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ORIGINAL PAPER

Incidence of *Ascaris suum*-specific antibodies in Austrian patients with suspected larva migrans visceralis (VLM) syndrome

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Abstract The pig roundworm, *Ascaris suum*, is commonly found in domestic pigs all over the world. The transmission to humans takes place by ingestion of infective *A. suum* eggs present in soil because pig manure is widely used as fertilizer. The possible role of *A. suum* in the human visceral larva migrans (VLM) syndrome has been discussed controversially during past decades, even though various case reports, particularly from Japan document pulmonal, hepatic and even cerebral symptoms caused by migrating *A. suum* larvae after ingestion of infected row meat (liver) or contaminated vegetables. We examined 4481 sera by *A. suum* immunoblot (As-IB) and 5301 sera by *Toxocara*-ELISA from patients with symptoms associated with the VLM syndrome during three consecutive years (2012–2014). The incidence of *A. suum*-specific antibodies was 13.2 %, the incidence of *T. canis* specific antibodies 12.9 % and from a part of the As-IB positive sera (n= 417) additional *Toxocara* serology was performed to demonstrate the specificity of our tests. Only 56 out of the 417 (13.4 %) sera showed antibodies to both helminth species demonstrating that double infections exist. Interestingly the age distribution of the patients showed that 2.8 % of the *Ascaris*-positive patients were younger than 21 years, while in the *Toxocara*-positive group 13.4 % were <21 years. These results are in accordance with a Dutch study suspecting different ways of transmission as cause for this interesting age distribution. Due to the fact that large amounts of untreated pig manure are used as fertilizer and that the expulsion of adult *A. suum* worms causing intestinal ascariosis is extremely rare in Central European countries, the zoonotic potential of *A. suum* is considerably underestimated. We suggest that the performance of reliable immunoserological tests, in all industrialized countries where pigs are raised and their manure is used as fertilizer, could help to assess the actual potential of *A. suum* as causative agent of the VLM syndrome in humans.

Keywords *Ascaris suum* · *Toxocara sp* · Immunoblot · Visceral larva migrans syndrome

Introduction

The pig roundworm, *Ascaris suum* is commonly found in domestic pigs all over the world, and considered to be the most prevalent helminth species in pigs in industrialized settings (Vlaminck et al. 2014). Because pig manure is widely used as fertilizer and the eggs of *A. suum* are extremely resistant to unfavorable environmental conditions, the transmission of the parasite to humans takes place by ingestion of infective *A. suum* eggs present in soil contaminated with pig feces (Roepstorff and Murrell 1997; Katakam et al. 2014; Pinelli et al. 2011). Even though the first case reports date from the 1970s, the possible role of *A. suum* in the human visceral larva migrans (VLM) syndrome was discussed controversially for decades (Petithory 1996; Maruyama et al. 1996). Phills et al. (1972) reported on four male students intentionally exposed to massive doses of *A. suum* ova during Carnival 1970, presenting themselves to an emergency department between 10 to 14 days later. All four showed pulmonary infiltrates, eosinophilia and elevated IgE immunoglobulin, but only the two less severely ill of them expelled a few worms in their stool and evolved minimal antibody responses while the

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others showed higher IgE levels, expelled no worms and were cyanotic and in acute respiratory failure on admission (Phills et al. 1972).

Two decades later Frans van Knapen (van Knapen et al. 1992) recommended considering other nematode larvae than the established dog roundworm *Toxocara canis* as causative agent of the VLM syndrome. Additionally various case reports, particularly from Japan document pulmonal, hepatic, and even cerebral symptoms caused by migrating *A. suum* larvae after ingestion of infected row meat (liver) or contaminated vegetables (Maruyama et al. 1996; Inatomi et al. 1999; Osoegawa et al. 2001; Sakakibara et al. 2002; Izumikawa et al. 2011). Finally, serological screening for specific *A. suum* antibodies carried out in the Netherlands, one of the largest pig producers in Europe, yielded a remarkable high seroprevalence in the "normal" population (Pinelli et al. 2011).

Since specific immunodiagnosis in humans is a prerequisite to learn more about the *A. suum*-induced VLM syndrome, we developed an immunoblot assay (As-IB) applicable in the routine laboratory (Schneider et al. 2015). Our As-IB uses larval secreted products (E/S) as antigen and shows a ninety-five percent specificity, demonstrating that cross-reactions with other helminths are rare.

