence (52.6%). The natural challenge conditions used in this field trial strengthen the likelihood of successful transmission control to both pig and human through a large-scale pig vaccination program. We believe this is a major contribution in anticysticercosis vaccine development as these rather simple yet protective peptides are potentially more cost-effective to produce and less variable in results than antigens that are more complex.

Key words: cysticercosis, synthetic peptides, vaccines
Abbreviate article title: T. solium cysticercosis vaccination

1. Introduction

Taenia solium cysticercosis is a common parasitic disease of the central nervous system of humans and one of the most frequent neurological disorders in several countries of Latin America, Africa and Asia, where it causes enormous human suffering and great economic loss [1,2,3]. It is also considered a re-emerging disease in the USA because of immigration of tapeworm carriers from endemic areas [4,5]. Autochthonous cases are still recognized in some countries of Eastern Europe, Spain and Portugal [6].

The life cycle of T. solium includes a larval phase (metacestode or cysticercus) that develops in both pigs and humans from ingested eggs contaminating the environment. When humans ingest improperly cooked pork meat infected with live cysticerci, the cysticerci develop to the stage of an adult intestinal tapeworm, which produces millions of eggs that are then shed to the environment in human faeces.

The hope of controlling transmission by massively improving socio-economic standards of the very poor in the developing countries is not foreseeable in the near future. Nevertheless, since the pig is an indispensable intermediate host, it is conceivable to curb transmission by reducing pig cysticercosis through their effective vaccination. Vaccination against metacestode infections with what seems a wide and capricious variety of vaccine preparations has been surprisingly effective (relative to the difficulties found in other antiparasite vaccines) in a number of different cestodes and hosts [7-13].

Developing an effective vaccine against T. solium pig cysticercosis is also being pursued by a number of scientists with promising results. However, most reports are based on experimental trials with very few pigs, that do not represent realistic transmission conditions [9,10,12,13] (i.e., in dose and frequency of challenge, age of the host in initial and subsequent challenges, genetic and nutritional status of pigs). On the other hand, when performed in the field, results are expressed only as yearly changes of in vivo prevalence of pig cysticercosis in the region by tongue inspection [14,15], but do not measure

the impact of vaccination upon infection intensity. In addition, changes in prevalence in the next couple of years after vaccination would imply an unlikely immediate and potent effect of vaccination upon the number of tapeworm-carriers in the rural communities. These experimental and preliminary field measurements of the promising effects of pig vaccination, demand a controlled field trial followed by thorough post-mortem counts of viable and damaged cysticerci to critically evaluate the vaccine's effects upon the prevalence and the intensity of pig cysticercosis in highly endemic rural areas.

The composition of the antigens to be included in a T. solium vaccine is a major issue. The use of crude antigen extracts from wild cysticerci collected haphazardly from naturally infected cysticercotic pigs can hardly be reproducible, as the mixtures usually include at least 20-50 or so different protein antigens recognizable in Western Blots [16], each carrying unknown numbers of peptidic and possibly glycosidic epitopes. Antigenic diversity among T. solium, although largely unexplored, has been shown to be significant [17] and could influence results. Further, some of the natural antigens have protective or infection-enhancing properties in murine T. crassiceps cysticercosis, whilst others are irrelevant [18,19]. With this in view, simplification of the vaccine composition was considered desirable. Purified natural antigens [9,18] and recombinant proteins of other cestodes [13,20] that have proved to be effective in homologous or heterologous systems are also being tried in T. solium pig cysticercosis. We have chosen to test synthetic peptides with a view of eventually expressing them in a more cost-effective biotech system (i.e. phage vectors, transgenic plant expression).

Because of the high costs and slow data retrieval involved in testing pigs in order to identify vaccine candidates, we first used the experimental disease caused in mice by another tapeworm (Taenia crassiceps) [21]. This murine parasite exhibits extensive antigen cross-reactivity [16] and cross-immunity with T. solium [22], and its metacestodes easily and rapidly develop in the peritoneal cavity of mice [21]. Thus, we identified and isolated antigens from T. crassiceps that effectively protect against experimental T. crassiceps murine

and experimental T. solium porcine cysticercosis [18]. Antibodies against these protective protein fractions were then employed to identify three recombinant antigens in a cDNA library [20]. One of them, namely KETc7, has been proved to effectively protect mice against T. crassiceps cysticercosis by DNA vaccination [23,24], via at least one known peptide stretch of 18 amino acids (GK-1) [25]. From the other two, KETc1 and KETc12, two additional peptides (KETc1/p of 13 and KETc12/p of 9 amino acids) were synthetically produced and confirmed in their capacity to protect mice [26]. The three protective peptidic epitopes identified were expressed in the different stages of T. solium, and represent different immunological targets in the parasite [26]

Thus, we decided to evaluate a vaccine against porcine T. solium cysticercosis that is composed of these three chemically synthesized peptidic epitopes (GK-1, KETc1/p, KETc12/p), under realistic field conditions of exposure to infection and under thorough assessment of the infection's prevalence and intensity in vaccinated and control pigs.

2. Materials and methods

2.1. Experimental design

The experimental design is that of a vaccinated and control study with 120 and 120 pigs in each group, distributed in matching pairs (one vaccinated and one control) in 70 village households. The study was performed under the natural exposure conditions of two different rural villages of Mexico that are endemic for pig-cysticercosis (prevalence =14 %). The response variables were prevalence (the number of pigs with at least one parasite/total number of pigs in the group) and intensity (the parasite load in each pig, classified as total cysticerci, viable or necrotic) collected from half the carcass of each pig in the study.

2.2. Selection of the communities

Two rural communities, Huatlatlauca and Tepetzetzintla, in the state of Puebla, Mexico, were selected. In both, the conditions which favour the transmission of T. solium were present, namely the high prevalence of

cysticercosis in live pigs (14 %, determined by tongue inspection), open air faecalism, local pork meat consumption and extensive domestic pig slaughtering, low personal hygiene and low sanitary conditions in household and village.

2.3. Vaccine

This vaccine consists of the following synthetic peptides: GK-1 (amino acids [aa] from 69 to 85 of KETc7; (Gly Tyr Try Try Pro Ser Asp Pro Asn Thr Phe Thr Ala Pro Pro Try Ser **Ala**); KETc1 (Ala Pro Met Ser Thr Pro Ser Ala Thr Ser Val Arg **Gly**) and KETc12 (Gly Asn Leu Leu Leu Ser Cys Leu **Gly**). They were prepared by manual stepwise solid-phase synthesis with N-tertibutyloxycarbonyl derivatives of L-amino acids on phenylacetamidomethyl resin (Sigma Chemical Co., St. Louis, Mo.). Bold letters indicate the extra amino acids that were coupled to the commercial resin. All peptides were 95% pure as judged by high-pressure liquid chromatography on analytical C18 reversed phase columns (3.9 by 150 nm; Delta Park; Waters). The correct amino acid sequence of each peptide was confirmed by protein sequencing on a pulsed liquid-phase protein sequencer (Applied Biosystems). The vaccine was transported refrigerated to the communities and immediately applied to the animals.

2.4. Pigs and vaccination

Previous research had shown that piglets in villages might be infected in the first few days after birth, as soon as they can follow their mothers in her search for food [27]. We were not certain that an incompletely developed immune system in newborn piglets would respond to vaccination, therefore, the sows of the piglets to be vaccinated were themselves vaccinated during gestation in the hope of reducing the risk of infection of the new-born. Thus, we purchased 49 pregnant sows in the selected communities. Twenty-five of these sows, those that were to breed the vaccinated piglets, were themselves vaccinated subcutaneously at the base of the ear during the last month of gestation with 500 μg of each peptide plus 300 μg of adjuvant (saponin). The remaining 24 pregnant sows, which were to breed the control piglets, were injected with 300 μg of adjuvant, at the same time and site of injection as the

vaccinated sows. It was estimated that one-hundred and twenty piglets per group (vaccinated and control) was adequate to evaluate the vaccine's effect with 95% confidence intervals at an expected prevalence of 14% of cysticercosis in the control group [28]. One-hundred and thirty eight piglets were vaccinated and 140 that received only adjuvant, served as controls. Of all these, 18 vaccinated and 20 control pigs died of causes not related to vaccination nor to cysticercosis and were excluded from the study. The piglets born to the vaccinated sows were vaccinated twice, at 60 and 90 days of age with 250 μg of each peptide plus 150 μg of saponin in saline isotonic solution at the base of the ear. Piglets born to the control sows received only 150 μg of saponin each at the same ages and under the same conditions as the vaccinated piglets and were kept as controls. After receiving the second dose of vaccine or adjuvant the piglets were distributed in matched pairs (one vaccinated, one control) among 70 villagers' households, preference being given to those villagers that had been previously identified as having owned infected animals. Each household received one or two matched pairs, thus, possible variations in management and exposure levels among the different households were controlled for all pairs. Villagers were instructed to treat and feed the experimental pigs as they usually do with their own pigs.

2.5. Necropsies

All pigs were humanely killed between the ages of 10 to 12 months. Half the carcass of each animal, together with liver, tongue, masseter muscle and heart were carefully dissected and all parasites counted. Additionally, the cysticerci were macroscopically classified in either viable or necrotic according to their macroscopic aspect (viable cysticerci being those well-limited neatly conserved cystic structures containing clear vesicular fluid, whilst necrotic cysticerci were no longer cystic but rather semi-solid caseous masses without discernible parasite structures). In addition, samples of parasitized tissues were fixed in buffered 10% formalin (pH 7.2). The samples were then processed for histological observation and stained with haematoxylin-eosin to confirm the macroscopic diagnosis by microscopy [27].

2.6. Statistical analysis

The statistical analyses used were the marginal χ^2 Mac Nemar test to compare the relative risk of being infected in vaccinated versus control pigs according to the results of necropsies in the 120 matched pairs. The non-parametric Mann-Whitney U test was used to compare the number of cysticerci recovered in both groups. The statistical programmes of EPI-Info and SSPS were used.

3. Results

Table 1 shows the effect of vaccination on the reduction of the number of cysticerci in infected pigs (15.8 to 7.5%). Table 2 summarises the distribution of infected pigs among vaccinated and controls groups. It also shows the number of total, viable and necrotic cysticerci found in each pig. Of the 120 control pigs, 19 (15.8%) were infected: 2 (10.5%) of these with 1 parasite, 13 (68.4%) with less than ten, and 6 (31.6%) with thousands of cysticerci. From these control pigs, 66565 cysticerci were collected, (63951 vesicular and 2614 necrotic). From the 120 vaccinated animals, 9 (7.5%) were infected with at least one parasite: 6 (66%) of these with less than ten, 2 (22%) with less than a hundred, and only one with 1286 cysticerci. Of these vaccinated pigs, 1369 cysticerci were collected (806 vesicular and 563 necrotic). At 95% level of confidence, results indicate 52.6% efficiency of the vaccine in reducing the number of infected pigs and 97.9% reduction of the total parasite load. The vaccine's reduction of parasite load relevant for transmission would be closer to 99%, if the necrotic cysticerci were of no consequence for transmission. The skewed distribution of the number of cysticerci found in each group should be noted: there being a number of low intensity cases (<10 cysticerci per pig) in both groups (15 in control and only 6 in vaccinated pigs), whilst massive intensities (>1000 cysticerci per pig) were more commonly found in the control pigs (6 vs 1).

4. Discussion

The prevalence of cysticercosis in the control pigs introduced in these rural villages very closely reproduced the previously estimated in vivo prevalence of pig cysticercosis, (15.8% obtained herein vs 14% previously reported [29]). This validates our approach and lends credence and significance to results in the vaccinated group.

The most direct and simple demonstration of the vaccine's effects is that it reduced the prevalence of cysticercosis among the vaccinated pigs by 52.6% and, most significantly, reduced the intensity of infection with viable cysts by 97.9%. That these potent effects were obtained with a mixture of synthetic peptides tested in realistic exposure conditions argues in favour of the effectiveness of vaccination against pig cysticercosis. It is also a strong technical argument towards the development of an effective, less variable, more stable and cost-effective vaccine based on peptides to be used on a wider scale. Even if it were the vaccine's only effects, the reduction in pig cysticercosis prevalence and parasite intensity is greatly beneficial for the villagers' economy because pork meat without cysticercosis sells at much higher prices. In addition, protecting pigs against cysticercosis by vaccination has the economic benefit of allowing the villagers' rustic low-cost form of rearing pigs to the only one they can afford, to continue. On the other hand, whether pig vaccination actually reduces the number of tapeworms that would eventually develop from lightly parasitized pigs will have to be ascertained in a study designed differently, as there is no known clear-cut correlation between the number of viable cysts in a pig and their probability of becoming a tapeworm upon ingestion by a human host.

