Vaccine 26 (2008) 2899–2905

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Inexpensive anti-cysticercosis vaccine: S3Pvac expressed in heat inactivated M13 filamentous phage proves effective against naturally acquired *Taenia solium* porcine cysticercosis

Julio Morales[a], José Juan Martínez[a], Karen Manoutcharian[b], Marisela Hernández[b], Agnes Fleury[c], Goar Gevorkian[b], Gonzalo Acero[b], Abel Blancas[b], Andrea Toledo[b], Jacquelynne Cervantes[b], Victor Maza[d], Fabrice Quet[e], Henri Bonnabau[e], Aline S. de Aluja[a], Gladis Fragoso[b], Carlos Larralde[b], Edda Sciutto[b,*]

[a] *Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, México, D.F. 04510, Mexico*
[b] *Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México, D.F. 04510, Mexico*
[c] *Instituto Nacional de Neurología y Neurocirugía, SSA, Insurgentes Sur 3877, México, D.F. 14269, Mexico*
[d] *Secretaría de Desarrollo Agropecuario, Dirección General de Ganadería, Gobierno del Estado de Morelos, Mexico*
[e] *Institut d'Epidémiologie Neurologique et de Neurologie Tropicale (EA3174), Université de Limoges, 2 rue du Dr. Marcland, Limoges 87025, France*

ARTICLE INFO

Article history:
Received 30 January 2008
Received in revised form 16 March 2008
Accepted 19 March 2008
Available online 10 April 2008

Keywords:
Vaccine
Taenia solium cysticercosis
Control

ABSTRACT

In search of reducing vaccine production costs', a recombinant M13 phage version of the anti-cysticercosis tripeptide vaccine (S3Pvac) was developed. The efficacy of S3Pvac-Phage vs. placebo was evaluated in a randomized trial that included 1047 rural pigs in 16 villages of Central Mexico. Three to five months after vaccination 530 pigs were examined by tongue inspection. At 5–27 months of age, 331 pigs (197 vaccinated/134 controls) were inspected at necropsy. Vaccination reduced 70% the frequency of tongue cysticercosis and, based on necropsy, 54% of muscle-cysticercosis and by 87% the number of cysticerci.

1. Introduction

Taenia solium cysticercosis still affects humans and rustic pigs living in Mexico [1–3] and in several other developing countries of Latin America, Asia and Africa [4–6], as well as in the countries of destiny of their migrant workers [7–9]. Albeit transitorily effective in focal situations, traditional measures to control cysticercosis transmission (i.e., health education, sanitation, meat inspection, . . .) are impractical to apply in the large scale and long enough to change the conditions that support parasite transmission [10]. Hopes of quicker, more general and lasting solutions, lie in the development of several technologically based approaches to control the endemia, while the definitive solution of social development finally comes [11]. Research for better and less costly diagnostic and therapeutic agents and protocols is nowadays getting some attention [12–14]. And so is the development of an effective vaccine against porcine cysticercosis, one that can meet with the conditions of low cost and feasible application to the millions of practically feral pigs exposed to acquire cysticercosis in impoverished nations [15].

The rationale for vaccination of pigs to curtail *T. solium* transmission rests on the expectation that reducing the number of infected pigs and/or their load of metacestodes would lead to a decrease in the number or viability of pig's cysticerci and thus to a reduction in the number of adult tapeworms, the stage of the parasite with the highest potential of spreading the infection [10].

In pursuit of such goal we first developed and successfully tested in the field the anti-cysticercosis S3Pvac synthetic vaccine composed by the protective peptides KETc12, KETc1 and the GK1 derived from the KETc7 [16]. However, the huge costs of synthetic peptide technology make S3Pvac production unaffordable in the massive amounts needed by nation-wide and sustained pig vaccination programs in the weak economies of endemic countries. Recombinant phage technology offered to cut down costs. S3Pvac recombinantly expressed in M13 filamentous phage (S3Pvac-Phage) exhibited high levels of protection against pig cysticercosis under experimental conditions [17]. However, when

Abbreviations: mo, months; vs, versus.

* Corresponding author. Tel.: +52 55 56223153; fax: +52 55 56223369.
E-mail address: edda@servidor.unam.mx (E. Sciutto).

0264-410X/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2008.03.042

approaching natural infections we realized that experimentation in pigs under controlled laboratory conditions could hardly imitate the natural pressures of cysticercosis transmission. The diversity of genetic backgrounds of the rural pigs, their meagre diets, their life-long continuous exposure to varied numbers of eggs coming from various tapeworms and the inevitable energy costs from the concomitant stress of living in the wild [18] could all lead to unreliable conclusions. The vaccine had to be tested under natural conditions of transmission and in the genetic type and lifestyles of the pigs exposed to infection [19].

Here, we inform of the efficacy of this novel vaccine candidate (S3Pvac-Phage) tested against naturally acquired pig cysticercosis in 16 rural communities of "Sierra de Huautla" in the State of Morelos, Mexico. The findings support the usefulness of this new, effective and inexpensive anti-cysticercosis vaccine.

2. Materials and methods

2.1. Construction of recombinant S3Pvac-Phage

Four different peptide-phage recombinants were prepared: (1) *from Taenia crassiceps* 96 aa long antigen KETc7 (FK7) (Manoutcharian et al., 1996) and (2) KETc7-derived peptide GK1 (FGK1) (aa 69–95, GYYYPSDPNTFYAPPYSA); (3) the *T. crassiceps* recombinant antigen-derived peptides KETc1 (APMSTPSATSVRG) (FKETc1) and (4) KETc12 (GNLLLSCLG) (FKETc12) [16]. They were all expressed on the phage surface by cloning the corresponding DNA fragments in phage/phagemid vectors as previously reported [17].