The aim of our present study was to investigate the incidence of *A. suum*-specific antibodies in Austrian patients with symptoms associated with the VLM syndrome and to compare it with the incidence of *Toxocara sp*. antibodies in patients with suspected VLM syndrome caused by *T. canis* during three consecutive years (2012–2014). We also compared sex, mean age, and age distribution of patients tested positive for either of the two helminth species. Furthermore, we calculated the number of patients showing antibodies to both helminths to demonstrate the specificity of our tests with a larger number of sera than in the previous study (Schneider et al. 2015). The results of this study should help to assess the potential extent of *A. suum* as a causative agent of VLM and the role of pigs and their manure as the main source of human *Ascaris* infections in Austria.

Material and methods

Serum samples

The Institute of Specific Prophylaxis and Tropical Medicine of the Medical University Vienna is Austria's Reference Laboratory for Parasitoses and the only institution in Austria that carries out serological screening for specific *A. suum* antibodies. A total of 4481 serum samples from patients with suspected VLM syndrome caused by *A. suum* were sent to our institute from 2012 to 2014 (group 1) and we performed the recently established As-IB on these sera (Table 1). In the same period, a total of 5301 sera from patients with suspected VLM syndrome caused by *T. canis* (group 2) were sent to our institute and we performed *Toxocara*-ELISA and *Toxocara* immunoblot (Tc-IB) on the Tc-ELISA positive sera (Table 2). The third group was included to demonstrate the specificity of our As-IB and consisted of 417 out of 590 sera from As-IB positive patients (group 1) with additional request for *Toxocara* serology (group 3) (Table 3).

Table 1 Number of sera tested for *Ascaris suum*-(group 1) specific IgG antibodies between January 2012 and December 2014

Year	Samples (*n*)	Positive (*n*)	Positive (%)
Ascaris			
2012	1113	111	9.97
2013	1393	136	9.76
2014	1975	343	17.76
2012–2014	4481	590	13.17

Parasitological–serological tests

Detection of anti-Ascaris suum IgG antibodies (As-IB)

As-IB was performed as previously reported (Schneider et al. 2015). Briefly, excretory–secretory (E/S) antigen derived from *A. suum* larvae was prepared using a modified procedure originally described by Savigny (1975) for the preparation of E/S antigens from *T. canis* larvae (TES). Electrophoresis was carried out on an 8–18 % gradient gel and (GE Healthcare) and the transfer membrane was Immobilone-P (Millipore). After activation of the Immobilon-P membrane it was cut into vertical strips and blocked with 5 % bovine serum albumin in phosphate buffered saline (PBS) for 30 min at 37 °C. All following steps were performed at 26–28 °C in an incubator shaker. The next step was three times washing with PBS for 3–5 min. The 1:750 dilutions of patients' sera in 0.1 % normal goat serum (Thermo Scientific) in PBS were incubated for 2 h followed by three washing steps. Colloidal gold-labeled anti-human IgG (EY Laboratories) with 20 nm particle size was the secondary antibody and after two hours incubation another two washing steps with PBS and short rinsing with distilled water followed; the reaction was enhanced by coprecipitation with silver (Silver Enhancing Kit, BBI international). The silver enhancing was performed in the dark for 30–40 min and stopped with distilled water. Positive serum samples showed the typical banding pattern with bands at 55, 75, 95, 115, and additional bands at approximately 130 kDa.

Detection of anti-Toxocara IgG antibodies (TES-ELISA and TES-IB)

For the diagnosis of VLM caused by *T. canis* we performed an ELISA based on the usage of excretory/secretory (ES)

Table 2 Number of sera tested for *Toxocara* sp. specific IgG antibodies (group 2) between January 2012 and December 2014 *Toxocara*

Year	Samples (*n*)	Positive (*n*)	Positive (%)
2012	1484	190	12.60
2013	1723	205	11.90
2014	2094	286	13.75
2012–2014	5301	681	12.88

antigens from *T. canis* larvae (TES) first described by de Savigny et al. (1979) and a TES- immunoblot (TES-IB) established by Magnaval et al. (1991). Serum samples were considered serologically positive if they showed clearly positive reactions in the ELISA and displayed the typical reactivity pattern in the TES-IB (Magnaval et al. 1991). The quality of our *Toxocara* serology has been periodically evaluated since 2005 by an external quality control assessment organized by the UK NEQUAS Microbiology and showed 100 % correct results so far. Unfortunately, no such quality assessment is available for *A. suum* serology.

