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Article

Usefulness of Fourier transform infrared (FTIR) spectroscopy to detect *Trichinella spiralis* (Owen, 1835) muscle larvae in ham and sausages made from the meat of an experimentally infected pig

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Abstract:

The aim of this work was to determine the usefulness of Fourier transform infrared (FTIR) spectroscopy to detect muscle larvae of *Trichinella spiralis* (Owen, 1835) in ham and sausages made from the meat of an experimentally infected pig. It was searched for the muscle larvae (ML) by conventional methods (artificial digestion and trichinoscopy stained with Mayer´s hemalum stain) and by FTIR spectroscopy. In addition, the infective capacity of the larvae found in swine products was analyzed. The parasite load was 8.5 ± 3 mL/g in ham and 4.5 ± 1.4 mL/g in sausage (P<0.0001). The spectra of the pig products prepared with the meat of an uninfected pig were different in the range of 1,700 to 900 cm^{-1} with respect to the spectra of the products from infected pigs. In this region, glycogen is the most abundant chemical group (1,200 and 900 cm^{-1}). The distance between classes between non-infested and infested products was 10.2 for ham and 5.52 for sausages (three is the minimum value to indicate class separation). The infective capacity of the larvae recovered from pig products decreased up to five times compared to that of the larvae obtained from experimentally infected mice. These results show that the FTIR spectroscopy is useful to determine the presence of *T. spiralis* larvae in the foods studied here. Further studies are needed to determine the influence of meat flavors on the detection of *Trichinella* by FTIR spectroscopy.

Key words: *Trichinella*, Meat-inspection, Sausage, Diagnosis, Infrared-spectroscopy.

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Introduction

Trichinella spiralis (Owen, 1835) is a parasitic nematode of worldwide distribution that causes the foodborne zoonosis named trichinellosis. Since various human clinical cases and outbreaks are related to eating pork or pork products (sausages) insufficiently cooked that host viable larvae[1-5], the International Commission on Trichinellosis continuously issues recommendations for the inspection and treatment of meat intended for human consumption[6]. In abattoirs, the artificial digestion using pepsin protease and hydrochloric acid is the standard assay for the detection of *Trichinella* larvae[7-9]. Notwithstanding inspection of pigs in public slaughterhouses is mandatory, some pigs remain without any sanitary control and, after slaughtering, the meat or meat products are domestically sold

without sanitary inspection. Thus, manufacturers exercise caution to improve the stability of raw pork meat using preservation techniques. However, the reproductive capacity of *Trichinella* muscle larvae has been reported not to be affected by, "wet-curing", "adobado" (meat spiced with chili) or cold storage of raw meat[10].

Sausages, in addition to being part of society's culinary customs, are also a way of preserving pork meat. There are some reports of human trichinellosis by ingestion of pork sausages[1,4,11,12] and, additionally, Forbes *et al*[13], reported that the larvae of *T. nativa* (Britov & Boev, 1972) maintained their infectivity in traditional northern raw and partially cooked sausages prepared with meat from experimentally infected seals. Since prepared sausages of unchecked pork could be a risk in the transmission of *Trichinella*, it is necessary to have diagnostic alternatives that can support the recommended techniques for meat inspection to make the definitive diagnosis of the parasite[7]. An alternative method, currently used in food analysis, is the Fourier transform infrared (FTIR) spectroscopy with attenuated total reflectance (ATR) and soft independent modeling of class analogies (SIMCA). The usefulness of FTIR spectroscopy has been previously reported to detect adulterations in pork meat products[14,15]. In recent times, someone of this research group developed the model for rat meat spiked with *T. spiralis* larvae and was able to detect three larvae in 10 g of rat meat; no interference was observed with antigens of *Ascaris suum* (Goeze, 1782) or *Taenia solium* (Linnaeeus, 1758)[16]. In a subsequent experiment, it was possible to identify the *T. spiralis* muscle larvae in pigs infected with different infective doses, from 812 to 13,000 larvae/pig[17]. However, the identification of larvae in meat products derived from infected pigs was awaiting. Thus, this work aimed to determine the usefulness of Fourier transform infrared (FTIR) spectroscopy to detect *Trichinella spiralis* muscle larvae in ham and sausages made from the meat of an experimentally infected pig.