The ligation mixtures of the recombinant phagemids FK7, FGK1 and FKETc12 (fused the foreign peptides to M13 cpVIII) were rescued and amplified by superinfection with M13KO7 helper phage (Invitrogen) from transformed *Escherichia coli* TG-1 cells as described previously [20]. KETc1 was cloned into the M13KE phage vector and the recombinant phage clone FKETc1 was amplified by infecting TG-1 cells as described elsewhere [20]. Recombinant phagemids/phage particles were recovered from the supernants of the cultures at 10^{12} to 10^{14} particles per ml and inactivated and sterilized by autoclave during 30 min at 121 °C. Each pig-dose of the S3Pvac-Phage vaccine contained, in a total volume of 2 ml, 10^{12} formaldehyde-heat inactivated phage particles of each of the four different recombinant phage particles.

2.2. Design of the vaccine trial

The objective of this work was to evaluate the efficacy of the S3Pvac-Phage vaccine on real conditions of use: that is, on piglets usually encountered in rural communities of Mexico. The vaccine trial lasted from April 2004 to July 2006. The procedures and experiments reported herein were conducted according to the principles set forth by the Mexican Ethical Committee for the care and use of farm animals.

2.3. Selection of rural area and communities

The rural area selected for the vaccination trial (Sierra de Huautla, Morelos, localized in the south of the state between coordinates 18° 20′ and 18° 31′ north altitude and 98° 51′ and 98° 53′ west longitudinal) had immediately before been shown was endemic for porcine cysticercosis. About a year before starting the vaccination program, from August to December 2003, tongue cysticercosis was found in 73 (13%) of the randomly selected 562 pigs from the 926 pigs older than 3 months living in 13 of the 16 communities planned to be included. The pigs' sample in this previous assessment of the local endemia represented 62% of the total population of pigs bred in these communities at that time, according to data from the Secretaría de Desarrollo Rural, Morelos. These previous findings documented active transmission of porcine cysticercosis in the communities and triggered the vaccination program which lasted from April 2004 to July 2006. This program included a total of 1047 pigs as shown in Table 1.

Table 1
Baseline characteristics

	Placebo ($n = 421$)	S3Pvac-Phage ($n = 626$)	*P*-Value
Age in month (mean ± S.D.)	3.08 ± 1.82	3.15 ± 2.34	0.60
Weight in kg (mean ± S.D.)	14.19 ± 8.28	14.62 ± 8.55	0.42
Sex, *n* (%)			
Male	198 (47.03)	305 (48.72)	0.59
Female	223 (52.97)	321 (51.28)	
Male castration, *n* (%)			
Yes	174 (87.88)	253 (82.95)	0.13
No	24 (12.12)	52 (17.05)	
Female castration, *n* (%)			
Yes	11 (4.93)	9 (2.80)	0.19
No	212 (95.07)	312 (97.20)	
Water supply, *n* (%)			
Well	80 (19.00)	142 (22.68)	0.14
River	236 (56.06)	356 (56.87)	
Tap-water	105 (24.94)	128 (20.45)	
Confinement, *n* (%)			
Loose	408 (96.91)	602 (96.17)	0.52
Tethered	13 (3.09)	24 (3.83)	
Latrine in owner's households, *n* (%)			
Yes	306 (72.68)	441 (70.45)	0.43
No	115 (27.32)	185 (29.55)	
Origin of pigs, *n* (%)			
Household	359 (85.27)	538 (86.08)	0.71
Purchased	62 (14.73)	88 (13.92)	
Destinations of pigs, *n* (%)			
Self-consumption	122 (18.98)	186 (29.71)	0.79
For sale	299 (71.02)	440 (70.29)	
Piglets' breed, *n* (%)			
Criollo	265 (62.95)	365 (58.31)	0.13
Other	156 (37.05)	261 (41.69)	

Table 2
Vaccination reduced 70% of the infected pigs diagnosed by tongue inspection at 7–9 months of age

Community	No of pigs included[a]	No of pigs inspected	% of infected pigs[b]	
			Control	Vaccinated
Ajuchitlan	136	113	6.6 (3/45)	4.4 (3/68)
Chimalacatlan	141	53	10.5 (2/19)	0 (0/34)
El Limon	90	29	27 (3/11)	11.1 (2/18)
Huautla	132	73	7.5 (3/40)	0 (0/33)
Xochipala	12	12	40 (2/ 5)	0 (0/ 7)
Huaxtla	24	9	25 (1/ 4)	0 (0/ 5)
El Tepehuaje	24	16	20 (1/ 5)	12.5 (1/11)
Huizaxtla	3	3	0	0 (0/ 3)
La Era	46	34	27.2 (6/22)	8.3 (1/12)
Los Elotes	24	13	0 (0/ 4)	0 (0/ 9)
Los Sauces	94	48	10.5 (2/19)	10.3 (3/29)
Quilamula	158	32	7.6 (1/13)	0 (0/19)
Rancho Viejo	44	25	33.3 (3/ 9)	6.2 (1/16)
San Jose de Pala	66	34	6.6 (1/15)	0 (0/19)
Santiopa	44	29	9 (1/11)	5.5 (1/18)
El Vergel	9	7	0 (0/ 1)	0 (0/ 6)
Total	1047	530	13 (29/223)	3.9 (12/307)

[a] Total number of not infected pigs of 3–4 months of age included in the vaccination trial according to tongue inspection.