The vaccination protocol starting in the gestating sows of the vaccinated piglets leads to the question of which of the vaccinations is the one relevant for the protective effects: the sows' or the piglets' or both?. While this point needs to be clarified, the success of the vaccination protocol suggests that vaccination of both pregnant sows and piglets should be considered. Experimentation is under way to determine the cellular and molecular immunological protagonists

responsible for the vaccine's efficiency against pig cysticercosis. Should results from T. crassiceps murine cysticercosis hold for T. solium in pigs, one would expect the participation of TH1-mediated immune responses under the strong influence of genetic and endocrine factors of the host [29, 30, 31].

Finally, it is also worth noticing the over-dispersed distribution of parasite intensities in both vaccinated and control groups. There are at least two modes: one of very light intensity infections (less than 10 parasites per pig) that is sensitive to vaccination (approximately 50% reduction in the vaccinated group), and another of massive intensity infections (with thousands or tens of thousands of parasites per pig), the most vulnerable to vaccination. An over-dispersed distribution of parasite intensities in naturally acquired pig cysticercosis has been documented before [29] and has led to suspect the involvement of host genetics in susceptibility and resistance to T. solium, as shown for T. crassiceps in mice [30]. Advisability of an extensive pig vaccination program for public health reasons would be strengthened by evaluation of the differential transmission roles of the pigs afflicted with few cysticerci versus the massively infected ones. There would be no question about the public health benefits of vaccination if the total number of cysticerci were the only thing that mattered.

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Table 1. Effect of pig vaccination against Taenia solium cysticercosis on the number of non-infected and infected pigs following field exposure.

	Pigs inoculated with:	
	Adjuvant (Controls)	Adjuvant + peptides (Vaccinated)
Non-Infected	101[a]	111
Infected	19	9
	15.8%[b]	7.5%

[a] Number of pigs per group. [b] Percent of infected pigs.

The relative risk of being infected in vaccinated pigs was significantly lower than in controls ($P < 0.05$, χ^2 Marginal Test).

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Experimental group	Number of Infected pigs	INTENSITY CLASS Number of cysticerci/pig 1-10	11-100	101-1000	1001-10000	> 10000	Total Necrotic/ Vesicular
Control							
Number of infected pigs in each intensity class	19	13	0	0	2	4	
Number of cysticerci in each pig		1,1,2,2,2,2,2, 3,3,3,3,4,9			2700,3500	13000, 14950, 15000, 17378	66565 2614/63951
Vaccinated							
Number of infected pigs in each intensity class	9	6	2	0	1	0	
Number of cysticerci in each pig		1,1,2,2,4,4,	34,35		1286		1364 563/806

he number of cysticerci recovered in controls was significantly higher than in vaccinated pigs (P < 0.05, Mann Whitney Test).

4.2 ARTICULOS EN COLABORACION

Synthetic peptide vaccine against Taenia solium pig cysticercosis: successful vaccination in a controlled field trial in rural Mexico

[c]M. Huerta, [b]A.S. de Aluja, [a]G. Fragoso, [a]A. Toledo, [b]N. Villalobos, [a]M. Hernández, [a]G. Gevorkian, [a]G. Acero, [d]A. Díaz, [e]I. Alvarez, [e]R. Avila, [a]C. Beltrán, [a]G. Garcia, [b]J.J. Martinez, [a]C. Larralde, [a]E. Sciutto*

[a]Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México.D.F., A.P. 70228. 04510; México. *Howard Hughes Medical Institute International Research Scholar. [b]Facultad de Medicina Veterinaria y Zootecnia, [c]Facultad de Medicina, Universidad Nacional Autónoma de México. México. D.F. 04510 México.

[d]Instituto Mexicano del Seguro Social, [e]Escuela de Medicina Veterinaria y Zootecnia, Benemérita Universidad Autónoma de Puebla, Puebla, México.

*Corresponding author. Tel.: (525) 6223818; fax: (525) 6223369; E-mail: edda@servidor.unam.mx.

Abstract

Taenia solium cysticercosis seriously affects human health when localised in the central nervous system (CNS) and causes great economic loss in pig husbandry in rural areas of endemic countries. Increasing the resistance to the parasite in the obligatory host pig may help in curbing transmission. Three synthetic peptides based on protein sequences of the murine parasite Taenia crassiceps, which had previously been shown to induce protection in mice against homologous challenge, were tested as a vaccine against T. solium cysticercosis in pigs. Vaccinated and unvaccinated piglets (240 in all) were distributed in pairs among the peasants' households of two rural villages in Mexico in which 14% of the native pigs were cysticercotic. Ten to twelve months later, the effect of vaccination was evaluated at necropsy. Vaccination decreased the total number of T. solium cysticerci (98.7%) and reduced the prevalence (52.6%). The natural challenge conditions used in this field trial strengthen the likelihood of successful transmission control to both pig and human through a large-scale pig vaccination program. We believe this is a major contribution in anticysticercosis vaccine development as these rather simple yet protective peptides are potentially more cost-effective to produce and less variable in results than antigens that are more complex.

Key words: cysticercosis, synthetic peptides, vaccines
Abbreviate article title: T. solium cysticercosis vaccination

1. Introduction

Taenia solium cysticercosis is a common parasitic disease of the central nervous system of humans and one of the most frequent neurological disorders in several countries of Latin America, Africa and Asia, where it causes enormous human suffering and great economic loss [1,2,3]. It is also considered a re-emerging disease in the USA because of immigration of tapeworm carriers from endemic areas [4,5]. Autochthonous cases are still recognized in some countries of Eastern Europe, Spain and Portugal [6].

The life cycle of T. solium includes a larval phase (metacestode or cysticercus) that develops in both pigs and humans from ingested eggs contaminating the environment. When humans ingest improperly cooked pork meat infected with live cysticerci, the cysticerci develop to the stage of an adult intestinal tapeworm, which produces millions of eggs that are then shed to the environment in human faeces.

The hope of controlling transmission by massively improving socio-economic standards of the very poor in the developing countries is not foreseeable in the near future. Nevertheless, since the pig is an indispensable intermediate host, it is conceivable to curb transmission by reducing pig cysticercosis through their effective vaccination. Vaccination against metacestode infections with what seems a wide and capricious variety of vaccine preparations has been surprisingly effective (relative to the difficulties found in other antiparasite vaccines) in a number of different cestodes and hosts [7-13].

Developing an effective vaccine against T. solium pig cysticercosis is also being pursued by a number of scientists with promising results. However, most reports are based on experimental trials with very few pigs, that do not represent realistic transmission conditions [9,10,12,13] (i.e., in dose and frequency of challenge, age of the host in initial and subsequent challenges, genetic and nutritional status of pigs). On the other hand, when performed in the field, results are expressed only as yearly changes of in vivo prevalence of pig cysticercosis in the region by tongue inspection [14,15], but do not measure

the impact of vaccination upon infection intensity. In addition, changes in prevalence in the next couple of years after vaccination would imply an unlikely immediate and potent effect of vaccination upon the number of tapeworm-carriers in the rural communities. These experimental and preliminary field measurements of the promising effects of pig vaccination, demand a controlled field trial followed by thorough post-mortem counts of viable and damaged cysticerci to critically evaluate the vaccine's effects upon the prevalence and the intensity of pig cysticercosis in highly endemic rural areas.

The composition of the antigens to be included in a T. solium vaccine is a major issue. The use of crude antigen extracts from wild cysticerci collected haphazardly from naturally infected cysticercotic pigs can hardly be reproducible, as the mixtures usually include at least 20-50 or so different protein antigens recognizable in Western Blots [16], each carrying unknown numbers of peptidic and possibly glycosidic epitopes. Antigenic diversity among T. solium, although largely unexplored, has been shown to be significant [17] and could influence results. Further, some of the natural antigens have protective or infection-enhancing properties in murine T. crassiceps cysticercosis, whilst others are irrelevant [18,19]. With this in view, simplification of the vaccine composition was considered desirable. Purified natural antigens [9,18] and recombinant proteins of other cestodes [13,20] that have proved to be effective in homologous or heterologous systems are also being tried in T. solium pig cysticercosis. We have chosen to test synthetic peptides with a view of eventually expressing them in a more cost-effective biotech system (i.e. phage vectors, transgenic plant expression).

Because of the high costs and slow data retrieval involved in testing pigs in order to identify vaccine candidates, we first used the experimental disease caused in mice by another tapeworm (Taenia crassiceps) [21]. This murine parasite exhibits extensive antigen cross-reactivity [16] and cross-immunity with T. solium [22], and its metacestodes easily and rapidly develop in the peritoneal cavity of mice [21]. Thus, we identified and isolated antigens from T. crassiceps that effectively protect against experimental T. crassiceps murine

and experimental T. solium porcine cysticercosis [18]. Antibodies against these protective protein fractions were then employed to identify three recombinant antigens in a cDNA library [20]. One of them, namely KETc7, has been proved to effectively protect mice against T. crassiceps cysticercosis by DNA vaccination [23,24], via at least one known peptide stretch of 18 amino acids (GK-1) [25]. From the other two, KETc1 and KETc12, two additional peptides (KETc1/p of 13 and KETc12/p of 9 amino acids) were synthetically produced and confirmed in their capacity to protect mice [26]. The three protective peptidic epitopes identified were expressed in the different stages of T. solium, and represent different immunological targets in the parasite [26]

Thus, we decided to evaluate a vaccine against porcine T. solium cysticercosis that is composed of these three chemically synthesized peptidic epitopes (GK-1, KETc1/p, KETc12/p), under realistic field conditions of exposure to infection and under thorough assessment of the infection's prevalence and intensity in vaccinated and control pigs.

2. Materials and methods

2.1. Experimental design

The experimental design is that of a vaccinated and control study with 120 and 120 pigs in each group, distributed in matching pairs (one vaccinated and one control) in 70 village households. The study was performed under the natural exposure conditions of two different rural villages of Mexico that are endemic for pig-cysticercosis (prevalence =14 %). The response variables were prevalence (the number of pigs with at least one parasite/total number of pigs in the group) and intensity (the parasite load in each pig, classified as total cysticerci, viable or necrotic) collected from half the carcass of each pig in the study.

2.2. Selection of the communities

Two rural communities, Huatlatlauca and Tepetzetzintla, in the state of Puebla, Mexico, were selected. In both, the conditions which favour the transmission of T. solium were present, namely the high prevalence of

cysticercosis in live pigs (14 %, determined by tongue inspection), open air faecalism, local pork meat consumption and extensive domestic pig slaughtering, low personal hygiene and low sanitary conditions in household and village.

2.3. Vaccine

This vaccine consists of the following synthetic peptides: GK-1 (amino acids [aa] from 69 to 85 of KETc7; (Gly Tyr Try Try Pro Ser Asp Pro Asn Thr Phe Thr Ala Pro Pro Try Ser **Ala**); KETc1 (Ala Pro Met Ser Thr Pro Ser Ala Thr Ser Val Arg **Gly)** and KETc12 (Gly Asn Leu Leu Leu Ser Cys Leu **Gly**). They were prepared by manual stepwise solid-phase synthesis with N-tertibutyloxycarbonyl derivatives of L-amino acids on phenylacetamidomethyl resin (Sigma Chemical Co., St. Louis, Mo.). Bold letters indicate the extra amino acids that were coupled to the commercial resin. All peptides were 95% pure as judged by high-pressure liquid chromatography on analytical C18 reversed phase columns (3.9 by 150 nm; Delta Park; Waters). The correct amino acid sequence of each peptide was confirmed by protein sequencing on a pulsed liquid-phase protein sequencer (Applied Biosystems). The vaccine was transported refrigerated to the communities and immediately applied to the animals.