Material and methods

Parasite

The parasite *Trichinella spiralis* (MSUS/ME/92/CM-92) was maintained in Wistar rats. The parasite was isolated in Mexico from a naturally infected pig in the 1970 and was typified in the 1990[18]. Each rat (250 g of body weight and 6 wk old), was infected orally with 23 muscle larvae (ML) per gram of body weight (equivalent to 6,000 ± 250 mL per rat). The experimental infection to obtain the larvae was approved by Comite de Etica y Cuidado de los Animales (CIECUA) of the Instituto de Ciencias Agropecuarias of the UAEH under the guidelines of Mexican regulation[19]. Muscular larvae were isolated by artificial digestion

with a solution of 0.5% pepsin (Sigma–Aldrich St. Louis, Mo; USA) in 0.2% hydrochloric acid. Afterward, larvae were counted at 100x magnification with bright field microscopy. The number of *T. spiralis* muscle larvae present in the sausages was also evaluated by artificial digestion.

Experimental infection

Two male York Landrace pigs *(Sus scrofa domestica* Linnaeus, 1758) of 4 wk old and 10 ± 0.2 kg were fed *ad libitum* with pelleted commercial food for the fattening stage (Agribrands Purina Mexico, Cuautitlán, Edo. Méx.). One pig was infected with 800 muscular larvae of *T. spiralis*, equivalent to a slight infection of 0.08 muscle larvae per gram of body weight; the other pig remained uninfected. The animals were maintained in separate corrals following the Mexican regulations[19,20]. The animals were slaughtered using a penetrating captive bolt gun at 12 wk post-infection and three samples of 10 g were obtained from five anatomical regions (rib, loin, leg, masseter and diaphragm). The samples were subjected to artificial digestion or to compression between two glasses and observation at 40x magnification (trichinoscopy) to search for parasites. The leg meat was used to make the ham, and the meat of loin, rib, and shank was used to make the sausage called "salchicha" in México, often similar to hotdogs, frankfurters, or wieners.

Pork products

The ham and sausage were prepared without seasonings at the School of Veterinary Medicine (Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México) according to traditional Mexican recipes and according to the Mexican regulation[21]. The ham was prepared from leg meat (605 g) without fat, tendons or ligaments. The meat was placed in a mincer to reduce the size of the meat pieces. The brine was prepared using sodium phosphate (9.0 g), salt (13.3 g), sugar (4.2 g), cure salt (12.0 g), sodium erythorbate (1.99 g), monosodium glutamate (0.18 g) and carrageenan (4.2 g) dissolved in water (350 ml). Then, the meat was placed in a container and the brine was added, then the container was covered with adhesive plastic and put in cooling conditions at 4 ^{o}C for 24 h. The next day, the cured meat was placed inside a knotted sheath with thread from the end, eliminating as much as possible the air to accommodate the meat. The preparation was placed into a vacuum machine to remove any air presence and then tightly knot the free end of the sheath. The ham was cooked entirely submerged in water at 80 ± 1 ^{o}C for 50 min and then cooled for 5 min in ice water. The sausage was made with the meat of loin, rib and shank without tendons or

ligaments. The meat (595 g) was minced twice with ice (110 g), salt (11 g) and salt of cure (1.7 g) to make an emulsion. At that point, pork fat (171.4 g) and ice (110 g) were added while the mincer was working. Subsequently, cornstarch (92.2 g) was slowly incorporated to form an emulsified paste. Without stopping the mincer, sodium erythorbate (1.3 g), ice (110 g) and sodium phosphate (5.2 g) were added. To prepare the sausage, the operation of the mincer was stopped, and the paste was placed at the bottom of the filler, which was fitted with a 1 cm diameter nozzle. The nozzle was covered with a layer of vegetable oil to insert the cellophane was knotted at the opposite end. Finally, the cellophane sheath was carefully filled, and it was split every 10 to 12 cm; each fraction was knotted. The sausage entirely submerged in water at 72 ± 1 ºC for 20 min and then cooled for 5 min in ice water. Fresh samples were subjected to artificial digestion and trichinoscopy to determine the number of muscle larvae in the ham and sausage.