Sex and age distribution

Sex, age, and results of the As-IB and TES-IB were logged into an Excel spreadsheet and analyzed to compare the incidences of antibodies to *A. suum*, to *T. canis* and to both helminths in Austrian patients with suspected VLM syndrome (Fig. 2). For statistical analysis of the seropositive patients' mean age we performed student's *t* test.

Results

Results of parasitological–serological tests

During the years 2012 to 2014, 590 (13.2 %) of the sera tested for *Ascaris* (*n*=4,481; group 1) and 681 (12.9 %) of the sera tested for *Toxocara* (*n*=5,301; group 2) antibodies reacted positive in the homologous test (Tables 1, 2). From 417 out of 590 As-IB positive patients *Toxocara* serology was requested as well and 56 (13.4 %) of these patients (group 3) showed positive results in the TES-ELISA and TES-IB as well (Table 3).

Table 3 Number of those *Ascaris*-positive sera from patients which were additionally examined for *Toxocara* antibodies (group 3)

Year	Samples (*n*)	Positive (*n*)	Positive (%)
2012	80	8	10.00
2013	104	10	9.51
2014	233	38	16.30
2012–2014	417	56	13.43

Age and sex distribution of *Ascaris* and *Toxocara* seropositive patients

In group 1, the mean age of the As-IB positive patients (*n*=590) was 53 (5–89) years and the gender distribution was 325 (55 %) females and 265 (45 %) males. In group 2, the mean age of the 681 *Toxocara* seropositive patients from the same period was 49 (2–90) years and 365 (54 %) were females and 316 (46 %) males. Statistical analysis (student's *t* test) showed that the patients mean age was not significantly different in these two groups ($p>0.05$).

The age distribution of the patients whose serum samples were sent to our institute with a request for the detection of specific antibodies to *A. suum* (*n*=4.481; group 1) or *T. canis* (*n*=5.301; group 2), respectively, is demonstrated in Fig. 1, showing that for both helminths, the majority of patients was between 40 and 70 years. The age distribution of the seropositive patients of group 1 and group 2 of each age group is demonstrated in Fig. 2 and shows that the majority of the *Ascaris*-positive patients (group 1) belonged to the age group 40 to 70 years, and that only 12 (2.8 %) were <21 years age. In the *Toxocara* seropositive patients (group 2) the age distribution was completely different, 78 (13.4 %) of the patients were younger than 21 years, the age group 21–30 years showed a slightly lower seropositivity (7.5 %), thereafter the percentage increased with age reaching its maximum with 22.7 % in the age group of 61–70 years (Fig. 2). None of the seropositive patients were older than 90 years, hence we terminated the age groups with 90 years although some seronegative patients were >90 years. Unfortunately the number of patients in the younger age groups was too small to gain statistical significant different mean ages e.g., for the age group 0–20 years. On the other hand, we assessed a highly significant ($p<0.00001$) different mean age of 43 and 39 years in the group of *Ascaris*-positive (*n*=431 in group 1) and the *Toxocara*-positive patients (*n*=454 in group 2), respectively, in patients younger than 61 years.

Discussion

Specific immunodiagnosis and detailed etiological inquiry is the only practical way to diagnose VLM patients because migrating larvae are very small and it is extremely unlikely to detect them in biopsies (Maruyama et al. 1997). The quality of the immunodiagnostic test for the detection of *A. suum*-specific antibodies is extremely demanding because somatic antigens of nematodes generally show high levels of cross-reactivity and are therefore not reliable tools (Pinelli et al. 2011; Fan and Su 2004). In our opinion, an immunoblot assay