[b] Number of infected pigs/total number of pigs inspected by tongue inspection in the control and in the vaccinated group in each of the 16 communities.

Sixteen rural communities were selected from the 25 in the area (Table 2). The conditions which favour the transmission of *T. solium* were present in all communities selected: a high prevalence of cysticercosis in live pigs (13%, as cited above), open air defecation, rustic pig rearing where pigs are allowed to roam free in search of food, extensive domestic pig slaughtering and local consumption of non-inspected pork with cysticerci.

2.4. Statistical design

Considering a vaccine efficacy of 50%, a minimum of 225 piglets in each vaccinated and control group would be required for confident statistical inferences [21,22]. In order to minimize differences related to hosts' genetic and exposure factors, half the members of each litter were immunized with S3Pvac-Phage vaccine described above and half with placebo (saline). The additional pig of litters with odd number of piglets was vaccinated. The owners were kept unawares of the treatment received by each pigs in the litter and were instructed to raise the pigs as accustomed.

The response variables were: (a) cysticercosis prevalence by tongue inspection; (b) cysticercosis prevalence at necropsy (the number of infected pigs with at least one parasite in masseters or tongue or diaphragm or heart/the total number of pigs inspected in the group); and (c) the number of cysticerci found in each individual pig carcass, macroscopically distinguishing whether the parasites were damaged or vesicular.

2.5. Pigs included in the study

For vaccination, 1127 piglets of 3–4 months of age and apparently healthy were eligible for inclusion. From these, 80 were excluded because they were found to be already positive for tongue cysticercosis. The remaining 1047 negative for tongue cysticercosis were included (Fig. 1). The owners of all piglets included accepted to participate in the study and gave their informed consent. All pigs included were labelled using a numbered microchip for their individual identification.

2.6. Pig-associated variables

The study recorded for each individual pig: age (months), weight (kg), and pregnancy (yes/no), male and female castration (yes/no), age (months) of castration, confinement (loose, tethered), source of animal drinking water supply (well, river, or tap water), latrine in the owners' household (yes/no) and whether it was positive or negative by tongue inspection. The pigs' sex, castration and pregnancy status were verified by veterinarian inspection. Pregnancy was confirmed by the birth of the piglets. Pregnant sows were slaughtered by their owners 2–3 months after parturition.