2.4. Pigs and vaccination

Previous research had shown that piglets in villages might be infected in the first few days after birth, as soon as they can follow their mothers in her search for food [27]. We were not certain that an incompletely developed immune system in newborn piglets would respond to vaccination, therefore, the sows of the piglets to be vaccinated were themselves vaccinated during gestation in the hope of reducing the risk of infection of the new-born. Thus, we purchased 49 pregnant sows in the selected communities. Twenty-five of these sows, those that were to breed the vaccinated piglets, were themselves vaccinated subcutaneously at the base of the ear during the last month of gestation with 500 μg of each peptide plus 300 μg of adjuvant (saponin). The remaining 24 pregnant sows, which were to breed the control piglets, were injected with 300 μg of adjuvant, at the same time and site of injection as the

vaccinated sows. It was estimated that one-hundred and twenty piglets per group (vaccinated and control) was adequate to evaluate the vaccine's effect with 95% confidence intervals at an expected prevalence of 14% of cysticercosis in the control group [28]. One-hundred and thirty eight piglets were vaccinated and 140 that received only adjuvant, served as controls. Of all these, 18 vaccinated and 20 control pigs died of causes not related to vaccination nor to cysticercosis and were excluded from the study. The piglets born to the vaccinated sows were vaccinated twice, at 60 and 90 days of age with 250 μg of each peptide plus 150 μg of saponin in saline isotonic solution at the base of the ear. Piglets born to the control sows received only 150 μg of saponin each at the same ages and under the same conditions as the vaccinated piglets and were kept as controls. After receiving the second dose of vaccine or adjuvant the piglets were distributed in matched pairs (one vaccinated, one control) among 70 villagers' households, preference being given to those villagers that had been previously identified as having owned infected animals. Each household received one or two matched pairs, thus, possible variations in management and exposure levels among the different households were controlled for all pairs. Villagers were instructed to treat and feed the experimental pigs as they usually do with their own pigs.

2.5. Necropsies

All pigs were humanely killed between the ages of 10 to 12 months. Half the carcass of each animal, together with liver, tongue, masseter muscle and heart were carefully dissected and all parasites counted. Additionally, the cysticerci were macroscopically classified in either viable or necrotic according to their macroscopic aspect (viable cysticerci being those well-limited neatly conserved cystic structures containing clear vesicular fluid, whilst necrotic cysticerci were no longer cystic but rather semi-solid caseous masses without discernible parasite structures). In addition, samples of parasitized tissues were fixed in buffered 10% formalin (pH 7.2). The samples were then processed for histological observation and stained with haematoxylin-eosin to confirm the macroscopic diagnosis by microscopy [27].

2.6. Statistical analysis

The statistical analyses used were the marginal χ^2 Mac Nemar test to compare the relative risk of being infected in vaccinated versus control pigs according to the results of necropsies in the 120 matched pairs. The non-parametric Mann-Whitney U test was used to compare the number of cysticerci recovered in both groups. The statistical programmes of EPI-Info and SSPS were used.

3. Results

Table 1 shows the effect of vaccination on the reduction of the number of cysticerci in infected pigs (15.8 to 7.5%). Table 2 summarises the distribution of infected pigs among vaccinated and controls groups. It also shows the number of total, viable and necrotic cysticerci found in each pig. Of the 120 control pigs, 19 (15.8%) were infected: 2 (10.5%) of these with 1 parasite, 13 (68.4%) with less than ten, and 6 (31.6%) with thousands of cysticerci. From these control pigs, 66565 cysticerci were collected, (63951 vesicular and 2614 necrotic). From the 120 vaccinated animals, 9 (7.5%) were infected with at least one parasite: 6 (66%) of these with less than ten, 2 (22%) with less than a hundred, and only one with 1286 cysticerci. Of these vaccinated pigs, 1369 cysticerci were collected (806 vesicular and 563 necrotic). At 95% level of confidence, results indicate 52.6% efficiency of the vaccine in reducing the number of infected pigs and 97.9% reduction of the total parasite load. The vaccine's reduction of parasite load relevant for transmission would be closer to 99%, if the necrotic cysticerci were of no consequence for transmission. The skewed distribution of the number of cysticerci found in each group should be noted: there being a number of low intensity cases (<10 cysticerci per pig) in both groups (15 in control and only 6 in vaccinated pigs), whilst massive intensities (>1000 cysticerci per pig) were more commonly found in the control pigs (6 vs 1).

4. Discussion

The prevalence of cysticercosis in the control pigs introduced in these rural villages very closely reproduced the previously estimated in vivo prevalence of pig cysticercosis, (15.8% obtained herein vs 14% previously reported [29]). This validates our approach and lends credence and significance to results in the vaccinated group.

The most direct and simple demonstration of the vaccine's effects is that it reduced the prevalence of cysticercosis among the vaccinated pigs by 52.6% and, most significantly, reduced the intensity of infection with viable cysts by 97.9%. That these potent effects were obtained with a mixture of synthetic peptides tested in realistic exposure conditions argues in favour of the effectiveness of vaccination against pig cysticercosis. It is also a strong technical argument towards the development of an effective, less variable, more stable and cost-effective vaccine based on peptides to be used on a wider scale. Even if it were the vaccine's only effects, the reduction in pig cysticercosis prevalence and parasite intensity is greatly beneficial for the villagers' economy because pork meat without cysticercosis sells at much higher prices. In addition, protecting pigs against cysticercosis by vaccination has the economic benefit of allowing the villagers' rustic low-cost form of rearing pigs to the only one they can afford, to continue. On the other hand, whether pig vaccination actually reduces the number of tapeworms that would eventually develop from lightly parasitized pigs will have to be ascertained in a study designed differently, as there is no known clear-cut correlation between the number of viable cysts in a pig and their probability of becoming a tapeworm upon ingestion by a human host.

The vaccination protocol starting in the gestating sows of the vaccinated piglets leads to the question of which of the vaccinations is the one relevant for the protective effects: the sows' or the piglets' or both?. While this point needs to be clarified, the success of the vaccination protocol suggests that vaccination of both pregnant sows and piglets should be considered. Experimentation is under way to determine the cellular and molecular immunological protagonists

responsible for the vaccine's efficiency against pig cysticercosis. Should results from T. crassiceps murine cysticercosis hold for T. solium in pigs, one would expect the participation of TH1-mediated immune responses under the strong influence of genetic and endocrine factors of the host [29, 30, 31].

Finally, it is also worth noticing the over-dispersed distribution of parasite intensities in both vaccinated and control groups. There are at least two modes: one of very light intensity infections (less than 10 parasites per pig) that is sensitive to vaccination (approximately 50% reduction in the vaccinated group), and another of massive intensity infections (with thousands or tens of thousands of parasites per pig), the most vulnerable to vaccination. An over-dispersed distribution of parasite intensities in naturally acquired pig cysticercosis has been documented before [29] and has led to suspect the involvement of host genetics in susceptibility and resistance to T. solium, as shown for T. crassiceps in mice [30]. Advisability of an extensive pig vaccination program for public health reasons would be strengthened by evaluation of the differential transmission roles of the pigs afflicted with few cysticerci versus the massively infected ones. There would be no question about the public health benefits of vaccination if the total number of cysticerci were the only thing that mattered.

Acknowledgments

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[a] Number of pigs per group. [b] Percent of infected pigs.

The relative risk of being infected in vaccinated pigs was significantly lower than in controls ($P < 0.05$, χ^2 Marginal Test).

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Experimental group	Number of Infected pigs	INTENSITY CLASS Number of cysticerci/pig 1-10	11-100	101-1000	1001-10000	> 10000	Total Necrotic/ Vesicular
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Number of infected pigs in each intensity class	19	13	0	0	2	4	
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Number of infected pigs in each intensity class	9	6	2	0	1	0	
Number of cysticerci in each pig		1,1,2,2,4,4,	34,35		1286		1364 563/806

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4.2 ARTICULOS EN COLABORACION

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Two Epitopes Shared by *Taenia crassiceps* and *Taenia solium* Confer Protection against Murine *T. crassiceps* Cysticercosis along with a Prominent T1 Response

ANDREA TOLEDO,[1] GLADIS FRAGOSO,[1] GABRIELA ROSAS,[1] MARISELA HERNÁNDEZ,[1] GOAR GEVORKIAN,[1] FERNANDO LÓPEZ-CASILLAS,[2] BEATRIZ HERNANDEZ,[3] GONZALO ACERO,[1] MIRNA HUERTA,[4] CARLOS LARRALDE,[1] AND EDDA SCIUTTO[1]*

Instituto de Investigaciones Biomédicas,[1] Instituto de Fisiología Celular,[2] and Facultad de Medicina, UNAM, México D. F.,[3] and Facultad de Medicina, Benemérita Universidad Autónoma de Puebla, Puebla,[4] México

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***Taenia crassiceps* recombinant antigens KETc1 and KETc12 have been shown to induce high level of protection against experimental murine *T. crassiceps* cysticercosis, an experimental model successfully used to test candidate antigens for use in vaccination against porcine *Taenia solium* cysticercosis. Based on the deduced amino acid sequence, KETc1 and KETc12 were chemically synthesized in linear form. Immunization with KETc1 induced 66.7 to 100% protection against murine cysticercosis, and immunization with KETc12 induced 52.7 to 88.1% protection. The elicited immune response indicated that both peptides contain at least one B-cell epitope (as demonstrated by their ability to induce specific antibodies) and one T-cell epitope that strongly stimulated the proliferation of T cells primed with either the free peptide or total cysticercal *T. crassiceps* antigens. The high percentage of spleen cells expressing inflammatory cytokines points to the likelihood of a T1 response being involved in protection. The protective capacity of the peptides and their presence in all developmental stages of *T. solium* point to these two epitopes as strong candidates for inclusion in a polyepitopic synthetic vaccine against *T. solium* pig cysticercosis.**

Taenia solium cysticercosis is a common parasitic disease of the central nervous system of humans in several countries in Latin America, Africa, and Asia, where it represents a major health and economic problem (2, 28). The life cycle of this parasite includes a larval phase (cysticercus) that affects both pigs and humans after the ingestion of *T. solium* eggs. The parasite's life cycle is completed when humans consume improperly cooked cysticercotic pork and the adult intestinal tapeworm develops and, in turn, produces millions of eggs that are shed in human feces. In regions of endemic infection, transmission is clearly related to prevailing low standards of personal hygiene and environmental sanitation control (i.e., open air fecalism) in areas where rustic rearing of pigs is practiced by the rural population (pigs roaming about freely in search of edibles and/or deliberately fed with human feces [11]). Regrettably, control of transmission by general improvement of the social, economical, and educational status in developing countries or by proper and strict meat inspection programs is not within reach in the near future. However, since the pig is an indispensable intermediate host, transmission could be hindered by lowering the prevalence of pig cysticercosis through vaccination. Development of an effective vaccine to be used in pigs is being pursued by a number of scientists, with promising results (9, 15–17).

Because of the high costs of experimentation in pigs, murine cysticercosis caused by *Taenia crassiceps* has been used to test and select promising antigens before they are tested in pigs (13, 21). Thus, it has been shown that total *T. crassiceps* antigens can cross-protect pigs against *T. solium* cysticerosis. However, the effects of vaccination with whole-antigen extracts were strongly dose dependent; besides, some antigens were found to be protective while others led to facilitation of the infection (22). Such complications with the use of whole-antigen extracts led us to redirect our research to the identification of individual protective antigens (14, 26). Using recombinant DNA technology, several vaccine candidates were identified in murine *T. crassiceps* cysticercosis with crude lysates of the respective clones as the immunogen (13, 14). One of them, KETc7, which has a protective capacity confirmed by DNA immunization (1, 20), includes at least one protective epitope of 17 amino acids (GK1). GK1 is also expressed in *T. solium* oncospheres (25), the parasite's developmental stage most vulnerable to immunological attack (19). Two additional protective clones, KETc1 and KETc12 (14), were also identified. Herein we report the protective capacity against *T. crassiceps* murine cysticerosis of the peptides deduced from these last two clones. Furthermore, we describe the localization of the peptides in each parasite stage of *T. solium* and *T. crassiceps*, the immune response they elicit in immunized mice—where T1 is most prominent—and propose them as additional components for a synthetic vaccine to be tested in pigs in an attempt to block *T. solium* transmission.