Detection of larvae in pork products stained with Mayer's hemalum

Samples 0.1 mm thick of ham and sausage (n= 20, each) were stained with Mayer's hemalum, as previously reported[22], but several modifications were made to the original method. Briefly, the samples were incubated in ether for 1 h at room temperature, then fixed in 10% formalin for 12 h. Afterward, they were stained with hemalum (Sigma Aldrich, St. Louis, MO, USA) for 15 min and subsequently the samples were dehydrated by consecutive passages through 70, 80, 90, 96 and 100% alcohol for 15 min each time. A final 20 min incubation in methyl salicylate was performed prior to mounting each slide with synthetic resin. The preparations were observed in light microscopy at 40X magnification, and the muscle larvae were counted.

Detection of *Trichinella spiralis* in pork products by FTIR spectroscopy

The ham and sausage spectra were obtained as previously described[16,17], using a FTIR spectrophotometer (Spectrum GX, Perkin Elmer Massachusetts, USA) equipped with a deuterated triglycine sulfate detector. The sampling station has an attenuated total reflection accessory (ATR) through which infrared radiation passes to a zinc selenide crystal. One-gram sample (n= 5) of each ham and sausage were placed on the sampling station. The samples were pressed on the surface of the glass to allow the infrared ray to pass through them and be reflected towards the spectrometer and, 64 readings (scans) were obtained from each analyzed sample. The spectra were acquired and processed with the Spectrum software version 3.01.00 (Perkin Elmer, Inc.). The spectra were scanned over a wave number range of

4,000–650 cm^{-1}, averaging 64 scans at a resolution of 4 cm^{-1}. The analysis region was 1,700 to 900 cm^{-1}.

Development of the SIMCA model

With the obtained spectra, the SIMCA model (Soft Modeling of Independent Classes Analogies) was elaborated for which 40 spectra of sausages infected and not infected with *Trichinella spiralis* larvae were used. The spectra were then subjected to Soft Independent Modeling of Class Analogy analysis (SIMCA) to determine the interclass distance between groups. The minimum value of the interclass distance must be greater than 3 so that the two analyzed populations are considered to be different[16,17].

Infective capacity of parasite

To determine the infective capacity of larvae recovered by artificial digestion of the infected ham and sausage, CD1 naive male mice (n= 10 per product) 5 wk old and 20 g, were orally infected with 50 ± 3 mL. In addition, five mice of the same age, sex, and weight were infected with an equal dose of militers obtained from a donor rat experimentally infected. At day 60 post-infection, mice were killed by cervical dislocation and then, submitted to artificial digestion to calculate the reproductive capacity index, *i. e.*, the number of larvae recovered from carcasses divided by the number of larvae used to infect mice[10,23]. The CIECUA approved the experimental infection protocol.

Statistical analysis

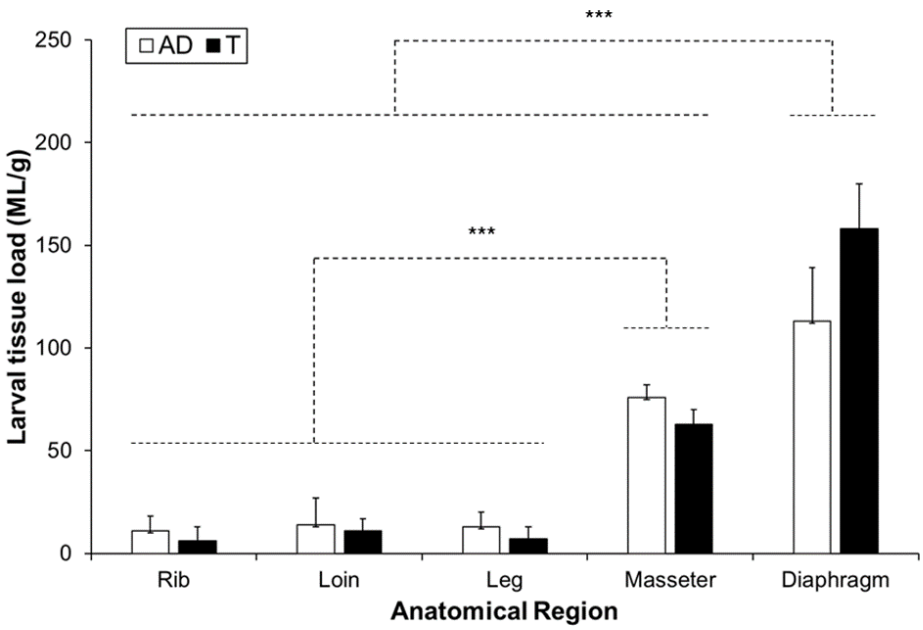
The parasite load in the experimentally infected pig was analyzed using the two-way ANOVA test followed by Bonferroni post-test. The parasite load in the pork products was analyzed by the unpaired Student´s T-test. The reproductive capacity index of larvae recovered from ham or sausage was analyzed with the one-way ANOVA test followed with the Tukey´s multiple comparison test. Analysis was performed with the GraphPad Prism version 6.01 for Windows (GraphPad Prism Software, version 6.01, La Jolla California USA).