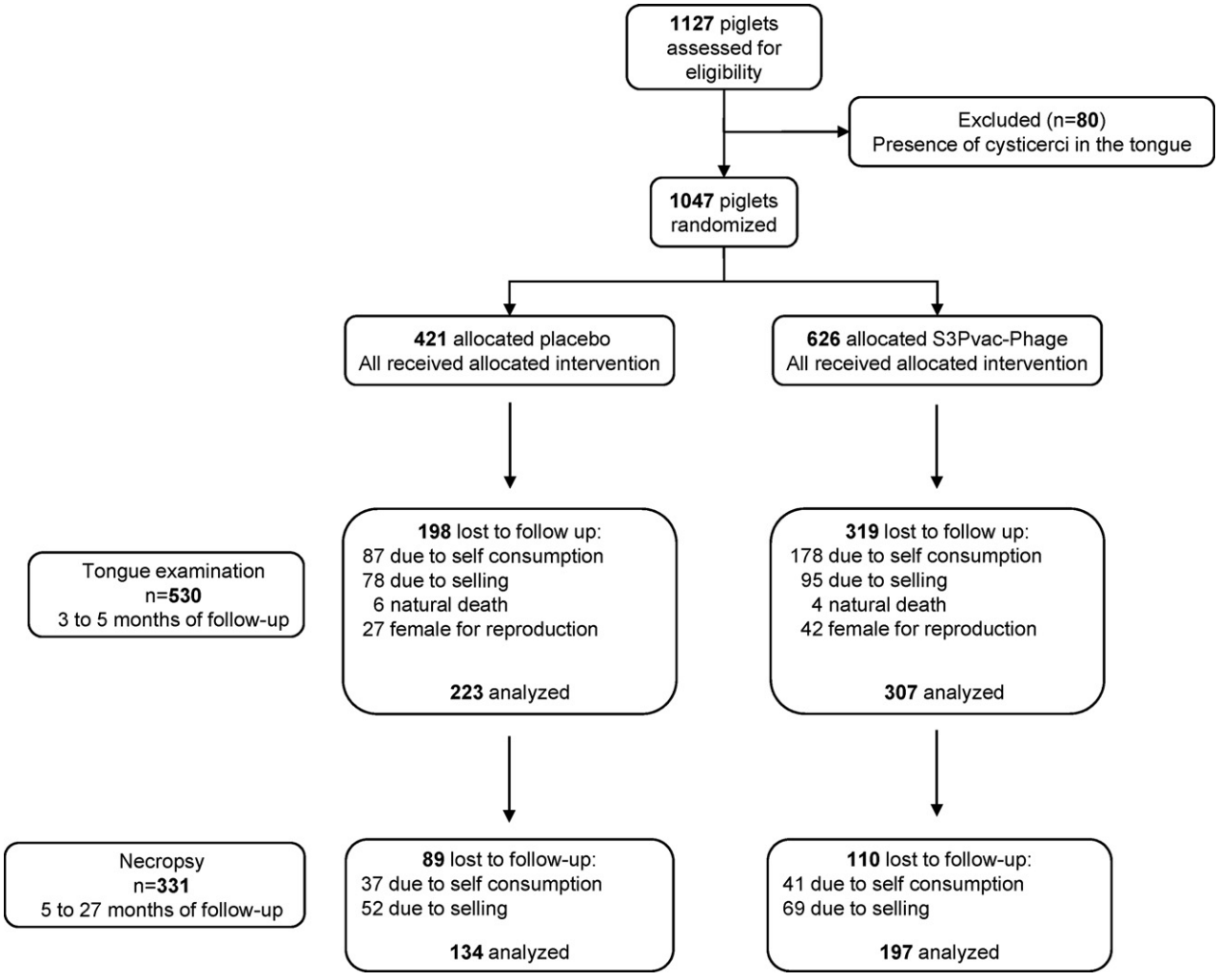

Fig. 1. Flow chart, vaccination survey in 16 Mexicans communities (April 2004–July 2006).

2.7. Vaccination of pigs

Half of the piglets in each litter received two subcutaneous injections of S3Pvac-Phage at 3–4 months of age and 1 month later, and the other half of the litter received only saline as placebo. When litters were composed by an odd number of piglets, the additional pigs were vaccinated. The pigs' age at vaccination (3–4 months) was chosen considering that high mortality (up to 50%) is expected in rural piglets before 2 months of age [19]. The S3Pvac-Phage vaccine composed by the four recombinant phages was autoclaved and adjusted to 4×10^{12} phage particles in 2 ml of media containing 0.05% of formaldehyde. Two millilitres of such vaccine preparation were injected subcutaneously at the base of the pigs' ears. The vaccine was maintained at 4–10 °C until used. Control pigs were each injected with 2 ml of saline only.

2.8. Follow-up of the vaccination trial

The 16 communities included in this study were visited weekly to look after the pigs included in the trial and to identify any modification in their localization in the community or disappearance due to their selling or death and to determine their reproductive status. For this purpose pigs were double-labelled using a microchip and an earring that permitted the visual identification of those included in the vaccination trial. After 7–9 months of age (3–5 months after vaccination), their tongues were inspected for cysticercosis. At 5–27 months of age, when their owners slaughtered the pigs for consumption, cysticercosis was diagnosed at necropsy by dissecting masseters, tongue, diaphragm, heart and the liver in search of visible cysticerci by making scalpel slices every half-centimetre. The number of cysticerci found in each pig was registered and the owners were instructed not to consume the meat if the pig was infected. Diagnosis of porcine cysticercosis was performed by six thoroughly trained technicians from Dirección General de Ganadería, directly supervised by one of veterinarians of our research team. Following the instructions of the veterinarians the infected carcasses were not consumed by the owners or were extensively cooked before consumed.

2.9. Statistical analysis

Data were processed in Excel 7.0 (Microsoft). Statistical calculations were performed using the computer program Statistica 9.0 (StataCorp LP, College Station, USA) and the statistical program of EPI-Info, SPSS (SPSS Inc, Chicago, USA) and SAS 9.1.3 (SAS Institute Inc., Cary, USA). Descriptive analyses were based on frequencies and percentages for qualitative variables and means and standard deviations for quantitative variables. Comparisons were made using the Student's *t*-test or the Mann–Whitney *U*-test. Frequencies were compared using the χ^2-test, with Yates' correction or two-tailed Fisher's exact tests when necessary. Adjusted relative risks (RRa) with the corresponding 95% confidence interval (95% CI) and *P* values were estimated by a multivariate logistical regression. We incorporated in the multivariate model, all variables of interest for which we found a *P* value of less than 0.25 in univariate analyses, by a backward stepwise procedure. All analyses of efficacy, using tongue examination or necropsy, as criteria of diagnosis were made at the end of study.

A two-sided *P* value of less than 0.05 was considered to indicate statistical significance.

3. Results

3.1. Sample size changes during the vaccine trial

A total of 1127 piglets of 3 months of age were examined by tongue inspection before vaccination. Eighty pigs (7.1%) were found infected and excluded from the study. Of the other 1047 not-cysticercotic piglets 626 were vaccinated and 421 were used as controls. Baseline characteristics of the 1047 pigs were not significantly different between control and vaccinated pigs (Table 1). Most male pigs ($n = 427$) and few sows ($n = 20$) were castrated by their owners at different times prior to slaughter arguing it quickens weight gain and improves the quality of the meat.

Changes in sample size occurred as the trial proceeded, as shown in Fig. 1 according to CONSORT Statement [23]. Because of the extremely harsh social and economic conditions of the communities involved, by the end of the trial, 49% of the pigs originally included were missing, mostly on account of their being consumed or sold. The diagram in Fig. 1 shows in detail the number and design allocation of the missing pigs.

Tongue inspections were made at 3–5 months after vaccination on 530 of the piglets included in the trial. Nearly half of the included pigs ($n = 517$) could not be inspected because they had been consumed or sold by their owners or were sacrificed without timely notice to the project's personnel (198 and 319, respectively in placebo and in the S3Pvac-Phage group). The residual 102 pigs (44 controls and 58 vaccinated) were not examined because of consumption. Necropsies were performed at 5–27 months of age on 331 pigs (197 and 134 pigs in the vaccinated and control group, respectively) because the residual 199 pigs (89 controls and 110 vaccinated) were consumed or sold without timely notice to the project's personnel.