MATERIALS AND METHODS

Peptides. Two *T. crassiceps*-derived peptides (14) that are shared by *T. solium* (24), KETc1 [APMSTPSATSVR(G)] and KETc12 [GNLLLSCL(G)], were synthesized by stepwise solid-phase synthesis with N^{α}-*tert*-butyloxycarbonyl derivatives of L-amino acids on phenyl-acetamidomethyl resin (Sigma Chemical Co., St. Louis, Mo.). The peptides were 95% pure as judged by high-pressure liquid

* Corresponding author. Mailing address: Departamento de Inmunología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México (UNAM), A.P. 70228, México D.F., 04510, México. Phone: (525) 6223818. Fax: (525) 6223369. E-mail: edda@servidor.unam.mx.

FIG. 1. Antibody levels determined by ELISA in individual control (C) and immunized (I) mice against *Tc*Ag. The mean level of antibodies was significantly higher in immunized mice than in controls. O.D., optical density.

chromatography in an analytical C_{18} reversed-phase column (3.9 by 150 mm Delta Pak [Waters]). The correct amino acid sequence of each peptide was confirmed by protein sequencing on a pulsed-liquid-phase protein sequencer (Applied Biosystems) at the National Institute of Cardiology, Mexico City.

Mice. BALB/cAnN mice, previously characterized as susceptible to cysticercosis (3), were used in vaccine trials. The original murine stock was purchased from M. Bevan (University of Washington) and then bred and kept in our animal facilities by the "single-line breeding" system for more than 30 generations. All mice used were males that were 5 to 7 weeks of age at the beginning of the experiments. The experiments reported herein were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals*, Institute of Laboratory Animals Resources, National Research Council, Washington, D.C.

Immunization of mice and serum collection. Groups of six to nine BALB/cAnN mice were subcutaneously immunized with two doses of 10 μg of each individual peptide in saponin (Sigma Chemical Co.) per mouse at a concentration of 100 μg/mouse as described elsewhere (25). This dose was determined as optimal in collateral experiments (data not shown). Ten days later, the mice were given a booster with the same immunizing dose of the same peptide in the same adjuvant as used before. Immune sera were obtained from each individual mouse before and after each immunization and stored at −70°C until individually tested for the presence of specific antibodies.

Parasites and cysticercal antigens. The ORF strain of *T. crassiceps* (4) has been maintained by serial passage in BALB/cAnN female mice for 15 years in our animal facilities. Cysticerci for infection were harvested from the peritoneal cavity of mice 1 to 3 months after inoculation of 10 nonbudding small cysticerci (2 to 3 mm in diameter) per animal. The soluble antigens were recovered from similar cysticerci by a previously described procedure (18). Whole *T. solium* cysticerci were dissected from skeletal muscle of highly infected pork carcasses 2 to 4 h after slaughter in an abattoir in Zacatepec, Morelos, Mexico, embedded in optimun-cutting-temperature compound (Miles, Inc.), and frozen at −70°C until used in immunofluorescence assays (see below). Segments from *T. solium* tapeworm and eggs were obtained from the feces of an infected man in Puebla, Mexico. The tapeworm was recovered after treatment with a single oral dose (2 g) of niclosamide (Yomesan; kindly supplied by Bayer). After being washed in saline plus antibiotics (100 U of penicillin per ml plus 100 μg of streptomycin per ml), several gravid proglottids were separated for immunofluorescence assays.

ELISA for antibody measurements. *T. crassiceps* whole soluble antigens (*Tc*Ag) were obtained as previously described (18) and used as the source of antigens in an enzyme-linked immunosorbent assay (ELISA) to measure the antibody response induced by peptide immunization by using a procedure described elsewhere (25).

Proliferation assay. Spleen cells from control and KETc1- and KETc12-immunized mice were harvested 15 days after the second immunization and cultured in RPMI 1640 medium supplemented with L-glutamine (0.2 mM), nonessential amino acids (0.01 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and fetal bovine serum FBS (10%). The cells were cultured with the appropriate concentration of concanavalin A (ConA) (5 μg/ml), KETc1 (50 μg/ml), KETc12 (10 μg/ml), or *Tc*Ag (10 μg/ml) and incubated at 37°C in a 5% CO_2 humidified atmosphere in flat-bottomed microtiter plates at a cell concentration of 2×10^5 cells per 200 μl of final volume. Then 10^5 peritoneal cells recovered from the

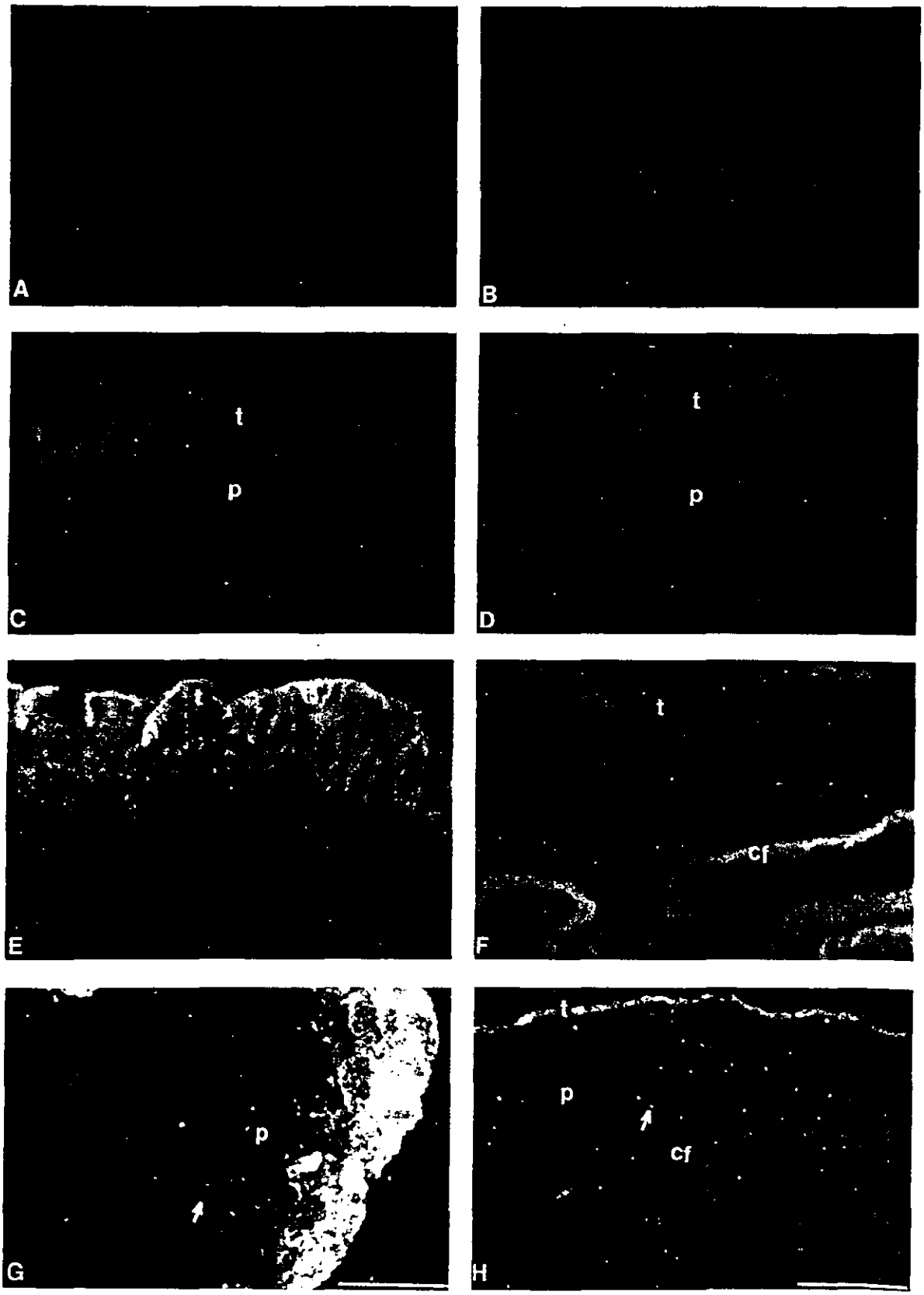
TABLE 1. Protective immunity against murine cysticercosis by immunization with KETc1 and KETc12 peptides

Group of experimental mice	Result for trial no.:											
	1			2			3					
	No. of cysticerci[a]	Parasite intensity[b]	% Protection[c]	No. of cysticerci	Parasite intensity	% Protection	No. of cysticerci	Parasite intensity	% Protection
Controls	50, 48, 40, 43, 47, 46	46.5 (41.1–51.8)		27, 24, 20, 24, 29, 20, 24, 27, 34	25.1 (21.5–28.7)		63, 55, 55, 73, 68, 97, 64	64.0 (47.7–82.3)	
KETc1 immunized	0, 0, 10, 36, 10, 19, 13, 18	11.5 (3.5–19.5)**	75.3	7, 10, 3, 4, 0, 0, 0, 0	3.0 (0.2–5.8)**	88.0	24, 39, 29, 18, 0, 19, 21	21.4 (11.0–32.1)*	66.7
KETc12 immunized	16, 0, 35, 10, 25, 22, 0	22.0 (2.3–41.7)*	52.7	3, 0, 1, 0, 17, 21, 0, 21, 8	7.0 (1.5–12.5)**	88.1	30, 14, 27, 21, 17, 11, 15	17.0 (11.4–25.7)**	73.4

[a] Individual number of cysticerci recovered 30 days after infection from each mouse.

[b] Mean (95% confidence interval) of individual parasite intensities in control mice injected with adjuvant alone or together with an immunizing peptide. Statistically significant differences between control and immunized mice at the 95% (*) or 99% (**) level.

[c] Percentage of protection with respect to controls.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same mice were added to each well in a volume of 50 μl. Peritoneal cells were obtained by ex vivo lavage with 5 ml of RPMI 1640 medium. After 72 h, the cultured cells were pulsed (1 μCi per well) for a further 18 h with [*methyl*-^{3}H] thymidine (Amersham Life Science, Little Chalfont, United Kingdom). Then all the cells were harvested and the amount of incorporated label was measured by counting in a 1205 β-plate spectrometer (Wallac).

Spleen cell phenotype analysis. After 3 days of in vitro culture with medium, *Tc*Ag, or peptides, splenocytes were harvested and CD8 and CD4 expression was determined by staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD8 (Pharmingen, San Diego, Calif.) and phycoerythrin-conjugated anti-CD4 (Pharmingen), respectively, by a previously reported procedure (25). Parallel samples of the cells were stained with the corresponding isotype control to account for nonspecific staining of the cells. Briefly, the cells were washed with phosphate-buffered saline (PBS) containing 10% gamma globulin-depleted FBS plus 0.02% NaN_3 and incubated with the indicated antibodies at 4°C for 30 min. After being washed, the splenocytes were resuspended in cold 1% formaldehyde in isotonic solution and analyzed with a FACScan instrument (Becton Dickinson, Palo Alto, Calif.). The results are expressed as a percentage of positive cells.

Cytokine measurements. For detection of intracellular cytokines, spleen cells were treated with medium, KETc1, KETc12, or *Tc*Ag and cultured for 60 h. To inhibit cytokine secretion, brefeldin A (2 μM) was added to the cell cultures 10 h before the assay. At harvest, the cells were centrifuged at 500 × *g* for 10 min and washed twice in ice-cold PBS containing 10% gamma globulin-depleted FBS plus 0.02% NaN_3. CD3 and interleukin (IL) expression were determined by two-color fluorescence-activated cell sorting (FACS) as previously described (25). Briefly, the cells were stained with biotin anti-CD3 (Pharmingen) and then streptavidin-FITC (Sigma) was added. Intracellular cytokines were assayed by using a cyto-Stain TM kit (Pharmingen) to fix and permeabilize the cells. To stain intracellular cytokines, fixed and permeabilized cells were incubated with phycoerythrin-conjugated monoclonal rat anti-IL-2, anti-IL-4, anti-IL-10, or anti-gamma interferon (INF-γ) (all from Pharmingen). Parallel samples of the cells were stained with isotype control to account for nonspecific cell staining. Then 10^5 cells were analyzed with a $CD3^+$ lymphocyte gate as defined by light scatter in a FACScan instrument. The results are expressed as a percentage of positive cells.