Results

Experimental infection of pig

The parasite load was determined in five anatomical regions (rib, loin, leg, diaphragm and masseter; n= 3 samples of each region) by artificial digestion and trichinoscopy (Figure 1). Statistical differences were observed among anatomical regions (P<0.0001) but the results obtained with the two detection methods were similar (P=0.4494). It should be noted that the anatomical regions with high commercial demand have three times less parasitic load than the masseter and five times less than the diaphragm, two of the preferred sites of encystment of *T. spiralis* in pigs.

Figure 1: Larval load of *Trichinella spiralis* in five anatomical regions of a pig experimentally infected

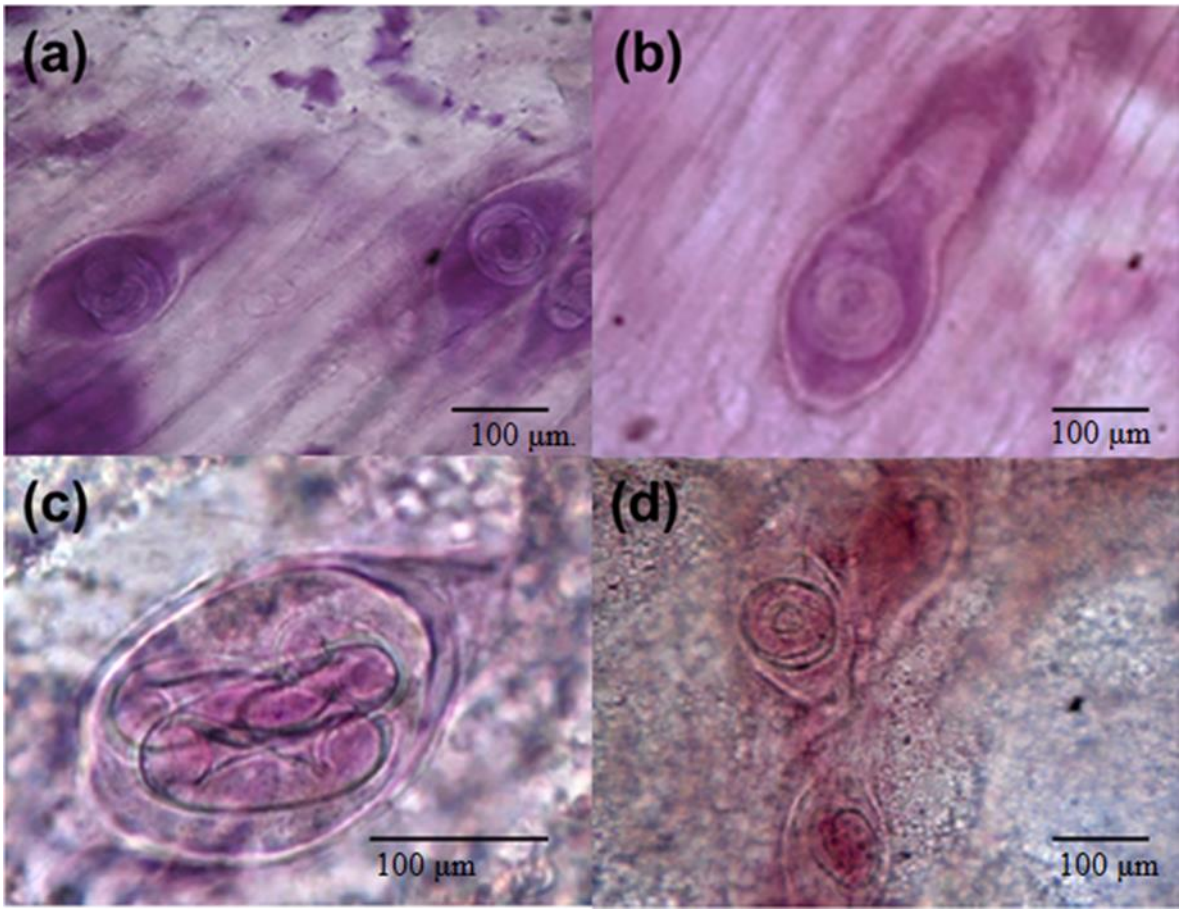


The graph shows the number of muscle larvae per gram of tissue (ML/g) obtained by artificial digestion (AD) or by trichinoscopy (T). A total of 3 samples, 10 g each one, was analyzed per anatomical region; the graph shows the average of 3 samples and their standard deviation.

Detection of larvae in pork ham and sausages using standard methods

