Advances in the diagnosis of *Ascaris suum* infections in pigs and their possible applications in humans

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SUMMARY

Ascariasis is one of the most common parasitic diseases in both humans and pigs. It has been shown to cause growth deficits in both species and to impair cognitive development in children. Notwithstanding its substantial impact on pig economy and public health, diagnosis of ascariasis has mostly relied on the detection of eggs in stool and further development of novel, more sensitive methods has been limited or non-existent. Here, we discuss the currently available techniques for the diagnosis of ascariasis in pigs, their caveats, and the implications of a new serological detection technique for the evaluation of both pig and human ascariasis.

Key words: *Ascaris suum*, *Ascaris lumbricoides*, ascariasis, diagnosis, serology, ELISA.

INTRODUCTION

In industrialized settings, *Ascaris suum* is the most important and prevalent helminth species in pigs. Although the majority of infections with *A. suum* are sub-clinical, the impact of ascariasis on pig growth and productivity can be substantial (reviewed by Thamsborg *et al*. 2013). In both experimental and field studies, the decreased health status of pigs, as a consequence of roundworm infection, is reflected by an average lower daily weight gain, feed conversion efficiency and meat quality (Hale *et al*. 1985; Stewart and Hale, 1988; Bernardo *et al*. 1990*a*; Kanora, 2009; Kipper *et al*. 2011; Knecht *et al*. 2012).

Good diagnostic tools are necessary to assess not only the presence but also the intensity of *A. suum* infections on a farm. This could then give an indication of the economic impact of the disease. Results of these diagnostic tests can also be employed to evaluate the effect of changing management practices such as anthelmintic treatments and alterations in pig housing on parasite epidemiology. However, today, the lack of proper diagnostic tools to identify farms with *Ascaris* problems in combination with the sub-clinical nature of the disease have created a lack of awareness towards this problem in farmers as well as veterinarians. Another important and often overlooked fact is that not only the presence of adult worms but also larval migration has a significant health impact (Stewart *et al*. 1984; Hale *et al*. 1985). However, with current diagnostic tools it not possible to correctly measure the intensity of larval exposure.

A large proportion of farmers seem to believe that the magnitude of worm infections on their own farm is insignificant, even though nearly all of them use anthelmintics to treat their stock (Dangolla *et al*. 1996; Wagner and Polley, 1997*a*; Theodoropoulos *et al*. 2001; Beloeil *et al*. 2003; Weng *et al*. 2005). Very rarely, pig farmers use the available diagnostic tools to investigate whether the actions they undertake to control ascariasis on their farm are actually effective.

The objective of the current review is to (1) discuss the current diagnostic tools used to detect the presence of *A. suum* in pigs, (2) to report a newly developed serological technique for the detection of *A. suum* infections in pig herds and (3) to consider the possible application of this technique in the field of human helminthology.

DIAGNOSTIC TOOLS: FROM WORMS TO SEROLOGY

Various methods are available to prove the presence of roundworm infections on a farm. First, post-mortem findings registered at the slaughterhouse can report the presence of adult worms in the small intestine or increased numbers of affected livers and lungs. Second, the analysis of pig stool to show the presence of parasite eggs is also often used. Finally, a newly developed antibody ELISA-test has now opened the door for more sensitive detection of roundworm exposure in fattening pigs.

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Presence of worms

At the slaughterhouse, it is possible to register the number of pig intestines harbouring adult worms. However, this is not a routine practice, and absence of worms does not guarantee absence of ascariasis on the farm. This is because intestinal larval stages are difficult to detect with the naked eye and pigs may have received anthelmintic treatment a few weeks prior to slaughter.

Lung lesions

Increased numbers of lung lesions have been associated with the presence of roundworm infections on farms in the past (Flesja and Ulvesaeter, 1980; Nakagawa *et al*. 1983; Bernardo *et al*. 1990*a*). Elevated percentages of lungs that show signs of pneumonia or pleuritis could therefore be an extra indicator of *A*. *suum* infections on a farm.