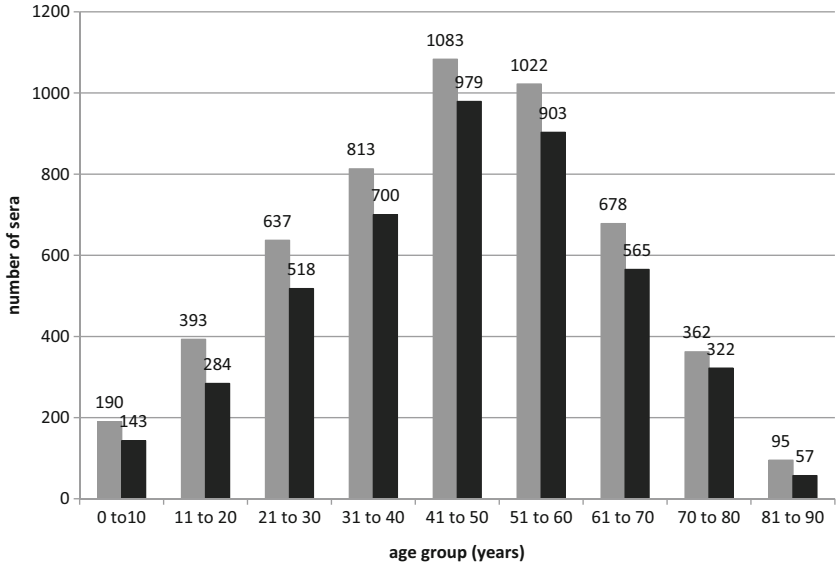

Fig. 1 Number of sera and age distribution of patients with request for *Ascaris* (*black bars*) or *Toxocara* (*gray bars*) serology between January 2012 and December 2014

(As-IB) based on larval secreted products (E/S) as antigen is the best choice for specific immunodiagnosis of the VLM syndrome caused by *A. suum* and was published recently (Schneider et al. 2015).

The aim of the present study was to investigate the incidence of *Ascaris* and *Toxocara* seropositive patients with symptoms associated with the VLM syndrome during three consecutive years (2012 to 2014). Our results demonstrate that the proportion of *Ascaris* seropositive patients (group 1) was slightly higher (13.2 %) than the percentage of *Toxocara* seropositive patients (12.9 %, group 2); (Tables 1 and 2). Group 3 (part of group 1) was introduced to demonstrate the potential of the performed serological tests to differentiate between the two helminth species. The fact that only 56 (13.4 %) out of the 417 As-IB positive sera (Table 3) showed antibodies against *T. canis* confirms as well the high specificity of the applied serological tests on one hand and is in accordance with other publications stating that double infection with *Ascaris* and *Toxocara* exists on the other hand (van Knapen et al. 1992, Pinelli et al. 2011).

Our first main topic to discuss was the comparison of the *Ascaris* and *Toxocara* incidences of the present study with data from similar studies. *A. suum* seroprevalence studies are rare, but a Dutch study applying an ES antigen based ELISA, (carried out between 1998–2009) reported that the percentage of *Ascaris* seropositive patients varied from 24.5 to 47 %, while the percentage of *Toxocara*-positive patients decreased from 17.7 to 8 % in the same period (Pinelli et al. 2011). The 12.8 % *Toxocara* seroprevalence of our patients with

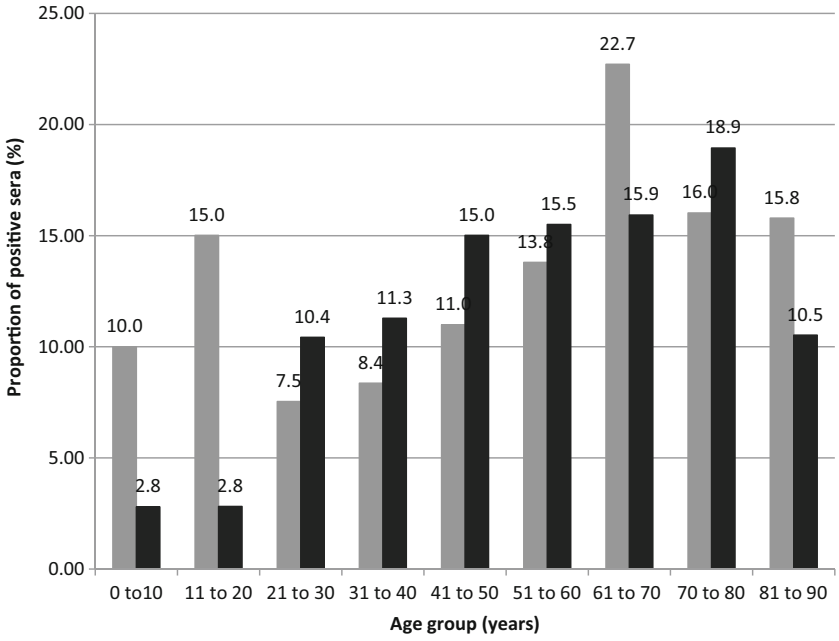

Fig. 2 Proportion of *Ascaris* (*black bars*) and *Toxocara* (*gray bars*) seropositive patients within different age groups, examined between January 2002 and December 2014