Detection of the muscle larvae in ham and sausages was carried out by artificial digestion and trichinoscopy. However, standard trichinoscopy did not allow to clearly identifying the parasites due to interference of the fat and starch contained in the products. Twenty ham samples (Figure 2, panels "a" and "b"), and 20 sausage samples (Figure 2, panels "c" and "d") were stained with Meyer´s hemalum to enhance the sharpness of the larval observation. The muscle larvae and the nurse cell were observed as purple structures surrounded by non-infected cells, which were of pink coloration. The parasite load in ham was of 8.5 ± 3 ML/g (mean ± SD) while, in sausage, the parasite load was 4.5 ± 1.4 ML/g (P<0.0001; Student´s T-test, two-tails).

Figure 2: Representative micrographs of *Trichinella spiralis* encysted larvae

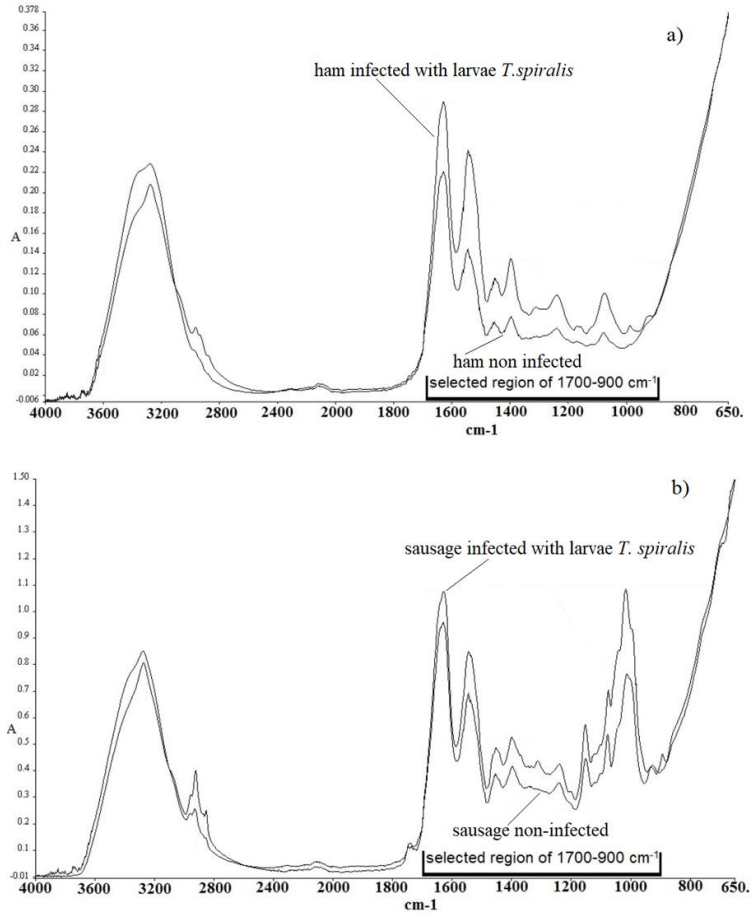


Trichinella spiralis encysted larvae stained with Mayer's hemalum in homemade ham (panels a and b, 100x magnification) and sausage (panels c and d, 400x and 100x magnification, respectively) prepared with meat from an experimentally infected pig.

