ELSEVIER

International Journal of Food Microbiology 48 (1999) 73–78

International Journal of Food Microbiology

Short communication

Factors affecting the efficiency of pooled sample digestion for the recovery of *Trichinella spiralis* from muscle tissue

H.R. Gamble*

U.S. Department of Agriculture, Agricultural Research Service, Livestock and Poultry Sciences Institute, Parasite Biology and Epidemiology Laboratory, Beltsville, MD 20705, USA

Received 19 October 1998; received in revised form 8 February 1999; accepted 8 February 1999

Abstract

Inspection for *Trichinella spiralis* in pork, horse and game meats is an important part of veterinary public health programmes in many countries. Variations of the pooled sample digestion test are the most widely used methods of inspection for this parasite. In this study, several aspects of the test, including sample preparation, reagent concentration and sample processing were examined for effect on test efficiency. Current methods using sample blending were equivalent or superior to sample grinding. Increasing the concentration of pepsin improved overall digestion slightly. The most critical factor affecting parasite recovery was sieve size. Currently used methods that employ a #80 (180 μm mesh) sieve limit the recovery of motile or dead parasites and therefore might decrease test sensitivity. It is recommended that a #45 (355 μm mesh) sieve be used to ensure optimal recovery of larvae.

Keywords: *Trichinella*; Pork; Artificial digestion; Meat inspection

1. Introduction

In many countries, meat inspection procedures include testing for *Trichinella* in animal species (pigs, horses) that are a source of infection for humans. Inspection procedures include methods that result in visual observation of the parasite in situ (trichinoscope examination) or following recovery of worms from muscle tissue (artificial digestion methods). The most widely used methods are those described in the Directives of the European Union (EU) (77/96/EEC, 84/319/EEC; Koehler and Grossklaus, 1972; Koehler, 1977), and most other countries follow procedures that are similar in principle (Gajadhar et al., 1996). However, there are significant technical differences among the various methods described in the EU Directives, as well as in methods employed elsewhere (Veterinary Code of the USSR).

While the theoretical sensitivity of testing a 1-g sample by artificial digestion is an infection level ≥ 1 larvae per gram (LPG) of tissue, the actual sensitivity is 3–5 LPG (Gamble, 1996, 1998a). This level of sensitivity could result in false negative results in samples that contain sufficient worms to

*Tel.: + 1-301-504-8300; fax: 1-301-504-6273.

E-mail address: rgamble@lpsi.barc.usda.gov (H.R. Gamble)

0168-1605/99/$ – see front matter
PII: S0168-1605(99)00017-3

cause human disease. Thus, test efficiency is important in assuring public health. Some of the factors that impact the efficiency of artificial digestion include: methods of sample preparation; composition of digestion fluid, including pepsin and hydrochloric acid concentrations; temperature and duration of digestion; and, methods of filtration and settling for recovery of freed larvae. In the present study, several of these variables were examined with respect to their impact on the efficiency of larval recovery.

2. Materials and methods

Trichinella spiralis (Beltsville pig strain, designated T-1 under nomenclature of the ICT, Trichinellosis Reference Centre, Rome, Italy) was maintained by serial passage in female Sprague-Dawley rats. For inoculation of pigs, muscle larvae were recovered from rats 35 days after infection by digestion in pepsin–HCl (Gamble, 1998b). Ten, six-month-old Duroc X York pigs were inoculated orally with 2500 infective larvae (L1). Thirty-five days after inoculation, pigs were killed by stunning with a captive bolt pistol followed by exsanguination. Loin musculature, which was chosen for this study because if its similarity to crus muscle and its availability in large quantity, was removed from each pig and stored at 5°C for up to one week prior to use in digestion studies.

The basic method used for digestion in this study was as described in the European Union Commission Directive 84/319/EEC, Annex, Part A.3.VI (Magnetic Stirrer Method for Pooled Sample Digestion). Modifications to this method were made for individual experiments.

For the comparison of sample preparation by blending versus grinding, pieces of loin muscle weighing 100 g were cut into 100 pieces of approximately 1 g each. Ten 100 g samples per treatment group were either blended, as described in EU Directives, or ground using a Hobart Model 4912 commercial meat grinder with a 2-mm pore size. Samples prepared in this way were processed in an identical manner using the Magnetic Stirrer Method with pepsin (NSF = National Standard Formulary 1:10,000; American Laboratories, Omaha, NE, USA) at concentrations of 0.5 and 1.0% (w/v). At the conclusion of digestion, the 2-l digest volume was passed through a #80 (180 μm mesh) sieve and the sieve was rinsed with an additional 100 ml of tap water. For each digest, the sieve was then scraped and back-flushed with water. The residual sample recovered from the sieve was blotted dry and weighed. All comparisons were performed on the same day to minimize variation.