suspected VLM syndrome is in accordance with previous studies from Austria and with the above mentioned study from The Netherlands (Deutz et al. 2005; Pinelli et al. 2011). but with 13.2 % *Ascaris* seropositive patients we have a lower incidence in Austria than in The Netherlands. There are several possible reasons for this difference between Austria and The Netherlands. In the first place—in contrast to The Netherlands—Austria does not belong to Europe's biggest pig producers and probably the amount of pig manure contaminated with infective *A. suum* eggs utilized as fertilizer is smaller. Other possible explanations are that although we received sera from all over Austria, the majority was from an urban population with a presumable lower risk of infection; that the knowledge about *A. suum* as cause of the VLM syndrome is still very limited in Austrian physicians; and finally that only ELISA with no confirmation by immunoblot was performed in the Dutch study bearing the risk of overlooking cross-reactions with other helminths. We suppose that the main cause of the different incidences between the two European countries, are not the test systems but rather differences in environmental contamination with the very resistant *A. suum* eggs.

The longevity of *A. suum* eggs is topic of numerous studies dealing with transmission of *A. suum* eggs in indoor and outdoor pig rearing systems (Katakam et al. 2013; Roepstorff et al. 2011). The mean prevalence of *A. suum* is 21.5 % in fatteners in northern European countries (Nejsum et al. 2012; Roepstorff et al. 1998). Unfortunately no information about Austrian pig farms is available. Several studies investigated the survival of *A. suum* eggs in pig slurry demonstrating that even after 10 months of storage at 5 °C, viable eggs could be detected in a concentration of more than 40 eggs per gram of liquid slurry (Katakam et al. 2013). This extreme resistance of *A. suum* eggs explains infection of the next generation of pigs on one hand and implies its zoonotic potential because pig slurry is widely used as fertilizer in Austria, the Netherlands and other industrialized nations.

The second topic to discuss was the age and the age distribution of our patients showing *Ascaris* or *Toxocara* seropositivity, respectively (Fig. 2). The mean age of *Toxocara*-positive patients (49 years) is only 4 years less than that of *Ascaris*-positive patients (53 years) and not significantly different ($p>0.05$). Although we have a different age distribution (Fig. 1) in our serum samples with demand for detection of specific antibodies to *A. suum* or *T. canis* (relatively few children), and a generally lower percentage of *Ascaris* seropositive patients than in the Dutch study, the age distribution of our seropositive patients is comparable for both helminthes (Pinelli et al. 2011). Figure 2 demonstrates that the majority of *Ascaris*-positive patients belongs to the age group 41 to 70 years while in *Toxocara* seropositive patients 13.4 % ($n=78$) are younger than 21 years, the age group 21–30 shows a slightly lower seropositivity (7.5 %), thereafter, the percentage increases with age reaching its maximum with 22.7 % for the age group of 61–70 years (Fig. 2). The similar age distribution pattern of *Ascaris* and *Toxocara* seropositive patients from Austria and from The Netherlands is a very interesting observation and we agree with the authors of the Dutch study suspecting different ways of transmission as cause for this interesting age distribution (Pinelli et al. 2011). Pinelli supposes that *Ascaris* infection is food associated while *Toxocara* infection is more likely transmitted through bad hand washing hygiene and pica, frequently seen in children (Pinelli et al. 2011). If infection with *A. suum* is food associated it seems likely that it happens in every age group and that reinfections might occur periodically and lifelong. Another interesting aspect is that genetic factors might influence the susceptibility of different persons to *A. suum* infections, comparable to findings for *Ascaris lumbricoides* in humans from endemic regions and *A. suum* in pigs (Dold and Holland 2011). In general, the susceptibility to *Ascaris* both in humans and pigs is poorly understood until now.