Considering the high number of piglets lost during the two stages of follow-up (tongue inspection, necropsy), analyses of efficacy were adjusted to ensure the statistical representation of the initial population. The variables of adjustment were identified using a logistic regression of the initial variables and the initial number of observations done (1047). Variables significantly linked with pig loss at tongue inspection were: origin (household, purchased), castration (yes, no), Piglets' breed (Criollo, other) and sex (male, female). Variables significantly linked with pig loss at the necropsy examination were: weight and castration (yes, no). Neither vaccination nor placebo were linked with loss at tongue inspection

3.2. Effect of vaccination as determined by tongue inspection

Table 2 shows the effect of vaccination on the number of infected pigs in the 530 pigs examined by tongue inspection at 7–9 months of age: cysticercosis was diagnosed in 12 pigs (3.9%) of the vaccinated group and in 29 pigs (13.0%) of the control group, corresponding to a vaccine efficacy of 70%. In the SP3Vac-Phage group an adjusted relative risk (adjustment on origin, castration, piglets' breed and sex as previously described) of 2.7 (CI_{95}: [1.8–4.2], $P < 0.0001$) was estimated, which means that cysticercosis frequency in vaccinated pigs are nearly threefold less that of the placebo group (number of observations used = 530, $R^2 = 0.32$).

3.3. Effect of vaccination as determined by necropsy

Table 3 shows that vaccination significantly decreases the number of infected pigs (13/197 = 6.6% in vaccinated group vs. 19/134 = 14.2% in control group, $P = 0.036$) and also significantly reduces the number of cysticerci counted at necropsy (8.4 ± 14.4 in

Table 3
Effect of vaccination on the number of pigs infected and the parasite load according to necropsy at 5–27 months of age

Community	[a]Control	[b]Number of cysticerci	[a]Vaccinated	[b]Number of cysticerci
Ajuchitlan	5/19	265	3/28	25
Chimalacatlan	2/9	71	2/10	5
El Limon	1/14	109	1/14	27
Huautla	1/31	69	2/28	21
Xochipala	0/1	0	1/7	6
Huaxtla	1/1	86	0/7	0
El Tepehuaje	0/2	0	0/1	0
Huizaxtla	0/0	0	0/2	0
La Era	1/7	107	1/4	2
Los Elotes	0/4	0	0/7	0
Los Sauces	1/11	40	3/23	49
Quilamula	2/12	44	0/15	0
Rancho Viejo	1/7	15	0/24	0
San Jose de Pala	3/10	182	0/13	0
Santiopa	1/6	60	0/14	0
El Vergel	0/0	0	0/1	0
Total	19/134	1048	13/197	135
Prevalence	14.2%		6.6%	
Mean ± S.D.		65.5 ± 74.2		8.4 ± 14.4

[a] Number of infected pigs/total number of pigs inspected by necropsy in the control and in the vaccinated group in each of the 16 communities.
[b] Number of cysticerci recovered in the control or vaccinated pigs infected in each community. Vaccine efficiency: 54.15% (reduction in the number of infected pigs) or 87.1% (reduction in the number of cysticerci).

Table 4
Effect of biological and exposure variables on infection determined by necropsy

	Infected	Infected	*P*-Value
Gender (male/female)	9/23	157/142	0.009
Gestation (yes/no)	12/11	20/123	0.0001
Castration in female (yes/no)	1/22	4/138	0.53
Castration in male (yes/no)	9/0	142/15	1
Month since castration in male	12.3 ± 4.2[a]	9.5 ± 5.4	0.12
Free roaming (yes/no)	32/0	289/10	0.6
Open water (yes/no)	27/5	243/56	0.8
Latrine (presence/absence)	15/17	266/73	0.001
Vaccination (yes/no)	19/13	115/184	0.036

[a] Mean ± S.D.

vaccinated group vs. 65.5 ± 74.2 in control group, $P = 0.013$). All the cysticerci detected were macroscopically vesicular. The relative risk of vaccinated pigs at necropsy was 2.3 (CI_{95}: [1.2–4.3], $P = 0.0113$, adjustment on weight and castration as previously described), which means that frequency of cysticercosis in vaccinated pigs is nearly half that of placebo controls (number of observations used = 331, $R^2 = 0.48$).

3.4. Relevance of biological and exposure factors on cysticercosis prevalence

Table 4 shows the different pig-associated factors and their relation with cysticercosis as determined by necropsy. In the univariate analysis, male ($P = 0.009$), absence of gestation ($P = 0.0001$), lower time since castration in males ($P = 0.004$), latrine in the owners' household ($P = 0.001$) and vaccination ($P = 0.036$) were related to a lower prevalence of cysticercosis. A multivariate logistic regression was performed including those factors that were related to infection with a significance under $P < 0.1$ in univariate analysis. The presence of latrine ($P = 0.039$), gestation ($P < 0.0001$) and vaccination ($P = 0.004$) were factors that significantly associated with protection according to this multivariate analysis.

3.5. Relevance of biological and exposure factors in the vaccine efficacy

Table 5 summarizes the effect of exposure and sexual factors on the vaccine efficacy as determined at necropsy and measured in terms of reduction of infected pigs and reduction in the number of the cysticerci in those infected. Only the three parameters signifi-

Table 5
Relevance of exposure and sexual factors in the vaccine efficacy in the 331 pigs diagnosed at necropsy

	Controls		Vaccinated		P^c	P^d
	[a]Frequency of infection	[b]($X \pm$ S.D.)	[a]Frequency of infection	[b]($X \pm$ S.D.)		
Sex						
Male	3/63	54 ± 16	6/103	11 ± 10	1	0.02
Female	16/71	55 ± 35	7/94	10 ± 14	0.007	0.008
Latrines						
Yes	8/97	47 ± 39	7/144	7 ± 6	0.29	0.019
No	11/37	61 ± 28	6/53	14 ± 17	0.05	0.003
Gestation						
Yes	10/13	51 ± 33	2/19	3 ± 1	<0.001	0.06
No	6/58	62 ± 41	5/76	13 ± 17	0.5	0.034

X: mean; S.D.: standard deviation.
[a] Number of infected pigs/total number of pigs.
[b] Mean of cysticerci ± S.D. in infected pigs.
[c] Difference in the frequency of infected pigs between control and vaccinated groups.
[d] Difference in the number of parasite between control and vaccinated groups.