To assess the influence of sieve size on worm recovery, *Trichinella spiralis* larvae, recovered by digestion of rats, were suspended in tap water at a density of 25 worms in 50 ml. For each replication, the 50 ml of tap water containing 25 worms was poured through a pre-wetted sieve; the sieve was then rinsed with an additional 50 ml of tap water. Larvae passing through the sieve were collected in conical pilsner glasses, allowed to sediment for 30 min and then counted. Sieves were back-flushed with 100 ml of tap water and any larvae collected were settled and counted in a similar manner. In addition, all sieves were examined microscopically following each replicate and worms remaining bound to the sieve were included with numbers recovered by back-flushing. Following each replicate, sieves were washed extensively in hot water and examined to ensure that no larvae remained. Sieves tested were 5-inch-diameter brass Tyler sieves, which conformed to ASTM, ANSI and ISO 565 specifications; sizes included #35 (500 μm mesh), #40 (425 μm mesh), #45 (355 μm mesh), #50 (300 μm mesh), #60 (250 μm mesh), #70 (212 μm mesh), #80 (180 μm mesh), #100 (150 μm mesh), #120 (125 μm mesh) and #140 (106 μm mesh) (W.S. Tyler, Mentor, OH, USA). The size specified for use in EU Directives corresponds to a #80 sieve. Each test consisted for five replications. In addition, each sieve was tested using worms that were maintained at 4°C, at 40°C and worms that were heat killed prior to testing (90°C for 1 min). Microscopically, worms at 4°C were coiled, worms at 40°C were motile and worms that were heat-killed were found in the characteristic C-shape. The number of worms recovered from each replicate was expressed as a percentage of the total number of worms found in the flow through, the back-flush and on the sieve. For each sieve size/worm treatment combination, the percentage recovery was averaged for comparison among treatments.

Settling time, following digestion, was determined using larvae recovered from rats as above. Larvae were suspended in tap water at a density of 100 L1/ml. Graduated cylinders (100 ml) were filled to the 100 ml mark (approximately 19 cm of fluid height) with standard digestion fluid. To prepare standard digestion fluid, 100 g of uninfected pork muscle were digested in artificial digestion fluid as described and the recovered fluids were used for the settling experiments. This fluid was used to more accurately represent the density of fluid through which larvae must settle. A 1-ml volume of larvae, containing 100 worms, was added to the top of each graduated cylinder containing digestion fluid. At 2 min intervals following the addition of larvae, the top 90 ml of the digestion mixture was removed by vacuum filtration; the number of worms in the top 90 ml and the remaining 10 ml was counted independently. For each time point, a total of five replicates was performed. Settling time was determined for larvae at 4°C (coiled) and 40°C (active). The number of larvae settled was converted to a percentage of total larvae, based on the counts obtained from the upper 90 ml and the lower 10 ml of fluid at each time point. Settled larvae were considered as those found in the bottom 10 ml of fluid. Mean values were determined for each time point based on the five replicates.

The most efficient method for recovering larvae, based on the results obtained here, was compared with the standard (magnetic stirrer) method described in the EU Directives. For this comparison, loin muscle from ten pigs that had received a high (2500 L1) or low (250 L1) dose of *T. spiralis* were digested. A total of three 100-g samples were digested from each pig, using each method. The optimized method differed from the standard method by the use of 1% pepsin (versus 0.5% pepsin) and the use of a #45 (355 μm mesh) sieve in place of a #80 (180 μm mesh) sieve. Both methods used blending as the method of sample preparation and settling times were as prescribed in the EU Directives. Mean values were compared for a total of 15 samples each, from the high and low dose pigs.

Comparison of variables was performed using an unpaired *t*-test or a two-way analysis of variance (ANOVA) (InStat, Graphpad, San Diego, CA, USA), depending on the number of variables examined. For ANOVA, Bonferroni *p* values were determined for comparisons of individual groups.