Experimental challenge. Metacestodes used in challenge infections were harvested from the peritoneal cavity of BALB/cAnN female mice carrying the ORF strain of *T. crassiceps* cysticerci. Ten small (diameter, ca. 2 mm), nonbudding larvae were suspended in 0.5 M NaCl–0.01 M sodium phosphate buffer (pH 7.2) and intraperitoneally injected into each challenged mouse using a 27-gauge needle (this procedure disrupts the cysticerci upon entry, but the fragments reorganize into cystic structures in a matter of a few days [24]). Mice were killed 30 days after infection, and the cysts found inside the peritoneal cavity were counted. In this form of infection, the parasites do not migrate to another location in the host. The variation in individual parasite intensities within groups of vaccinated and control mice was attributed to differences in the infectivity of each parasite inoculum. In consequence, each experiment measuring levels of immunity by parasite intensity always included a group of nonimmunized mice to assess the infectivity of each inoculum. Thus, the effects of immunization measured in each experiment were contrasted with the control group.

Immunolocalization of KETc1 and KETc12 protein. *T. crassiceps* cysticerci and *T. solium* specimens (cysticerci and gravid proglottids) were placed on ice in a 50-ml conical plastic-bottom centrifuge tube containing ice-cold PBS. All tissues were treated to prepare slides as previously reported (20). The slides were rehydrated and blocked with 1% bovine serum albumin (BSA) in PBS plus 0.1% Triton X-100 (pH 7.2) (PAT) for 1 h. A second blocking in cysticercus-infected tissue sections was performed with sheep anti-mouse IgG (whole antibody, Amersham) diluted 1:100 in PBS plus 0.1% BSA, and then the samples were incubated for 1 h at 4°C. Slides of *T. solium* tapeworm and eggs were incubated 1 h at 4°C with horse serum diluted 1:100 in PBS plus 0.1% BSA as a second blocking agent. The solutions were removed, and the slides were overlaid with the appropriate sera from noninfected (negative control), infected (positive control), or anti-KETc1- or anti-KETc12-immunized mice diluted 1:10,000 in PBS plus 0.1% BSA, incubated overnight at 4°C, and then washed twice in PBS (pH 7.2). Finally, sections were incubated with FITC-labeled goat anti-mouse immunoglobulin G (Zymed) diluted 1:50 for 1 h at room temperature. The slides were washed twice and mounted with aqueous mounting solution (Zymed). Preparations were observed with an epifluorescence microscope Olympus BH2-RFCA.

Statistical analysis. Statistical comparison of individual parasite intensities between groups was performed by the Kruskal-Wallis nonparametric analysis of variance ANOVA test because many mice contained zero parasites in the immunized groups and because parasite intensity is a discontinuous variable (0, 1, 2, . . . *n* parasites). Data were considered statistically significant at $P < 0.05$. A Student-Newman-Keuls multiple-comparison test was used to measure the statistical significance between the immune response elicited in vaccinated and control mice.

RESULTS

Protective effect of peptide immunization against *T. crassiceps* cysticercosis. The effect of peptide immunization on the number of cysticerci recovered from mice immunized with KETc1 and KETc12 or adjuvant alone (controls) is shown in Table 1: 66.7, 75.3 or 100% protection was induced using KETc1 as immunogen, and 52.7, 73.4, or 88.1% protection was induced using KETc12 as immunogen. Some mice were completely protected (no parasites) by immunization with either KETc1 or KETc12.

Antibody response induced by KETc1 and KETc12 immunization. To test for the presence of a B-cell epitope(s) within the two peptides, the levels of induced anti-KETc1 and anti-KETc12 specific antibodies were assessed. *T. crassiceps* cysticercal antigens (Fig. 1) as well as each of the peptides were used as antigens in ELISAs (data not shown). Figure 1 shows low but detectable levels of serum antibodies in both KETc1- and KETc12-immunized mice.

Immunolocalization of KETc1 and KETc12 in the parasite. Pooled sera with the highest antibody levels induced by KETc1 and KETc12 immunizations were used to immunolocalize the native antigen in both *T. crassiceps* and *T. solium* (Fig. 2 and 3). KETc1 and KETc12 were expressed in the tegument of *T. crassiceps* cysticerci, albeit with different distributions. KETc1 was restricted to the tegument of both cysticerci (Fig. 2E and F), while in *T. solium* it was found in the most external part of the tegument and also in the cuticular folds of the spiral canal (Fig. 2F). KETc12 (Fig. 2G and H) was very abundant in both metacestodes. Nevertheless, the *T. crassiceps* tegument showed an intensely positive wall surface and parenchyma, especially around the calcareous corpuscules (Fig. 2G). KETc12 was also detected in the oncosphere of the egg as numerous points (Fig. 3G), in contrast to KETc1, which was almost negative. Both epitopes were present in tapeworm tissue: KETc1 was very abundant on the most external side of the tegument (Fig. 3F), and KETc12 was distributed along the tegument's depth (Fig. 3H). When sera from infected mice were used, all structures were fluorescent (Fig. 2C and D and 3C and D). The specificity of these antibody reactions was demonstrated by the lack of reactivity of normal mouse serum with the used tissues (Fig. 2A and B and 3A and B).

FIG. 2. Immunofluorescent staining of *T. crassiceps* (A, C, E, and G) and *T. solium* (B, D, F, and H) cysticerci. Sections of 6 μm were processed and incubated with pooled sera from noninfected mice (A and B), *T. crassiceps*-infected mice (C and D), and KETc1-immunized (E and F) and KETc12-immunized (G and H) mice. The tegument (t) and the parenchyma (p) are evident in both cysticerci (C and D). In *T. crassiceps* cysticerci (E), KETc1 antigen shows a protruding and intensely positive signal in the tegument, while in *T. solium* cysticerci (F) it is clearly evident in the cuticular folds of the spiral canal (cf). KETc12 is quite abundant in both metacestodes; it is evident in the tegument and in the parenchyma of *T. crassiceps* (G) as well as in the tegument, parenchyma, and flame cells (arrows) of *T. solium* (H). Bar, 40 μm.

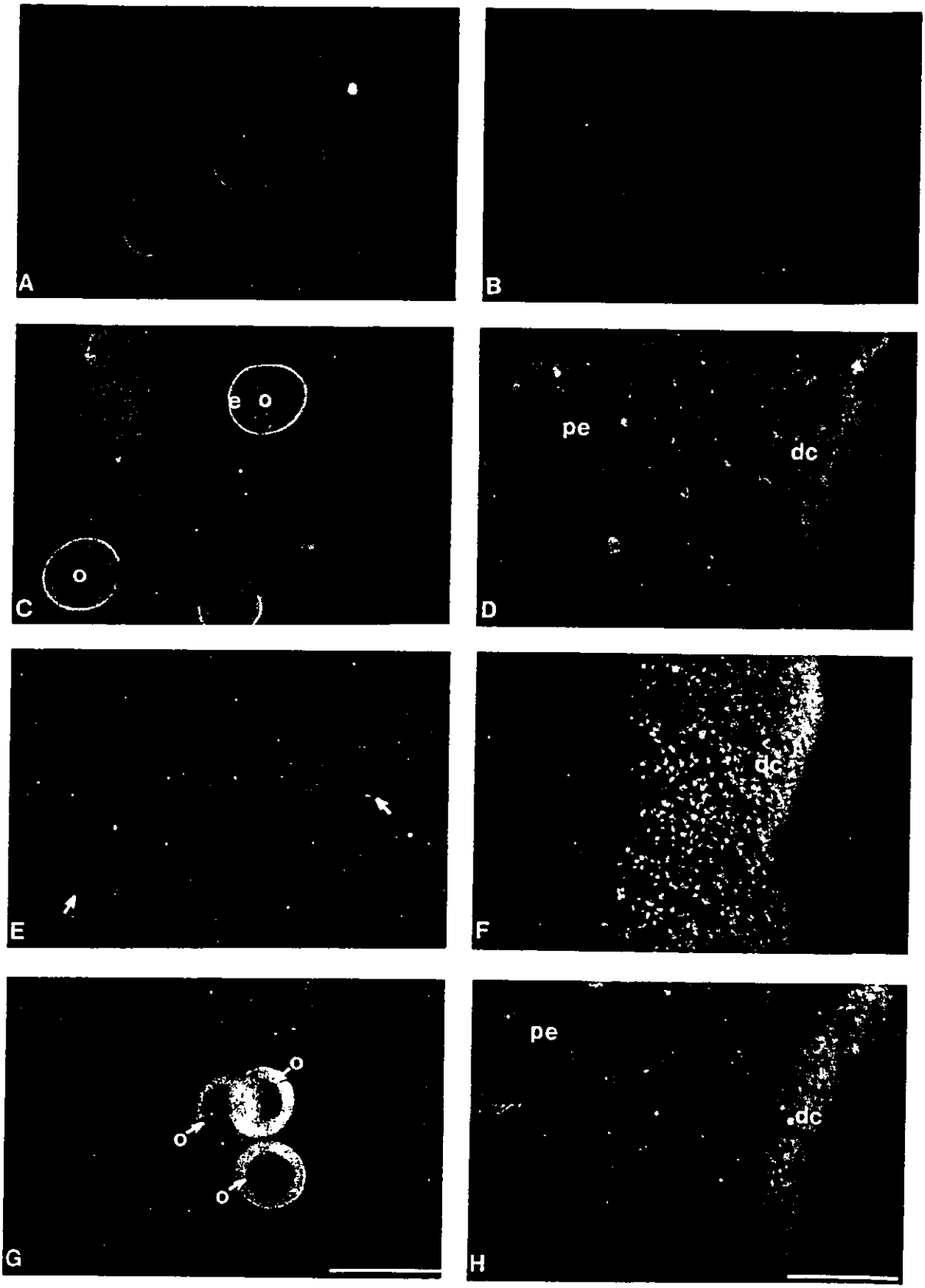
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TABLE 2. T-cell proliferative response of splenocytes from control and KETc1- and KETc12-immunized mice

Source of cells	Amt of [^{3}H]thymidine (cpm) incorporated after in vitro incubation with			
	Media	ConA	Peptide	*Tc*Ag
Controls	1,086 ± 370	183,643 ± 16,482	1,342 ± 248	754 ± 113
KETc1 mice	2,734 ± 1,364	119,030 ± 2,675	7,289 ± 2,675[b]	9,941 ± 1,802[b]
KETc12 mice	1,560 ± 262	155,125 ± 9,816	6,321 ± 2,021	10,450 ± 2,238[b]

[a] Mean ± standard deviation of the [^{3}H]thymidine incorporated after 3 days of culture of splenocytes from three individual mice assayed separately. The data are representative of three repeat experiments, each performed in triplicate.

[b] Significantly increased proliferative responses were achieved when cells from immunized mice were stimulated with both peptides and whole *Tc*Ag. Comparisons between immunized and control values were considered statistically significant at ($P < 0.05$).

Assessment of T-cell epitopes on the KETc1 and KETc12 peptides. The proliferative response of spleen cells from mice immunized with KETc1 or KETc12 or saponin alone is reported in Table 2. Spleen cells from mice injected in vivo with free peptides or saponin were stimulated in vitro with the corresponding peptide, *Tc*Ag, or ConA in previously determined optimal concentrations. Table 2 shows that in vitro stimulation with KETc1 or KETc12, as well as with cysticercal antigens, induced a significantly greater proliferative response in cells from immunized mice than in those from control mice. Cells from mice injected with saponin (controls) showed no proliferative response above background levels.

Figure 4 shows that stimulated cells increased from 3.5 or 4.5% to 8–16.3% when the cells were primed with *Tc*Ag or the appropriate peptide, respectively. Stimulated cells were enriched in both $CD4^+$ and $CD8^+$ cells by factors of 1.2 to 2.0 for $CD4^+$ and 3.9 to 4.9 for $CD8^+$.

The proportion of cells capable of producing IL-2, IL-4, IL-10, and INF-γ was determined by FACscan analysis after intracellular staining for cytokines. The results are shown in Table 3. The proportion of cells producing IL-2 and INF-γ were significantly higher in KETc1-, KETc12-, or *Tc*Ag-stimulated cells than with media alone and more so in immunized than in control mice. The levels of IL-4- and IL-10-expressing cells were also increased but to a lesser extent.