Liver lesions

Currently the most applied method to measure roundworm exposure post-mortem is to look at the degree of white spot formation on swine livers. The development of so-called 'white spots' or 'milk spots' is an immunological response of the host following larval migration through the liver tissue. These white spots are characterized by interlobular depositions of fibrous tissue and cellular infiltrates and are typical of *Ascaris* infections (Nakagawa *et al*. 1983; Perez *et al*. 2001). This technique is therefore used in many studies that wish to report the *A*. *suum* prevalence. Recent results published from Sweden, England and New Zealand, checking 2·4, 0·8 and 6·2 million pigs, respectively indicated that 5, 4·2 and 9·2% of pigs had livers showing white spots (Lundenheim and Holmgren, 2010; Sanchez-Vazquez *et al*. 2012; Neumann *et al*. 2013).

Usually, the number of pigs showing liver lesions far exceeds the number of pigs in which adult worms can be recovered from the intestine (Bernardo *et al*. 1990*c*). This is the consequence of natural immune responses of the pigs causing the expulsion of more than 90% of the *A*. *suum* immature stages from the intestine before they have the chance to develop into adult worms (Roepstorff *et al*. 1997; Helwigh and Nansen, 1999; Masure *et al*. 2013*b*). This process takes place approximately 17 days post-infection. As a consequence, many pigs will never harbour adult worms even though they are continuously reinfected while others who were less successful in clearing the larvae will harbour numerous adults. This will eventually result in the strong aggregation of *Ascaris* populations within the pig population (Polley and Mostert, 1980; Roepstorff *et al*. 1997; Boes *et al*. 1998). This, again, highlights the importance of a diagnostic tool that can also detect larval exposure. Nonetheless, when milk spots are present, it is highly likely that pigs have undergone recent *A*. *suum* infections (Wagner and Polley, 1997*b*).

On the other hand, an absence of white spots does not necessarily mean that pigs were not infected before. In pigs undergoing continuous exposure to migrating larvae, the number of white spots on the liver will increase until 6–9 weeks after start of exposure, after which there is a gradual decline towards lower levels (Eriksen *et al*. 1992). In continuously exposed pigs, a natural immunity at the level of the intestine builds up which is called the pre-hepatic barrier (Eriksen, 1982; Urban *et al*. 1988; Masure *et al*. 2013*a*). This immunological response at the level of the gut prevents larvae from successfully starting their migration through the body, thereby preventing the formation of liver white spots. Whether the build-up of the pre-hepatic barrier is already completed in fattening pigs is doubtful but probably depends on the infection intensity to which they had been exposed during fattening. Additionally, it has also been shown that white spots start to resolve after about 2–3 weeks post infection (Eriksen *et al*. 1980; Roepstorff *et al*. 1997). Hence, the number of liver white spots is a poor indicator of long-term *A*. *suum* exposure as it only reflects recent larval migration. Livers might therefore look normal or only mildly affected at slaughter even though pigs have been exposed to significant numbers of infective larvae during the course of their life.

Furthermore, the visual assessment of livers is rather subjective. The decision on whether or not an abnormality on the liver is considered a true white spot depends on the perception of the person doing the assessment. This combined with the high speed of slaughter makes it easy to miss certain livers that show only a few white spots. This supports the variability in the white spot counts and increases doubt on the reliability and uniformity of the data on liver lesions at slaughter. Even though recent reports show a low percentage of total affected livers (approximately 5–10%) (Lundenheim and Holmgren, 2010; Sanchez-Vazquez *et al*. 2012; Neumann *et al*. 2013), it is however highly likely that a much higher percentage of pigs will have suffered from ascariasis.

Faecal egg counts

For the detection of *A*. *suum* in pigs, it is also possible to perform faecal egg counts and determine the number of eggs per gram of stool (EPG) (Roepstorff, 1998). The coprological tests are easy to perform and do not require expensive equipment. However, they are time-consuming and labour-intensive, thus not the ideal tool for the screening of large sample sets. Additionally, the interpretation of the results is not always as straightforward as could be expected since false-negative results are very common.

False negative results are possible when only immature worms are present or when only worms

of a single sex are present. It has been shown that as much as 23% of pigs that harboured worms in their intestine did not excrete any eggs (Boes *et al*. 1997). Furthermore, due to the strong aggregation of the adult *Ascaris* worms in pigs, only a minority of pigs in the population are expected to have worm eggs in their faeces. As a consequence, a substantial number of animals need to be screened in order to reduce the chance of falsely classifying a farm as *Ascaris* negative.