3. Results and discussion

The weight of undigested meat from ten replicate digests performed with samples prepared by blending and digested in 0.5% pepsin was 2.372±0.803 g (mean±standard deviation) and the weight of undigested meat from ten replicate digests performed with samples prepared by grinding and digested in 0.5% pepsin was 3.868±1.466 g (mean±standard deviation). These values were statistically different ($p = 0.011$) using a two-tailed unpaired *t*-test. The weight of undigested meat from ten replicate digests performed with samples prepared by blending and digested in 1.0% pepsin was 1.433±0.986 g (mean±standard deviation) and the weight of undigested meat from ten replicate digests performed with samples prepared by grinding and digested in 1.0% pepsin was 3.309±0.847 g (mean±standard deviation). These values were statistically different ($p = 0.0002$) using a two-tailed unpaired *t*-test. Sample preparation varies from country to country (Zimmermann, 1983). EU Directives require sample blending; however, Russian regulations consider grinding through a 2-mm pore plate to be a superior method of sample preparation (Veterinary Code of the USSR; A. Bessonov, pers. comm.). In this study, we found blending to be equal or superior to grinding, as evidenced by the amount of residual tissue following digestion. Blending resulted in a 28.7% reduction in the amount of residual tissue following digestion, as compared with grinding, or viewed another way, resulted in digestion of another 1.5 g of tissue.

The data used for sample preparation comparison were further analyzed for effect of pepsin concentration. Samples prepared by blending and digested with 1% pepsin had statistically less residual muscle remaining on sieves as compared with blended samples digested with 0.5% pepsin ($p = 0.03$). No statistical differences were observed between ground samples digested with 1.0 or 0.5% pepsin ($p = 0.31$). In EU prescribed methods, pepsin concentration varies from 3.5 to 5 g/l of digestion fluid. Other published methods (Gamble, 1998b) suggest

using 10 g/l in a similarly acidified environment. We confirm here that the use of a 1% pepsin solution can result in significantly less undigested tissue when compared with 0.5% pepsin, under certain conditions. Whether or not this benefit of increased digestion of tissue at an increased cost of materials offsets the need for improved sensitivity should be considered in light of the prevalence of infection. It is possible that too much pepsin could digest larvae, particularly if they are otherwise damaged. It is suggested that pepsin concentrations be adjusted to use the minimal concentration that results in complete digestion of muscle tissue.

The mean values (±standard deviation) for data collected from digestion experiments in which the size of the sieves used for filtration was varied is presented in Table 1 and statistical comparison of these values is shown in Tables 2 and 3. In most cases, significant changes in worm recoveries were not found between sequential sieve sizes. However, variation did occur within sieve sizes depending on the physical state of the worms. Worms maintained at 4°C were more readily recovered from sieves with smaller openings and live worms passed more easily through sieves than did dead worms. When two commonly used sieve sizes [#80 (180 μm mesh opening) and #45 (355 μm mesh opening)] were compared directly, the larger sieve (#45) was significantly more effective than the smaller sieve (#80) for recovery of both motile (40°C) larvae and dead larvae. No statistically significant differences were observed between these sieve sizes when larvae were coiled (4°C). EU Directives prescribe a 177-

Table 2
Differences in recovery of *Trichinella spiralis* larvae following passage through sieves of various sizes

Sieve Comparison	Differences in recovery when worms are:		
	at 4°C	at 40°C	Dead
35 vs. 40	ns[a]	ns	ns
40 vs. 45	ns	ns	ns
45 vs. 50	ns	ns	ns
50 vs. 60	ns	ns	ns
60 vs. 70	ns	ns	$p < 0.05$
70 vs. 80	ns	ns	ns
80 vs. 100	ns	ns	ns
100 vs. 120	$p < 0.001$	$p < 0.05$	ns
120 vs. 140	$p < 0.001$	ns	ns
45 vs. 80	ns	$p < 0.01$	$p < 0.001$

[a] ns = no significant difference.

Table 3
Differences in recovery of coiled, motile or dead *Trichinella spiralis* larvae following passage through sieves of various sizes

Sieve size	Differences in recovery when worms are:		
	at 4 vs. 40°C	at 4°C vs. Dead	at 40°C vs. Dead
35	ns[a]	$p < 0.05$	$p < 0.05$
40	ns	$p < 0.05$	$p < 0.05$
45	ns	$p < 0.001$	$p < 0.001$
50	ns	$p < 0.001$	$p < 0.001$
60	ns	$p < 0.001$	$p < 0.001$
70	$p < 0.05$	$p < 0.001$	$p < 0.001$
80	$p < 0.01$	$p < 0.001$	$p < 0.001$
100	$p < 0.01$	$p < 0.001$	$p < 0.001$
120	$p < 0.05$	$p < 0.001$	$p < 0.05$
140	ns	ns	ns

[a] ns = no significant difference.