DISCUSSION

Our results show that KETc1 and KETc12 induce protection against experimental murine *T. crassiceps* cysticercosis and that both are B- and T-cell epitopes. The protective capacity and the immunity induced by these two peptides closely resemble those induced by GK1, a previously reported protective epitope also shared by *T. crassiceps* and *T. solium* (25). It should be noted that there are two forms of expressing protection; i.e., reduction in parasite intensity and proportion of totally parasite-ridden mice. Because *T. crassiceps* cysticerci multiply asexually in the peritoneal cavity of infected mice, the reduction in parasite intensity is highly dependent on the time of assessment after infection; the effects of vaccination tending to disappear in late infections. Sterile immunity is attained only if the initial inoculum is totally destroyed by the immune system response. These and previous results are in accordance with the notion that if a single *T. crassiceps* cysticercus evades or survives the initial immune attack of the murine host, it will multiply and eventually reach very high parasite intensities indeed (3). This initial immune attack would appear must successful if a strong T1 response is induced, as we have shown here and others have shown previously (23). In older infections, when massive parasite intensities ($>10^3$) are achieved, T2 responses predominate and perhaps downregulate T1 (23). These two peptides would appear to touch off the protective T1 response more efficiently than the T2 response.

The immunologic assays performed in our experiments indicate the immune mechanisms involved in infection control. It has been repeatedly stated that protection induced by vaccination against *T. crassiceps* murine cysticercosis is T1 related whereas antibodies and other T2 molecules are less effective (1). In this study, results point in the same direction: while antibodies are erratically and weakly induced by both KETc1 and KETc12, IL-2 induction is noticeably increased 5.7- to 10.1-fold in immunized mice relative to control mice. The same is true for IFN-γ, the characteristic inflammatory cytokine, which activates macrophages in the vicinity of the parasite and triggers their well-known damaging effects (25). In addition to the preponderance of the inflammatory interleukins IFN-γ and IL-2, the low profiles of IL-4 and IL-10, which inhibit the proliferation of the T2 responses, could well explain the low levels of antibodies elicited by both peptides. Protective immunity in the context of a T1 response has also been related to innate resistance conditions (23, 27). Despite the low levels of specific antibodies induced by both peptides, their possible protective role cannot be excluded. This is of particular relevance considering the recent finding of the capacity of anti-GK1 antibodies to block *T. solium* cysticercus conversion to tapeworms (5). The fact that more than 50% of human neurocysticercosis patients make antibodies against KETc1 and KETc12 (8) strengthens our interest in these two epitopes.

Based on the different anatomic distribution of KETc1 and

FIG. 3. Immunofluorescence staining of the *T. solium* oncosphere (A, C, E, and G) and proglottid tegument (B, D, F, and H). Sections of 5 μm were processed and incubated with pooled sera from noninfected mice (A and B), *T. crassiceps*-infected mice (C and D), and KETc1-immunized (E and F) and KETc12-immunized (G and H) mice. It is evident that the oncosphere (o) and the distal cytoplasm region (dc) (C and D, respectively) stain positively. Some structures of the perinuclear cytoplasm region (pc), like the protoplasmic extensions of the tegumental cells, are also apparently positive. KETc1 antigen is almost negative in the oncosphere and appears as little positive spots (arrows); in contrast, in adult tissue (F) it is quite evident in the distal cytoplasm region of the tegument. The KETc12 antigen is only slightly present in the oncosphere (o) but is quite conspicuous in the distal cytoplasm region and in the perinuclear cytoplasm region of the adult tissue. Bar, 40 μm.

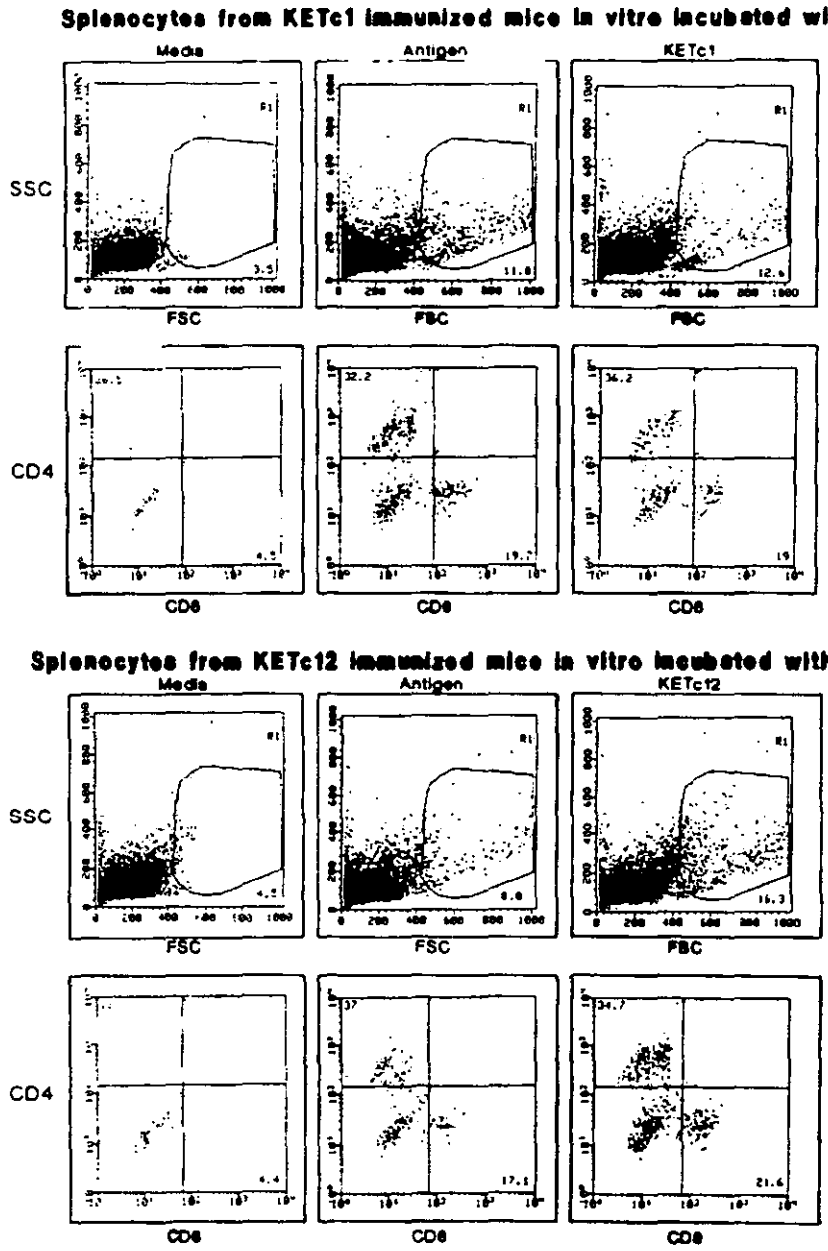

FIG. 4. Flow cytometer analysis of spleen cells from KETc1- and KETc12-immunized mice with or without in vitro stimulation with the respective peptide or antigen (*Tc*Ag) R1 denotes the region of proliferating cells in the SSC/FSC plot (side-scatter/forward-scatter plot), and the number below indicates the percentage of cells in this region. $CD4^+$ and $CD8^+$ cell percentage expression was determined in the defined R1 gate

KETc12 in *T. solium* cysticerci and in oncospheres, which also differ from that of GK1, it would appear that all three peptides should be included in a vaccine against pig cysticercosis to maximize the number of targets. In spite of the risk associated with extrapolating from *T. crassiceps* and mice to *T. solium* and pigs, optimism about vaccine development prevails because of the many examples of effective immunity induced against different cestodes in diverse hosts. Also, the extensive similarities among cestode infections in terms of their natural history, pathology, and antigenic composition all point to possibly similar effects of vaccination (6, 7, 10). In fact, different sources of protective antigens have been successfully used as vaccines against porcine cysticercosis, one using recombinant antigen from *Taenia ovis* (12) and the other using antigen from *T. crassiceps* itself (13).

In the hope of increasing the efficiency of vaccination, it is advisable that KETc1 and KETc12 plus GK1, all of which induce high levels of protection in the murine model of cysticercosis and are present at all stages of *T. solium* development, be considered as candidates for inclusion in a mixed poly-

TABLE 3. Percentage of $CD3^+$ splenocytes expressing different intracellular cytokines with or without in vitro stimulation[a]

Stimulation	% of cytokine-expressing cells from mice immunized with[b]							
	KETc1				KETc12			
	IL-2	IL-4	IL-10	INF-γ	IL-2	IL-4	IL-10	INF-γ
None (media)	1.4	0.7	0.6	0.6	1.6	0.9	1.2	0.7
Peptide	8.1	4.4	3.0	11.2	9.4	3.2	4.8	14.5
Cysticercal antigens	13.0	4.0	3.6	6.7	16.2	2.6	4.2	17.0

[a] Pooled splenocytes from three KETc1 or KETc12 immunized mice were analyzed for intracellular cytokines 60 h after in vitro stimulation.
[b] The percentages of $CD3^+$ cells expressing the four different interleukins (not included) were below 1% in splenocytes from control mice with and without in vitro stimulation. Data are representative of two different experiments using different mice.

epitopic synthetic vaccine to be used against *T. solium* cysticercosis in pigs.

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V. Discusión.

En este trabajo se reporta la evaluación en condiciones naturales de transmición (estudio en campo) de 3 péptidos producidos en forma síntetica KETc1, KETc12 y GK1, contra la cisticercosis porcina por *T. solium* en cerdos rusticos. Para ello fue necesario: 1) identificar la comunidad rural donde la cisticercosis porcina es endémica. 2) conocer la edad óptima de vacunación, 3) conocer si existe inmunidad adquirida por calostro, 4) diseñar un protocolo de vacunación y evaluar el efecto de la vacuna ante un desafio natural.

La vacuna evaluada consiste en 3 péptidos GK1, KETc1 y KETc12 evaluada en su capacidad protectora en un modelo experimental de cisticercosis murina. Estos péptidos perteneces a Ag recombinates representativos de fracciones antigénicas protectoras en contra de la cisticercosis murina y porcina (Sciutto *et al.*, 1995: Manoutcharian *et al.*, 1996).

Se aplicaron los tres péptidos sintéticos y se evaluó su capacidad de protección en condiciones naturales de transmisión. La evaluación en campo resultó crítica considerando que las condiciones experimentales podrían no representar adecuadamente las condiciones naturales de transmisión, en especial tomando en cuenta que los cerdos rústicos en general están desnutridos, son genéticamente heterogéneos y están expuestos a desafíos múltiples de dosis variables.

Como la vacuna fue dirigida a modificar la prevalencia de la cisticercosis porcina, con el fin de reducir al máximo el tamaño de la muestra de cerdos que fueron vacunados, fue necesario conocer la prevalencia de cisticercosis porcina en las zonas rurales en estudio, la que se determinó en las comunidades de Huatlatlauca Puebla. Este conocimiento resultaba indispensable para identificar una población adecuada en donde evaluar la vacuna contra la cisticercosis porcina. Para determinar la prevalencia se utilizó el diagnóstico en pie por exploración de lengua y detección en la misma de la presencia del parásito.

Adicionalmente, se determinó la presencia de anticuerpos específicos contra el cisticerco en cerdos con el fin de conocer la relación entre el diagnóstico serológico y el diagnóstico en lengua. Un 14 % de cerdos cisticercosos de un total de 80 examinados en los meses de junio de 1996 a diciembre de 1997 se les determinó cisticercos en lengua. Estos cerdos cisticercosos no presentaron niveles de anticuerpo anticisticerco pero si parásitos detectables en lengua. Además del conjunto de cerdos seropositivos ninguno presento cisticercos detectables en lengua. Estos resultados señalan que no existe correlación entre el diagnóstico serológico y el realizado por exploración en lengua.

Para optimizar la aplicación de la vacuna se determinó la edad óptima de inmunización que resultó de 60 día. Si bien sólo se exploraron 2 edades (40 y 60 días) obtubimos diferencias en cuanto a que los cerdos de 40 días respondieron significativamente menos que los de 60 días (artículo 1). Estas observaciones permitieron elaborar un protocolo de vacunación para evaluar la vacuna en cerdos criados rústicamente, quienes están más expuestos a la cisticercosis desde sus primeros días de vida. En cerdos de granja se reporta que a los dos meses son maduros inmunológicamente (Aluja et al., 1996). Sin embargo, en cerdos rústicos no existe información al respecto.