Detection of larvae in pork ham and sausages using FTIR spectroscopy

Figure 3 shows the FTIR spectra of ham (panel a) and sausage (panel b), obtained with 30 samples of each pork product. Spectrum differences between infected and non-infected pork products were observed in the range of 1,700 to 900 cm^{-1}. Since each peak in the spectrum is related to functional chemical groups, all biological samples have a "fingerprint" spectrum related to their chemical composition. The soft independent modeling of class analogy (SIMCA) showed 100 % recognition rate and 100 % rejection rate. Spectra from non-infected ham or non-infected sausage were classified as normal pork product (100 % recognition) while, the spectra from infected ham or infected sausage were rejected (100 %) because the interclass distance between non-infected and infected pork products was of 10.2 for ham and 5.2 for sausage (Table 1).

Figure 3: Representative spectra obtained by MID-FTIR-ATR for the detection of *Trichinella spiralis* muscular larvae in homemade ham and sausage from an experimentally infected pork



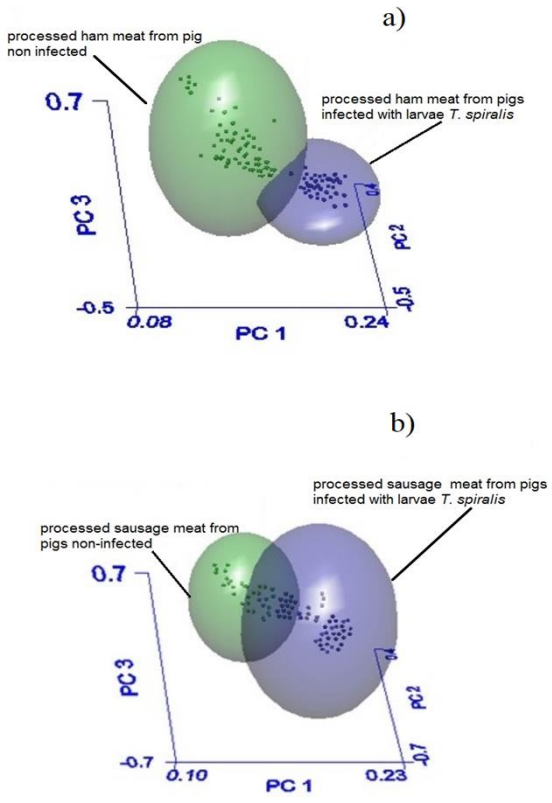
Panel (a) shows the spectra of infected and non-infected ham, while panel (b) shows the spectra of infected and non-infected sausage. Absorbance (A) is presented as a function of wavelength.

Table 1: Interclass distance and percentages of recognition and rejection rates (SIMCA model) between *T. spiralis* ham and sausage infected and non-infected pigs

Class	ID	Recognition rate (%)	Rejection rate (%)
Ham infected Ham non-infected	10.2	100 (30/30)	100 (30/30)
Sausage infected Sausage non-infected	5.52	100 (30/30)	100 (30/30)

Figure 4 shows the three-dimensional analysis of non- and infected pork products. Panel (a) shows the ham, and panel (b), the sausage. The three-dimensional image of non- and infected products seems to overlap because they share in common all the raw material for making the sausages, but they also have elements that are not shared (points within the figure), that is, components of *Trichinella* larva.