Table 6
Weight of the control and vaccinated pigs included in the trial

		Age (months)			P^a	P^b
		3–4	7–9	12		
Controls						
Infected	11	18 ± 10.6	43.2 ± 24.4	52.1 ± 11.9	0.006	0.29
Not-infected	71	15.1 ± 8.5	45.4 ± 20	63.4 ± 18.4	<0.0001	<0.0001
Vaccinated						
Infected	6	6 ± 2	30.8 ± 18.5	41.7 ± 7.5	0.004	0.2
Not-infected	127	14.8 ± 8.9	43.9 ± 19.8	61.2 ± 19.1	<0.0001	<0.0001

Increase of the weight of 215 pigs in which cysticercosis was diagnosed by tongue inspection and necropsy.
[a] Differences in the weight between 3–4 and 7–9 months.
[b] Differences in the weight between 7–9 and 12 months of age.

cantly related to cysticercosis (Table 4) were used for this analysis: the sex of the pigs, if female had or not one gestation, and if the pigs' owners had or not latrines in their houses. As Table 5 shows, the vaccine significantly reduced the percent of infected pigs in females but not in males. The vaccine also reduced the frequency of cysticercosis in females that that had been pregnant and to similar extents in pigs whose owners had or had not latrines at home. In addition, the vaccine significantly reduced the number of cysticerci in vaccinated pigs disregarding any of the other variables considered.

3.6. Weight gain and age

Table 6 shows the weight gain of pigs in which tongue and necropsy diagnosis coincided. Their weights increased similarly between 3–4 and 7–9 months of age, regardless of whether they were vaccinated or controls and whether infected or not. However, after 7–9 months of age, a non-significant 28% increase in weight was detected in control or vaccinated pigs if they had acquired cysticercosis, while those healthy still significantly increased their weight by 39%.

4. Discussion

This study shows the extent of the protective capacity of the S3Pvac-Phage vaccine against naturally acquired porcine cysticercosis under realistic conditions of transmission in a highly endemic region of Mexico [1].

A high number of pigs were lost for follow-up despite close weekly surveillance. This is neither surprising nor novel for in-the-field evaluation trials in underdeveloped regions [15]. Undisciplined human behaviour is emblematic of communities under pressure by harsh social and economic conditions, as they occur in rural Mexico. However, the number of pigs lost during the trial from the control and the vaccinated group did not significantly differ at tongue examination ($P = 0.21$) nor at necropsy ($P = 0.90$), indicating unbiased withdrawal of the pigs. That is, infected pigs were not preferentially withdrawn from the initial sample.

However, in order to examine the effect of pig loss upon the validity of our statistical conclusions, an independent research group (co-authors from Université de Limoges), expert in statistical analysis, was invited to more thoroughly examine the database, reduce bias and improve the robustness of the conclusions reached. All their statistical approaches performed (Student's *t*-test, Mann–Whitney *U* or χ^2 with Yates' correction or two-tailed Fisher's exact tests or the logistic analysis) confirmed that vaccination significantly reduced the prevalence and intensity of porcine cysticercosis in the sample studied by tongue inspection and by necropsy at time of slaughter. Also, when using the most rigorous maximum Bias Approach to statistical analysis of trials with numerous missing data, in two of its modalities, it confirmed significant protective effects of vaccination. However, no differences in prevalence due to vaccination were significant in its most stringent modality of considering as cysticercotic all missing pigs, vaccinated or not. An unlikely possibility since similar numbers of vaccinated and not vaccinated pigs were documented as missing and because it would imply a cysticercosis prevalence level close to 50% among the included pigs. Their results showed that S3Pvac-Phage significantly reduced the prevalence of cysticercosis among the vaccinated pigs by 54.2% and, most significantly, reduced the intensity of infection with vesicular cysts by 87.1%. It is important to note that a pig was considered infected regardless of its total parasite load: the pig was scored as positive even if only one cysticercus was found in its muscles. Thus, the higher efficacy of a vaccine in reducing the parasite load is a more sensitive response variable than the stringent goal of sterile immunity. The latter may perhaps be of use for negotiations dealing with international import of potentially contaminated meats but, for lowering transmission in a defined endemic region, a reduction of 87% in the number of parasite larvae that may eventually transform into tapeworms could significantly impact the endemia to the low levels of impending extinction of the parasite [24].

The efficacy of the S3Pvac-Phage vaccine reported in this study is similarly high to that obtained using the synthetic first version of the anti-cysticercosis vaccine (S3Pvac) when also tested under realistic conditions of rural Mexico [16]. We believe this is a major achievement since the significant lower cost of this new version makes its application feasible for extensive control programs in undeveloped countries.

Other important findings are also disclosed by this trial.