En el desarrollo de un protocolo de vacunación se deben considerar las posibilidades de infección en la población a la cual la vacuna se aplicará. En este sentido, los cerdos en las comunidades se exponen al parásito tan pronto como comienzan a convivir en un medio altamente endémico. Proteger a cerdos mal nutridos y además de pocos días de nacidos parece poco viable, de acuerdo con los resultdos obtenidos en este estudio. Se exploró la posibilidad de vacunar a las cerdas gestantes con el propósito de proteger a los lechones en etapas tempranas a través del calostro, por medio de la transferencia de inmunidad de la madre a la cría. La vía por la que los anticuerpos maternos

llegan al feto dependerá de la naturaleza de la barrera placentaria de la especie animal. En el cerdo, el tipo de placentación es epiteliocorial con seis capas que separan la circulación materna de la fetal y que impide la transferencia de inmunoglobulinas casi en su totalidad. Sin embargo esta ausencia de transferencia de Igs en la etapa fetal, no es sorprendente considerando que el cerdo es la especie que, en porcentaje, tiene la más alta concentración de inmunoglobulinas en el calostro, sobre todo del tipo IgA y en menor proporción IgG (Rickard *et al.,* 1982). El calostro representa las secreciones acumuladas en las últimas semanas de la gestación dentro de la glándula mamaria, así como proteínas procedentes de la circulación sanguínea por efecto de los estrógenos y la progesterona. La transferencia pasiva de inmunidad en contra de la fase larvaria de céstodos fue observada desde hace varios años Miller, 1932; Kerr, 1934; Campbell, 1938 mencionados por Rickard, 1982. A partir de entonces, la capacidad protectora del calostro se ha comprobado, en *Taenia taeniaeformis* en ratas y con *Hymenolepis nana* en ratones. En ratas, para la protección contra *Trichinella spiralis* se demostró que se puede reducir significativamente la carga parasitaria (hasta en un 28%) y al experimentar con intercambio de crías de madres inmunizadas con no inmunizadas, se comprobó que esta protección se realizó totalmente a través del calostro (Kumar *et al.,* 1989). También quedó demostrada la capacidad de protección que ofrece el calostro en contra de metacéstodos de *Taenia saginata* en becerros que fueron alimentados con el calostro de vacas que durante la gestación fueron inmunizados por vía parenteral utilizando oncósferas activas de *T. saginata* como antígeno, obteniendo después del desafío una degeneración de más del 80% en los metacéstodos de los animales protegidos (Lloyd *et al.,* 1976).

Los resultados más prometedores se consiguieron en la inmunidad a través del calostro contra *T. ovis* utilizando corderos neonatos. Sin embargo, han existido experimentos en los que no se consiguió la respuesta esperada: Gemmell (1969) trabajando con *Taenia hydatigena*; Urquhart (1961) utilizando *T. saginata*. Diversas causas pueden explicar estos resultados: el estado

inmune del animal, la fase y el tipo de céstodo utilizado, las características de raza del ovino y los antígenos empleados. En cerdos no se han hecho estudios al respecto.

En el presente estudio sobre la transferencia de inmunidad a través del calostro, se observaron que la vacunación indujo la formación de anticuerpos (IgA e IgG) anticisticerco en la cerda vacunada, mismos que se transfirieron a los lechones. Los anticuerpos en el calostro se detectaron desde las 8 y hasta las 48 horas posparto, (por problemas técnicos no fue posible llevar a cabo más determinaciones). Los niveles de anticuerpos en el calostro de la cerda vacunada conrespecto a la no vacunada fueron significativamente superiores ($P<0.05$). Con la prueba de *t.* Student. En vista de esos resultados consideramos vacunar a las cerdas gestantes para proteger a los lechones a través del calostro durante las primeras etapas de vida. Con el proposito de explorar esta posibilidad solo se dispuso de dos cerdas gestantes, una vacunada y otra no, tomandoles suero y calostro durante las primeras 48 horas. Los resultados sugieren que el calostro contiene suficientes anticuerpos por lo menos hasta las primeras 48 horas después del parto, para proteger a las crías. El pequeño número de cerdas gestantes y el hecho que solo se presentan datos hasta las 48 horas post-parto no autorizan a llegar a conclusiones definitivas a este respecto. El resultado de los ensayos con los sueros de los mismos lechones de la madre vacunada, indican una protección de las crías por lo menos hasta 30 días comparándolas con las crias de la cerda no vacunada.

Esta información nos permitió sugerir que podíamos aumentar la protección de la población de cerdos tempranamente después del nacimiento a través de la vacunación de cerdas gestantes. El protocolo de vacunación implicó vacunación de las cerdas gestantes y de sus lechones a los 60 días de edad previa determinación de ausencia de anticuerpos maternos al tiempo de la primera inmunización.

Todos los experimentos de vacunación con los antígenos sínteticos

fueron realizados en cerdos provenientes de cerdas rústicas adquiridos en el mercado de Tepeaca y mantenidos conjuntamente en la comunidad de Huatlatlauca, Puebla. El protocolo del esquema de vacunación se explican ampliamente en la publicación (Huerta *et al.*, 2000). Los cerdos vacunados y sus controles fueron sacrificados entre los 10 y 12 meses de edad y se les practicó la necropsia para determinar el número y el estado histológico de los cisticercos, observándose un mayor porcentaje de carga parasitaria en los cerdos controles respecto a los vacunados. Se observaron en el grupo control 18 cerdos infectados de 120 en total mientras que en el vacunado 9 de 120. Por lo tanto la vacuna redujo el número de cerdos infectados un 50%. Los cisticercos recuperados de cerdos vacunados presentaron un mayor grado de daño tanto macro como microscópico, indicando que la vacuna tiene una capacidad preventiva y posiblemente cisticida. Al respecto de la capacidad cisticida cabe la posibilidad de que el número de cisticercos instalados fue menor en los cerdos vacunados por una respuesta inmune más intenza capaz de dañar al parásito.

Finalmente este trabajo de investigación conjunta evidencias que nos permiten proponer esta vacuna sintética como una herramienta realista para el control de esta enfermedad.

Conclusiones.

Los resultados son alentadores, considerando que los resultados indican que la vacuna:

1. reduce el número de cerdos infectados.

2. reduce el número de cisticercos instalados.

3. reduce el porcentaje de cisticercos vesiculares Vs cisticercos caseosos y calcificados.

Los tres péptidos KETc1, KETc12 y GK1 a una dosis de 500 μg/cerda

gestante en el último tercio de la gestación y de 250 μg/cerdo a los 60 y 90 días de edad redujeron un 98.7% la carga parasitaria, en condiciones naturales de transmisión obteniendo niveles de protección de un 50%.

Por lo anterior consideramos, GK1, KETc1 y KETc12 como candidatos idóneos para ser constituir en una vacuna polivalente en contra de la cisticercosis porcina, capaz de disminuir el establecimiento y desarrollo del parásito. Para su recomendación masiva será necesario evaluar el efecto de la vacuna a través del tiempo y el efecto que ésta tiene en la reducción de las tasas de prevalencia en cisticercosis porcina y humana a mayor escala.

El Proyecto se ha realizó en el marco de un Convenio de Colaboración entre la Benemérita Universidad Autónoma de Puebla y la Universidad Nacional Autónoma de México, participando la Facultad de Medicina y la Escuela de Medicina Veterinaria de la BUAP y el Instituto de Investigaciones Biomédicas y la Facultad de Medicina Veterinaria y Zootecnia de la UNAM, SSA.

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VII. Apéndices.

Materiales y métodos.

5. Estudio post-mortem.

Se determinó la presencia y número de cisticercos en forma macroscópica y observación microscópica de una muestra de los cisticercos identificados. Se cuantificó el número de parásitos en las masas musculares de la mitad de cada canal de cerdo con base en el estudio de Vargas *et al.,* 1986 quienes demostraron que no hay diferencias estadísticas en la distribución de los cisticercos de una mitad de canal a la otra. Además se identificó la fase histopatológica de todos los cisticercos en las canales que tenian menos de 10 cisticercos y 10 muestras de cada pieza de los cerdos muy parasitados como: lomo, pierna, lengua, brazuelo, intercostales, soaps, etc. (Aluja *et al.*, 1995., Vargas *et al.*, 1994).

De acuerdo al daño degenerativo observado en las larvas, estas se consideraron en dos grupos uno viable e infectivo, grado 0, 1, 2 y 3 y el otro no viable y no infectivo grados 4, 5 y 6. Los resultados se resumen en el cuadro 8 y Figura 10. En el cuadro 8 se anotan además observaciones de algunos hallazgos de otros parasitos encontrados.

Para este proyecto se planeo dar una compensación al dueño y el compromiso de que se les compraría el cerdo si llegaba el tiempo de sacrificarlo. La carne no infectada se les regaló a la comunidad para hacer más amistoso su apoyo a este proyecto.

Durante el desarrollo del proyecto los participantes del mismo supervisaron la existencia de los cerdos, registrando los datos que resultaron de:

a) Detección de Ac totales por ELISA utilizando líquido vesicular de cisticerco de *T. solium* como antígeno.

La fuente de información fue primaria, se presentaron los datos en tablas

tetracóricas, de asociación, se presentaran gráficos de correlación y lineales. Se analizó la información con paquetes estadísticos Statical, SSPS, Grad Pad, Stadistical. El análisis estadístico se efectuó utilizando la prueba Chi-cuadrada, Mantel Heanzel con el programa SYSTAT.

APÉNDICE DE RESULTADOS.

3. Se evaluó la capacidad de transferir inmunidad a través de calostro de madres a hijos en dos cerdas gestantes, una vacunadas y otra no vacunada, encontrándose que la vacunación indujo la formación de anticuerpos (IgA e IgG) anticisticerco en el calostro de la cerda vacunada (Figura 4 y 5), mismos que se transfirieron a los lechones (Figura 8). Estos antícuerpo se detectaron desde las 8 y hasta las 42 horas posparto en calostro (Figura 6). En los lechones se mantuvieron hasta los 30 días de edad, última fecha que se evaluaron antes del desafío. Se identificaron en los sueros de lechones de cerdas vacunadas mayores títulos de IgG, que de IgA en los lechones de cerdas no vacunadas (Figura 6 y 7). Con la prueba de *t.* Student se observo significativamente superiores los niveles de anticuerpos en los lechones provenientes de la cerda vacunada que de la cerda no vacunada. Al contar el número de cisticercos instalados, no se observó diferencias significativas entre la carga parasitaria promedio de lechones provenientes de cerdas vacunadas respecto a los de cerda no vacunada ($P<0.05$). Sin embargo se observaron, una menor carga parasitaria respecto al promedio esperado en tres de los 6 cerdos de la camada proveniente de la cerda vacunada, (Cuadro 5). En el estudio histopatologico no se observaron diferencias estadísticas respecto a los grados de viabilidad. Los resultados se resumen en las Figura 4 a la 8 , y en los Cuadros 5 y 6.

Figura 4.

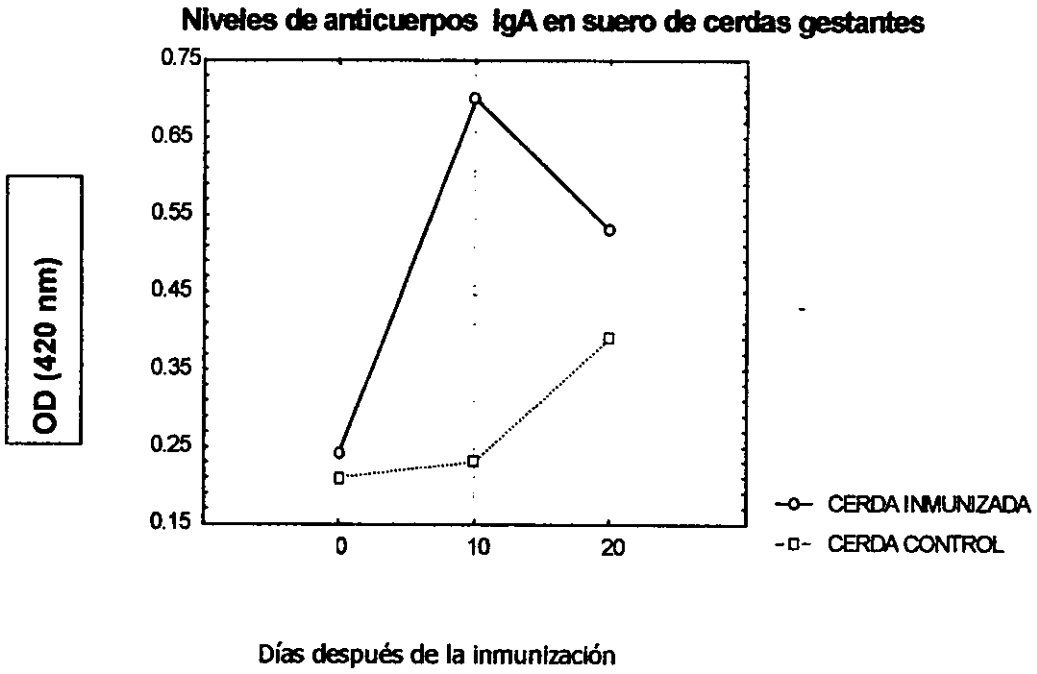


Figura 5.