Figure 4: Three-dimensional component analysis score plots

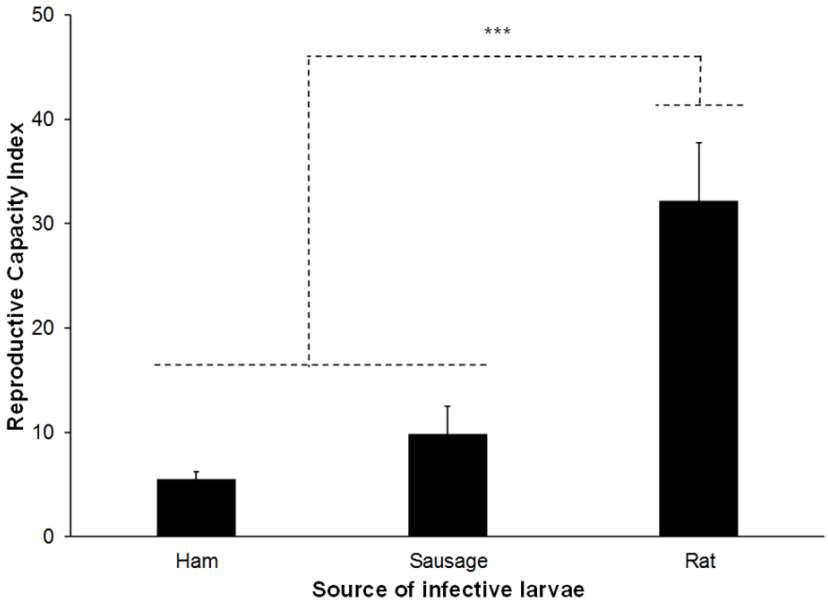


Score plots were generated by the optimized SIMCA models for the different of ham (panel a) and sausage (panel b) infected and non-infected with *Trichinella spiralis*

Reproductive capacity

Only 2 out of 10 (20 %) mice administered with larvae recovered from ham were found to be infected, while in the group given larvae recovered from sausage, 3 mice out of 10 (30 %) were infected. Figure 5 shows that the reproductive capacity index (RCI) of larvae recovered from ham was 5.5 times lower than that of larvae recovered from infected rats. The RCI of larvae recovered from sausage was 3 times lower than that of larvae recovered from infected rats.

Figure 5: Comparison of the reproductive capacity index of *Trichinella spiralis* larvae from pork products prepared with the meat of a pig experimentally infected



Differences among groups were evaluated with a 0.05 level of significance one-way ANOVA followed by Tukey's test for between-groups comparison (P<0.0001).

Discussion

Although sanitary inspection of pigs is mandatory in public slaughterhouses, many products are prepared from "backyards" pigs and then marketed without health inspection. The search for *Trichinella* in pork products is complicated because diverse parts of the pig are used to make sausages, and it is known that these parasites do not distribute homogeneously in the different anatomical regions of their host[2,5]; data obtained in this research confirm this

observation. Pork is one of the main sources of protein for the Mexican population; in fact, it is the second most-consumed meat in the country and the main ingredient in many culinary recipes. Coincidentally, the regions with the highest economic demand (leg, loin, rib, shank, among others) are those with the lowest parasite load. However, there is no anatomical part of the pig that is not either exploited for consumption as a main food or combined with other elements; sausages are a good example because are usually prepared with fat, viscera or blood.

Since scarce alternatives to search for parasites in pork products are available, here it was examined the utility of FTIR spectroscopy to detect *Trichinella spiralis* muscle larvae in ham and sausage of an experimentally infected pork. This technique had already been tested in *Trichinella* experimentally infected pigs[17], and no interference was observed with antigens of *Ascaris suum* or *Taenia solium*[16]; therefore, the next step was to identify the diagnostic utility of FTIR spectroscopy in pork products. The infrared spectroscopy has already applied successfully for quality control, adulterant detection, and origin denomination of wine, honeybees, olive oil, spirit drinks and beer, dairy products, fish, beef meat, and clenbuterol, among others[24]. Previous experiments have shown FTIR spectroscopy is capable of recognizing up to 3 larvae of *Trichinella* in 10 g of meat with a confidence limit of 99 %[16]. Analysis of data reported here suggests that *T. spiralis* larvae were successfully identified in infected pork products. The spectra of non-infected ham and sausage showed the characteristic bands of fatty acids, proteins, and glycogens already reported[25,26], and the spectra of infected pork products were similar to those previously described in meat obtained from experimentally infected pigs[17]. At this region, the carbohydrates, mainly glycogen, are the most abundant chemical group (1,200 and 900 cm^{-1}) and, as a point of interest, it is known that once *Trichinella* establishes in skeletal muscle, the larva transforms the muscle cell into a nurse cell, and from this location, continuously releases excretory and secretory products or ESP. These products contribute to establishing parasitism[27] and are important in the induction and modulation of the host immune response[28]. The ESP contains many functional proteins, which are glycoproteins, some of them bearing multi-antennary N-glycans caped with a monosaccharide named tyvelose[29]. The results obtained in this work show that the FTIR spectroscopy could be an additional alternative to the inspection procedures established for the meat trade for human consumption.