Table 1
Recovery of *Trichinella spiralis* larvae following filtration through sieves of various sizes

Sieve #	μm mesh	Number of larvae recovered when larvae were:		
		Coiled (4°C)	Motile (40°C)	Dead
35	500	97.1±2.9[a]	99.2±2.3	87.0±7.2
40	425	98.3±2.4	98.1±2.8	82.2±11.9
45	355	96.8±3.3	96.6±4.8	67.0±9.7
50	300	97.9±2.9	96.3±4.6	45.0±5.0
60	250	99.1±1.7	85.9±7.9	47.5±13.7
70	212	97.8±3.2	75.2±6.4	21.9±14.6
80	180	93.0±4.3	63.1±12.3	18.6±11.9
100	150	88.8±16.0	45.1±11.8	6.7±2.9
120	125	38.4±9.6	20.8±10.0	2.9±4.3
140	106	0	6.4±5. 2	1.1±2.0

[a] Mean (±standard error) percentage of larvae that passed through the sieve.

μm mesh (#80) sieve, while other methods (Gamble, 1998b; Veterinary Code of the USSR) recommend a larger mesh size. This study confirms that sieve sizes across a range of 180 to 450 μm openings do not influence recovery when larvae are tightly coiled. Therefore, the efficiency of digestion methods in which larvae are chilled prior to filtering would be unaffected by different sieves in this range. However, significant differences in recovery of motile and dead worms were found when sieve sizes were varied across the same range. The most significant finding was that, under the conditions tested, recovery of motile (40°C) worms was reduced from 96.6% using a #45 (355 μm mesh) sieve to 63.1% using a #80 (180 μm mesh) sieve. In EU Methods III and VI, (Directive 84/319/EEC), worms would be motile during the recovery stages due to warm temperatures used during the digestion process. Methods IV and V require the addition of ice following digestion, however, it is uncertain if lowering of temperature is sufficient to cause coiling of worms. Thus, it is possible that all currently described EU methods might be less efficient due to the use of a sieve size that is too small for larvae to pass through consistently.

The results of larvae settling times at both 4°C and 40°C are presented in Fig. 1. Significant differences ($p < 0.05$) in the percentage of worms settled were only seen during the first two time intervals (0 to 2 min and 2 to 4 min of settling time) for both temperatures. However, significantly greater ($p < 0.05$) numbers of motile larvae (40°C) had settled at the 4 and 6 min time intervals, as compared with coiled larvae (4°C). EU Directives and other published methods (Gamble, 1998b) specify minimum settling times of 20–30 min. Based on the data presented here, settling times as described in currently used methods are more than adequate for efficient recovery of larvae and, in fact, could be shortened to reduce the overall test time.

Tissue samples from *T. spiralis*-infected pigs were digested using standard (0.5% pepsin and #80/180 μm mesh sieve) and optimized (1.0% pepsin and #45/355 μm mesh sieve) conditions. The results of worm recoveries are presented in Table 4. Fewer, but not significantly different, numbers of worms were recovered from 'low dose' pigs using the standard method compared with the optimized method. The optimized method resulted in recovery of a significantly greater number of larvae (43.3%) from 'high dose' pigs compared with the standard method. The results of these studies suggest that improvement of pooled sample digestion would be effected by adoption of a 1% pepsin concentration and use of a larger sieve size for filtration steps.