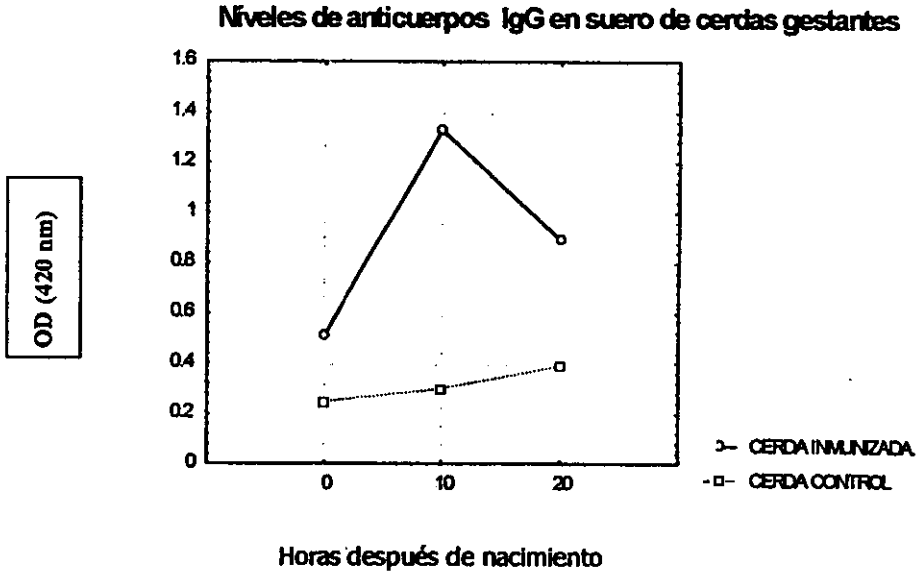


Figura 6.

Níveles de anticuerpos Ig A en calostro

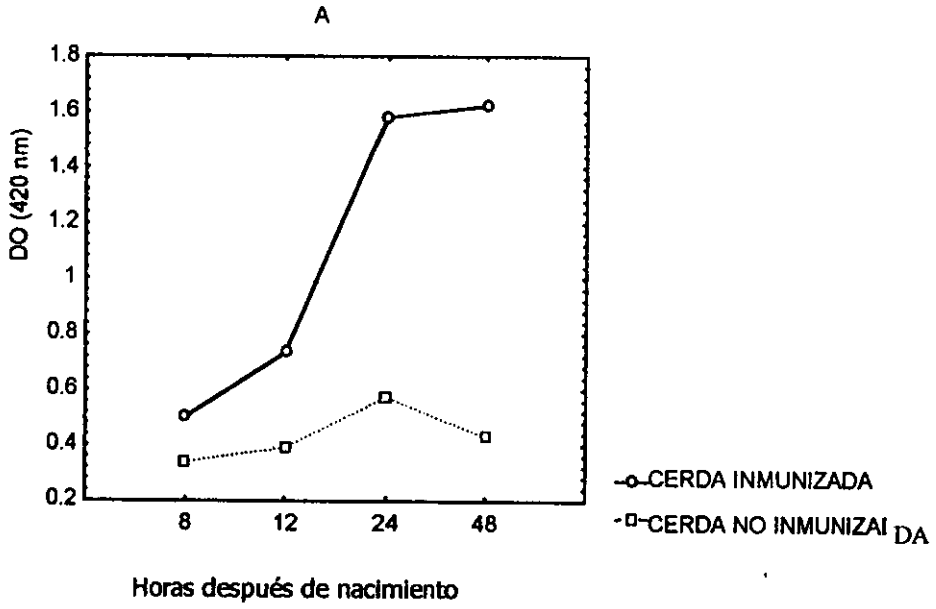


Figura 7

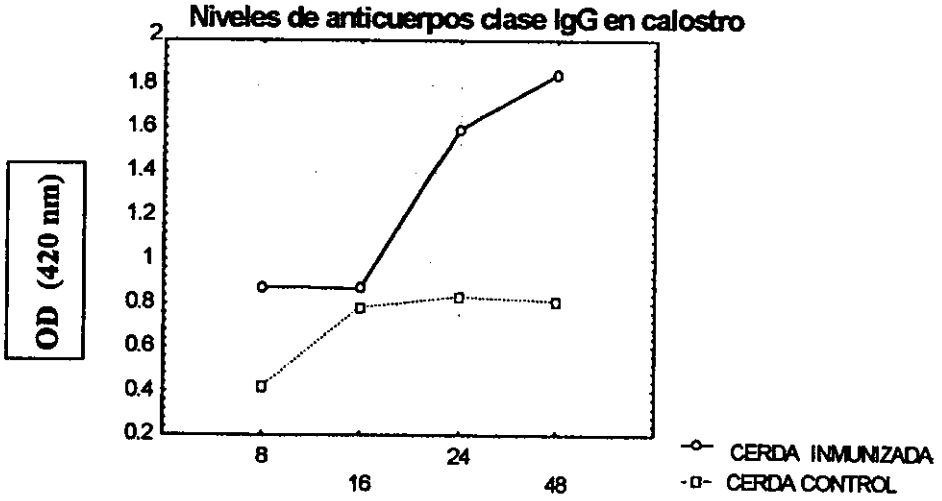
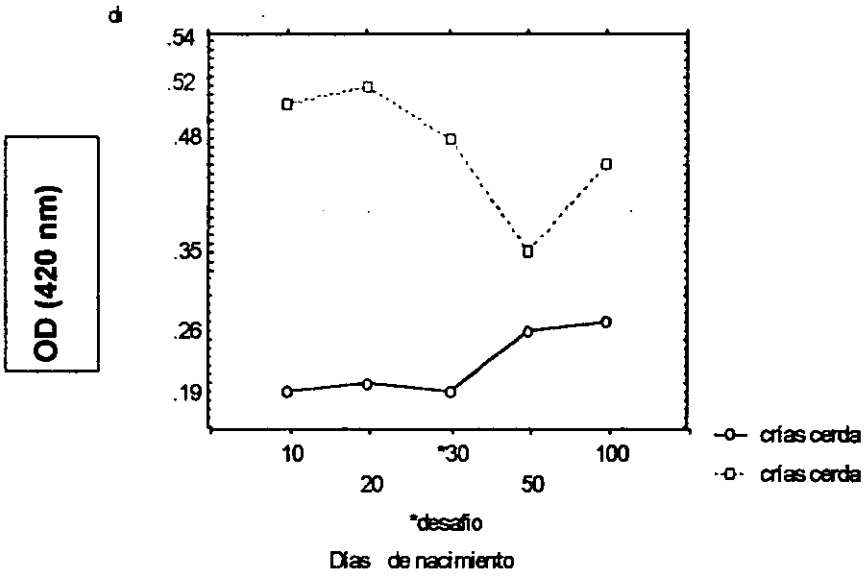


Figura 8.

Niveles de anticuerpos IgG en crias



Cuadro 5.

Carga parasitaria en crías de cerda inmunizada y control

controles	No de parasitos
1	103
2	138
3	140
4	155
5	372
vacunados	181 ± 108.1
1	315
2	85
3	413
4	48
5	197
6	86
	190.6 ± 146.6

* **Los lechones fueron desafiados a los treinta días de edad con 50.000 huevos de *T. solium*.**

5. Estudio de la fase histopatológica de los cisticercos, localizados en los diferentes tejidos de los cerdos vacunados y no vacunados. Se observo que tanto los cerdos infectados experimentalmente como de los desafiados en forma natural, mostró diferencias estadíticas $P< .05$ con la prueba X^2.

Respecto a su viabilidad, los cisticercos se dividieron en dos grupos de acuerdo a la clasificación histológica reportada por (Vargas *et al.*, 1986):

a) Vesiculares. Cisticercos con transparente fluido vesicular, que son infectantes. En este grupo se incluyeron las clasificaciones histológicas de cisticerco grado 0,1, 2 y 3.

b) Cisticercos caseosos y calcificados con exudado caseoso y calcificado con un gran daño y reacción inflamatoria importante. En este grupo se incluyeron los grados histológicos 4, 5 y 6.

Cuadro 6.

Distribución de lapoblación de cisticercos recuperados en cerdos controles y vacunados infectados naturalmente.

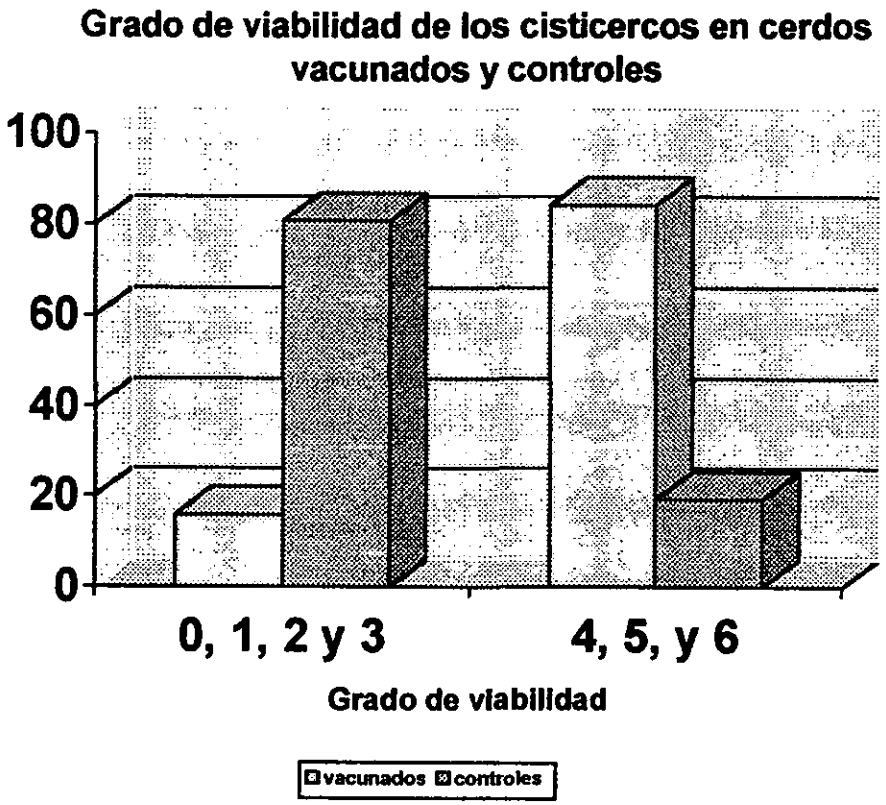
Cerdos	*Viable	** No viable	Total de cisticercos	Observaciones
Control N=18	322 (80.7%)	77 (19.3%)	399 (83%)	*Sarcocystis*
Vacunados N=9	8 (15.9 %)	74 (84.1%)	82 (17%)	

***Cisticercos vesiculares con tegumento integro, con reacción inflamatoria leve.**

****Cisticercos dañados, con reacción inflamatoria granulomatosa.**

Distribución calculada en la muestra de cisticercos provenientes de cerdos controles y vacunados en un total 1879 laminillas observadas. En 10 de los 18 cerdos controles se observaron *Sarcocystis sp* y cisticercos *Tenuicollis*.

Figura 9


Grado de viabilidad de los cisticercos en cerdos vacunados y controles
100
80
60
40
20
0
0, 1, 2 y 3
4, 5, y 6
Grado de viabilidad
vacunados
controles