The vaccine significantly reduced the number of cysticerci in pigs exposed to different levels of exposure: high (absence of latrines) or low (presence of latrines). Indeed, in the absence of vaccination, cysticercosis prevalence changed from 8.2 to 29.8% if the owners of the pigs had or not latrines in their households, the relevance of improving sanitary conditions in the rural areas. However, vaccination plus latrines dropped down the overall cysticercosis prevalence further, from 29.8 (without both variables) to 4.8% (with both of them): a prime example of positive interactions between control measures aimed against porcine cysticercosis.

The roles of sexual factors upon cysticercosis prevalence and vaccination effects are also emphasized by this study. A higher prevalence of cysticercosis was found in females (22.5%) vs. castrated male pigs (4.8%). The vaccine did not modify the percentage of male pigs totally protected possibly because castration inhibits the effective TH1 mediated immune response to vaccination, as it happens in murine cysticercosis [25]. Nevertheless, the vaccine significantly reduced the number of cysticerci established in both females and castrated males to a similar relative extent (81% of reduction vs. controls). The increase of cysticercosis prevalence in pregnant sows is also intriguing. Ten of the thirteen pigs that were pregnant in the control group became infected. Albeit the low number of pigs included in this group, the significant relation between pregnancy and cysticercosis points to a role of hormonal factors in cysticercosis, very much in line with the consistently higher prevalence of cysticercosis in sows found in different epidemiological studies [1,26]. Nonetheless, the vaccine managed to reduce the parasite loads even in the more permissive pregnant sows. Clearly, the relevance of sexual factors merits further studies considering their prominent role under experimental conditions in murine cysticercosis caused by the closely related cestode *Taenia crassiceps* [25] and earlier epidemiological hints coming from porcine and human cysticercosis [26–30].

Another finding of practical importance is that vaccination did not reduce the weight gain of pigs but cysticercosis did (Table 6). So that, the net effect of vaccination not only lowers prevalence of porcine cysticercosis but also implies economic benefit by allowing for a normal weight gain in the protected pigs.

In summary, this article exhibits clear evidence of the usefulness and limits of the newly developed and less costly vaccine S3Pvac-Phage under realistic conditions of porcine-cysticercosis transmission.

Acknowledgements

The authors thank Cesar Medina Rojas, Roberto Oregón Morales, José López Reyeros, Ubaldo Leal Ponciano, David García Herrera, Oscar Cruz Bartolomé, Juan Antonio Hernández, Diana Hernández, Mario Kemler Valencia, Antonio Linares Ibáñez, Humberto Cisneros, Julián Rodríguez, Alfredo Blas Brito, Salvador Linares Lara, Conrado Canovas Olmos, Francisco Gadea, Elias Salinas Granados, Herminio Jiménez, Nelly Peña H., Manuel Alejandro, Julián Rodríguez Cabrera, Silvia Vargas and Mercedes Baca for their technical support. The authors also thank the program ECOS-North, (Evaluation-orientation de la Coopération Scientifique, France, Amérique Latine) which permitted the collaboration of a French research group comprised by some authors (Quet F and Bonnabau H) under the leadership of Pierre Marie Preux. This investigation was partially supported by Consejo Nacional de Ciencia y Tecnología 185946, 46953-m, 2004-01-040, the Fundación Produce, Morelos A.C. and Secretaria de Desarrollo Rural del Estado de Morelos, México, CEAMISH-Universidad Autónoma del Estado de Morelos, LISIG-UAEM, Autoridades Municipales de Tlaquiltenango, Tepalcingo y Ciudad Ayala del Estado de Morelos, CBTA No. 8 de Xoxocotla and the Dirección General de Personal Académico, Universidad Nacional Autónoma de México IN-221905.