The third topic was the unresolved taxonomic question about the validity of the present classification of *A. suum* and *A. lumbricoides* because human and pig *Ascaris* worms are morphologically indistinguishable and show only 3–4 % nucleotide differences in the mitochondrial genome (Anderson et al. 1993). Several studies support the recent proposal that *A. suum* and *A. lumbricoides* represent host-associated subpopulations of the same species (Nejsum et al. 2005; Leles et al. 2012, Shao et al. 2014). Although both have a strong affinity to their natural hosts, few studies report that *A. suum* can develop to the adult stage in the human host (Phills et al. 1972; Anderson 1995; Nejsum et al. 2005). The performed As-IB is based on *Ascaris* worms collected from pigs and intended to improve the diagnosis of VLM in humans but cannot (and will not) answer the taxonomic questions about the validity of the present classification into *A. suum* and *A. lumbricoides*. Intestinal *Ascaris* infections are extremely rare in Austria; in the period 2012– 2014, we registered 13 patients with adult *Ascaris* worms and two patients with *Ascaris* eggs out of 19,700 (0.00076 %) stool samples. In general, no sera from these patients were provided but we included all available sera ($n=3$) from patients with intestinal ascariosis in a previous study to investigate the potential cross-reactivity between pig and human *Ascaris* (Schneider et al. 2015). The fact that two out of three patients infected by presumably *A. lumbricoides* showed no reaction and only one reacted positive in our As-IB allows several conclusions. In our opinion it points to the fact that intestinal *Ascaris* infestations do not necessarily lead to the production of specific antibodies and that our As-IB presumably does not differentiate between *A. suum* and *A. lumbricoides*. Since we do not perform molecular biological investigation of worms, we cannot rule out that one of these few worms is in fact *A. suum;* but the number of adult worms is negligible compared to the 13, 2

% patients with symptoms associated with the VLM syndrome showing *A. suum*-specific antibodies. A recent study comprising 536 adult *Ascaris* worms from human and pig hosts from different geographical settings identified that worms collected from humans in Europe (in contrast to those from humans from Asia or Africa) are genetically clustering to those collected from swine (Betson et al. 2014). But the expulsion of adult worms is a poor indicator for the actual *A. suum* exposure in developed countries and analyzing only adult worms, the zoonotic potential of *A. suum* might be underestimated (Nejsum et al. 2012; Betson et al. 2014). We agree with the authors stating that probably different host preferences might lead to visceral larval migration instead of intestinal ascariosis causing greater morbidity as in hosts that are well-adapted to the parasite (Nejsum et al. 2005; Betson et al. 2014). A study from the UK reported on a focus of locally acquired *A. suum* infection in humans in Cornwall, especially in children <5 years of age (Bendall et al. 2011). Molecular biological investigation of 11 *Ascaris sp*. worms expelled by humans yielded pigs as source of infection and modifications of animal husbandry and fecal waste disposal were recommended (Bendall et al. 2011).

Unfortunately, none of the above mentioned studies, correlating human ascariosis in industrialized countries with pig manure, comprised specific serological testing for antibodies against *A. suum*. Serological screening and detailed etiological inquiry of patients and their family members would be very interesting to learn more about *A. suum* as causative agent of the VLM syndrome or human (visceral) ascariosis, respectively, in non-endemic areas for *A. lumbricoides* with high hygienic standards.

Presumably it is not a mere coincidence that the majority of publications about *A. suum* as a causative agent of the VLM syndrome originate from The Netherlands and Japan, respectively. The Netherlands are one of Europe's biggest pig producers and vegetables as well; hence, the prerequisites for human infection are quite obviously complied. In Japan, pig manure is used as fertilizer as well, and additionally the habit of consuming raw meat (including raw liver) was described in some severely ill patients (Kim et al. 2002; Izumikawa et al. 2011). But the risk for human infection exists as well in other industrialized nations where pigs are raised and their manure is used as fertilizer. Anthelmintic treatment of herds alone bears the risk that anthelmintic resistance will develop and for organic farms routine preventive treatment is no option (Roepstorff et al. 2011). Pretreatment of pig manure used as fertilizer has to be discussed to interrupt the route of transmission of viable *A. suum* eggs into the food chain (Roepstorff et al. 2011). We suggest that the problems in the immunodiagnosis and the lack of reliable commercially available tests are the main reasons for the limited knowledge and acceptance of *A. suum* as causative agent of the VLM syndrome in humans.

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