Previously, it has been shown that the SIMCA (soft independent modeling of class analogies) model is capable of differentiating between *Trichinella* and other worms such as *Ascaris* and *Taenia*[16]. All three worms are frequently found in pigs, and are transmissible to humans; however, only *Trichinella* is microscopic (1.2 mm), while the others can be seen with the naked eye, the adult *Ascaris* worm measures more than 15 cm and the *Taenia* larva measures at least 0.5 cm. Therefore, it is desirable to have an alternative diagnostic method to avoid the transmission of *Trichinella* between pigs and humans. It is also desirable that these methodologies can be applied in epidemiological studies to know the prevalence and

distribution of *Trichinella*. Although the product quantity to be analyzed for epidemiological surveillance purposes remains to be assessed, here there were obtained results with only 5 g for each pork product.

As far as was known, there are many different types of seasoning frequently used to preserving and improving the flavor of meat, but there are scarce data about their spectroscopy. Thus, further studies should be done to determine if any seasoning could have a spectrum similar to that of *Trichinella*, which could cause the report of "false-positive" samples. Such a study is significant since it has been previously shown that some seasonings do not influence the infectivity of *Trichinella*[10].

In contrast, the analysis of sausages by trichinoscopy and artificial digestion was complicated due to interference with fat and starch; some samples had to be stained to improve detection of the parasite. To the best of our knowledge, this is the first report in which Mayer´s hemalum staining is used as an aid in the identification and diagnosis of *T. spiralis*. It was took advantage of the contrast between the parasites and the non-infected myocyte to count the muscle larvae. The staining properties of the muscular larvae and the nurse cell have been widely described from hematoxylin-eosin stained histological sections[30] and Giemsa stained compressed muscle[31]. Although in this work, sections of ham and sausage were stained to demonstrate the presence of muscle larvae, it seems that the use of dyes could be an alternative to support the intentional search of the larva in meat samples, where the nature of the product masks or conceals the presence of parasites.

The results made evident that ham and sausage preparation limits the infective capacity of *Trichinella* larvae. Most probably, almost all larvae die during the preparation of pork products. Kotula *et al*[32] reported that the *Trichinella* muscle larvae are not infective in pork chops (2.54 cm thick) cooked to an internal temperature of 66 to 77 ºC in a conventional oven. This internal temperature was reached after 35 to 43 min of cooking. Nowadays, the International Commission on Trichinellosis recognizes cooking as one of the three acceptable means to inactive *Trichinella*[33]. Here, it was cooked the ham with water at 80 ± 1 ºC for 50 min and the sausage at 72 ± 1 ºC for 20 min. Difference in cooking time between ham and sausage consists in that meat of sausages are pre-cooked during 20 min (between 60 and 70 ºC); thus, meat does not lose consistency or plasticity and the sausage can be assembled appropriately. According to culinary recipes, to prepare the food, sausages must have additional final cooking (boiled or fried) for 10 to 30 min; the results show that some larvae remained alive. This can be explained considering that heat inside the ham and sausage was not consistently distributed or, in the case of the sausage, it must be due to the cooking time was short. The results of the infective capacity show that the RCI obtained with the larvae recovered from the ham was lower than that obtained with the sausages.

This data is important since there are several reports of human trichinellosis by ingestion of pork sausages[1, 34-37], turning sausages from meat without sanitary inspection into a probable source of *Trichinella* transmission. This information is also important because, in some countries, such as Mexico, the "salchicha" ranks first in the list of consumption of sausages in the country, followed by ham, “chorizo” and mortadella. In 2011, the consumption per capita in Mexico was of 7.8 kg and in 2017 was of 8.6 kg[38].

Conclusions and implications

Here, it was successfully used FTIR spectroscopy for the detection of *Trichinella spiralis* muscle larvae in pork products (ham and sausage) and discrimination of non-infected products from those infected with the parasite. The usefulness of this methodology could be extended to detect other etiological agents. Implementing the method for the routine inspection of pork products would reduce the time of sample analysis (5 min), in comparison to 10-30 min for trichinoscopy and 1 to 3.5 h for artificial digestion. Other advantages are the amount of sample needed for the analysis, the sample processing without previous treatments, the rapid obtaining of results. Besides, the methodology is an environmental-friendly process. However, limitations of this methodology are the cost of the equipment and the infrastructure necessary for its operation.

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Conflicts of interest

None of the authors have a conflict of interest with respect to this publication.

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