References

[1] Morales J, Martinez JJ, Garcia-Castella J, Peña N, Maza V, Villalobos N, et al. *Taenia solium*: the complex interactions, of biological, social, geographical and commercial factors, involved in the transmission dynamics of pig cysticercosis in highly endemic areas. Ann Trop Med Parasitol 2006;100(2):123–35.
[2] Rodriguez-Canul R, Fraser A, Allan JC, Dominguez-Alpizar JL, Argaez-Rodriguez F, Craig PS. Epidemiological study of *Taenia solium* taeniasis/cysticercosis in a rural village in Yucatan State, Mexico. Ann Trop Med Parasitol 1999;83:57–67.
[3] Fleury A, Morales J, Bobes RJ, Dumas M, Yanez O, Pina J, et al. An epidemiological study of familial neurocysticercosis in an endemic Mexican community. Trans R Soc Trop Med Hyg 2006;100:551–8.
[4] Montano SM, et al., Cysticercosis Working Group in Peru. Neurocysticercosis: association between seizures, serology, and brain CT in rural Peru. Neurology 2005;65(2):229–33.
[5] Rodriguez-Hidalgo R, Benitez-Ortiz W, Praet N, Saa LR, Vercruysse J, Brandt J, et al. Taeniasis–cysticercosis in Southern Ecuador: assessment of infection status using multiple laboratory diagnostic tools. Mem Inst Oswaldo Cruz 2006;101(7):779–82.
[6] Preux PM, Druet-Cabanac M. Epidemiology and aetiology of epilepsy in sub-Saharan Africa. Lancet Neurol 2005;4(1):21–31.
[7] DeGiorgio CM, Medina MT, Duron R, Zee C, Escueta SP. Neurocysticercosis. Epilepsy Curr 2004;4:107–11.
[8] De la Garza Y, Graviss EA, Daver NG, Gambarin KJ, Shandera WX, Schantz PM, et al. Epidemiology of neurocysticercosis in Houston, Texas. Am J Trop Med Hyg 2005;73(4):766–70.
[9] Sorvillo FJ, DeGiorgio C, Watermant SH. Deaths from cysticercosis United States. Emerg Infect Dis 2007;13:230–5.
[10] Sciutto E, Fragoso G, Fleury A, Laclette JP, Sotelo J, Aluja A, et al. *Taenia solium* disease in humans and pigs: an ancient parasitosis disease rooted in developing countries and emerging as a major health problem of global dimensions. Microbes Infect 2000;2:1875–90.
[11] Pawlowski ZS, Allan J, Sarti E. Control of *Taenia solium* taeniasis/cysticercosis: from research towards implementation. Int J Parasitol 2005;35:1221–32.
[12] Nash TE, Singh G, White AC, Rajshekhar V, Loeb JA, Proano JV, et al. Treatment of neurocysticercosis: current status and future research needs. Neurology 2006;67(7):1120–7.
[13] Levine MZ, Lewis MM, Rodriquez S, Jimenez JA, Khan A, Lin S, et al. Development of an enzyme-linked immunoelectrotransfer blot (EITB) assay using two baculovirus expressed recombinant antigens for diagnosis of *Taenia solium* taeniasis. J Parasitol 2007;93(2):409–17.
[14] Serpa JA, Yancey LS, White Jr AC. Advances in the diagnosis and management of neurocysticercosis. Expert Rev Anti Infect Ther 2006;4(6):1051–61.
[15] Sciutto E, Rosas G, Hernandez M, Morales J, Cruz-Revilla C, Toledo A, et al. Improvement of the synthetic tri-peptide vaccine (S3Pvac) against porcine *Taenia solium* cysticercosis in search of a more effective, inexpensive and manageable vaccine. Vaccine 2007;25(8):1368–78.
[16] Huerta M, de Aluja AS, Fragoso G, Toledo A, Villalobos N, Hernandez M, et al. Synthetic peptide vaccine against *Taenia solium* pig cysticercosis: successful vaccination in a controlled field trial in rural Mexico. Vaccine 2001;20:262–6.
[17] Manoutcharian K, Diaz-Orea A, Gevorkian G, Fragoso G, Acero G, Gonzalez E, et al. Recombinant bacteriophage-based multiepitope vaccine against *Taenia solium* pig cysticercosis. Vet Immunol Immunopathol 2004;99:11–24.
[18] Del Rey A, Roggero E, Randolf A, Mahuad C, McCann S, Rettori V, et al. IL-1 resets glucose homeostasis at central levels. Proc Natl Acad Sci USA 2006;103(43):16039–44.
[19] Sciutto E, Morales J, Martínez JJ, Toledo A, Villalobos MN, Cruz-Revilla C, et al. Further evaluation of the synthetic peptide vaccine S3Pvac against *Taenia solium* cysticercosis in pigs in an endemic town of Mexico. Parasitology 2007;134:129–33.
[20] Manoutcharian K, Terrazas LI, Gevorkian G, Acero G, Petrossian P, Rodriguez M, et al. Phage-displayed T-cell epitope grafted into immunoglobulin heavy-chain complementary-determining regions: an effective vaccine design tested in murine cysticercosis. Infect Immun 1999;67(9):4764–70.
[21] Lemeshow S, Hosmer DW, Janella K, Lwanga SK. Adequacy of sample size in health studies. John Wiley & Sons Ltd.; 1990.
[22] Elashoff JD, Lemeshow S. Sample size determination in epidemiologic studies. In: Ahrens W, Pigeot J, editors. Handbook of epidemiology. Berlin/Heidelberg: Springer; 2004. p. 559–94.
[23] Moher D, Schulz KF, Altman DG. For the CONSORT Group, The CONSORT statement: revised recommendations for improving the quality of reports of parallel-group randomised trials. Lancet 2001;357:1191–4.
[24] Heesterbeek JA, Roberts MG. Threshold quantities for helminth infections. Math Biol 1995;33(4):415–34.
[25] Morales-Montor J, Larralde C. The role of sex steroids in the complex physiology of the host–parasite relationship: the case of the larval cestode of *Taenia crassiceps*. Parasitology 2005;131(Pt 3):287–94.
[26] Morales J, Velasco T, Tovar V, Fragoso G, Fleury A, Beltran C, et al. Castration and pregnancy of rural pigs significantly increase the prevalence of naturally acquired *Taenia solium* cysticercosis. Vet Parasitol 2002;108:41–8.
[27] Peña N, Morales J, Morales-Montor J, Vargas-Villavicencio A, Fleury A, Zarco L, et al. Impact of naturally acquired *Taenia solium* cysticercosis on the hormonal levels of free ranging boars. Vet Parasitol 2007;149(1–2):134–7.
[28] Del Brutto OH, García E, Talámas O, Sotelo J. Sex-related severity of inflammation in parenchymal brain cysticercosis. Arch Intern Med 1988;148(3):544–6.
[29] Larralde C, Montoya RM, Sciutto E, Diaz ML, Govezensky T, Coltorti E. Deciphering western blots of tapeworm antigens (*Taenia solium, Echinococcus granulosus*, and *Taenia crassiceps*) reacting with sera from neurocysticercosis and hydatid disease patients. Am J Trop Med Hyg 1989;40:282–90.
[30] Fleury A, Dessein A, Preux PM, Dumas M, Tapia G, Larralde C, et al. Symptomatic human neurocysticercosis—age, sex and exposure factors relating with disease heterogeneity. J Neurol 2004;251(7):830–7.