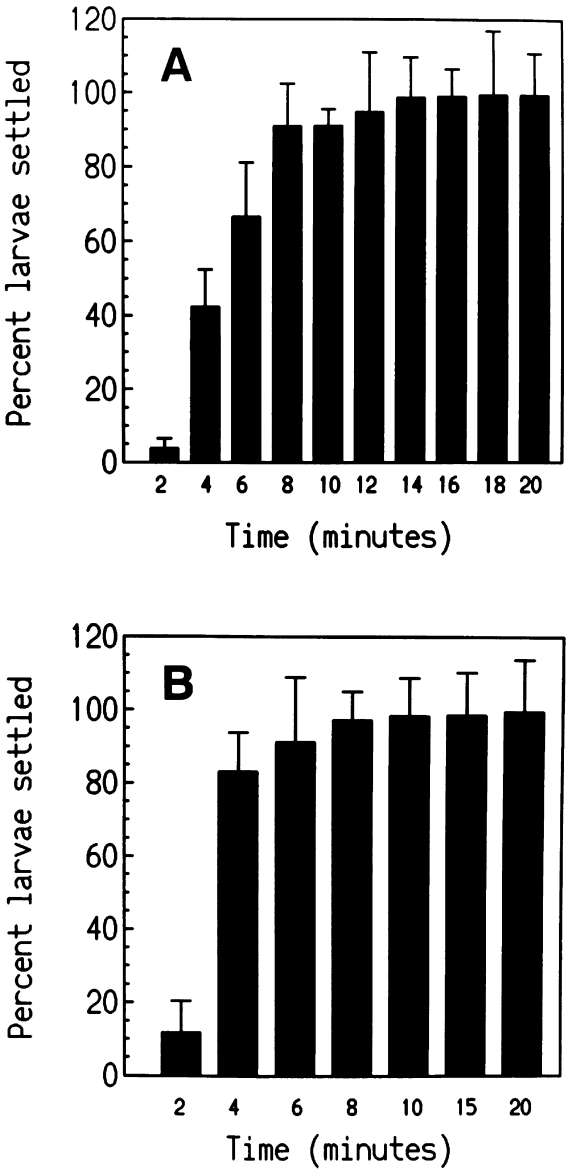

Fig. 1. Comparison of settling times of *Trichinella spiralis* larvae that were maintained at either 4°C (panel A) or 40°C (panel B). Values are expressed as means±standard deviation for five replicates per treatment.

Table 4
Recovery of *Trichinella spiralis* following pooled digestion using standard (0.5% pepsin and #80 sieve) and optimized (1.0% pepsin and #45 sieve) conditions

	# Larvae recovered using:		*p* value
	Standard method	Optimized method	
Low-dose pigs	15.53±1.56[a]	16.67±1.49	ns[b]
High-dose pigs	264.87±32.51	379.53±34.37	0.018

[a] Mean (±standard error) number of larvae recovered from three samples taken from five pigs (15 samples in total).
[b] No significant difference.

4. Conclusions

Methods of testing for *Trichinella spiralis* in pork, horse and game meats vary among countries and these variations influence test efficacy. In the studies performed here, several variables in test performance were compared to determine how they affect the test results. Of the variables tested, sample preparation by blending versus grinding resulted in only minor differences in the overall test efficiency, as did increases in pepsin concentration. Settling times were more than adequate for all methods and could be shortened from those currently used to reduce the overall test time. The use of #80 (180 μm mesh) sieves reduced the recovery of motile larvae. It is recommended that the use of #80 (180 μm mesh) sieves be limited to those methods in which recovered larvae are tightly coiled (i.e., if digestion fluid is chilled). Recovery of *Trichinella* larvae using artificial digestion methods, as described in the EU Directives, would be improved by increasing the sieve size to >300 μm mesh.

Acknowledgements

The authors thanks Mr. James McCrary and Ms. Everlie Bolton for excellent technical assistance in the performance of these studies.

References

European Economic Community, 1977. Commission Directive 77/96/EEC. Official Journal of the European Communities 26, 67–77.

European Economic Community, 1984. Commission Directive 84/319/EEC. Journal of the European Communities 167, 34–43.

Gajadhar, A.A., Forbes, L.B., Rajic, A., 1996. The double separatory funnel procedure for the detection of *Trichinella* larvae in pork. Official Protocol, Agriculture and Agri-Food Canada, Version 1.0, November 14 1996, 18 pp.

Gamble, H.R., 1996. Detection of trichinellosis in pigs by artificial digestion and enzyme immunoassay. J. Food Prot. 59, 295–298.

Gamble, H.R., 1998a. Sensitivity of artificial digestion and enzyme immunoassay methods for inspection of trichinellosis in pigs. J. Food Prot. 61, 339–343.

Gamble, H.R., 1998. Trichinellosis. In: OIE Manual of Standards for Diagnostic Tests and Vaccines, Ch. 3.5.3, pp. 477–480.

Koehler, G., 1977. Zur effektivität der verdauungsmethode beim nachweis der trichinellose des schlachtschweines. Fleischwirtschaft 57, 421–423.

Koehler, G., Grossklaus, D., 1972. Untersuchungen zur diagnose der trichinellose am schlachtschwein. Archiv fuer Lebensmittelhygiene 4, 77–81.

Tret'Yakov, A.D. (Ed.), Veterinary Code of the USSR: Provisions, Guidelines, Instructions, Directions and Rules on Veterinary Matters, Vol. II, 1972. Kolos, Moscow.

Zimmermann, W.J., 1983. Surveillance in swine and other animals by muscle examination. In: Campbell, W.C. (Ed.), *Trichinella* and Trichinosis, Plenum Press, New York, pp. 515–528.