False-positive faecal samples are also often detected. This is usually the result of coprophagia and/or geophagia and their prevalence and magnitude depends on different management and housing factors (Boes *et al*. 1997). The number and range of false-positive *A. suum* egg counts in pigs can be considerable, but in general, EPG levels lower than 200 should be considered false-positives (Boes *et al*. 1997). The detection of false positive samples is not important since the diagnosis of ascariasis is on a farm level. Knowing which individual animal has adult worms or not has no implications for the treatment strategies since anthelmintic treatment is always applied to all pigs from that same herd.

In continuously exposed pigs, the quantities of eggs that are shed seem to be correlated with the number of adult worms in the intestine (Bernardo *et al*. 1990*b*; Nejsum *et al*. 2009*a*). However, regardless of the dose regimen, the numbers of worms that end up in the small intestine are generally inconsistent and independent of the intake of infective stages (Eriksen *et al*. 1992). Furthermore, there seems to exist an inverse relationship between the number of adult worms found in the intestine and the amount of eggs given during a single experimental infection dose (Andersen *et al*. 1973; Roepstorff *et al*. 1997). Consequentially, the number of adult worms, and therefore the EPG, are not representative of the amount of migrating larvae the pig has been exposed to. Nor does it reflect their possible attribution to production losses.

Generally, the numbers of infected pigs identified by coprological investigation represent an underestimation of the true parasite prevalence or infection intensity on a farm (Vlaminck *et al*. 2012) and prevalence studies using coprological data should therefore be interpreted with the necessary caution.

Serology

Another, more convenient and well-established way to screen for certain pathogens in the pig industry is the use of serological tests. Many ELISA tests are available for the detection of the most common porcine ailments (e.g. Salmonellosis, *Mycoplasma hyopneumoniae* infection, PRRS virus, porcine circovirus, swine influenza virus).

Because of the natural immune responses active in most pigs which expel the majority of larvae after they accomplish their hepatotracheal migration in the gut (Roepstorff *et al*. 1997; Helwigh and Nansen, 1999), there usually is no correlation between the adult worm load and antibody levels against parasite antigens in naturally or trickle-infected pigs (Roepstorff and Murrell, 1997; Nejsum *et al*. 2009*b*). As a result, ELISA values actually reflect both the number of adult parasites that reside in the pig's intestine as well as the degree of larval exposure. In theory, the use of a serological method could overcome the difficulties associated with the traditional methods of roundworm diagnosis in pigs (i.e. examination of livers or stool samples) and be more specific for the detection of *A. suum* infections.

The possible application of ELISA tests for the diagnosis of *A. suum* infections in pigs has been investigated in the past. Both adult and larval extracts or excretory/secretory products and some purified adult proteins have been evaluated (Urban and Romanowski, 1985; Lind *et al*. 1993; Yoshihara *et al*. 1993; Bogh *et al*. 1994; Roepstorff, 1998; Frontera *et al*. 2003). Although most of these tests were shown to be effective in diagnosing *A. suum* infection, no apparent steps were taken for future practical application of the developed ELISAs.

More recently, a vaccination experiment using the purified *A. suum* haemoglobin antigen (AsHb) revealed its possible use as a diagnostic antigen for the detection of *A. suum* infected pigs (Vlaminck *et al*. 2011). Further investigation and evaluation of AsHb as a diagnostic antigen showed a high diagnostic sensitivity and specificity (99·5 and 100% respectively) in experimentally infected pigs (Vlaminck *et al*. 2012). The ELISA test could detect total IgG antibodies produced against AsHb from 6–8 weeks post-infection onwards. When evaluated in the field, this AsHb-based ELISA, currently marketed in Europe under the name SERASCA®, showed superior sensitivity for the detection of *A. suum* infections in comparison to stool examination and percentages of condemned livers. The excretion of parasite eggs generally occurred in the oldest fattening pigs, with a maximum of 30% of pigs sampled secreting eggs whereas up to 90–100% of the pigs from the same age group were seropositive (Vlaminck *et al*. 2012). In a more recent investigation, serological analysis and the number of affected livers per batch of slaughtered pigs from different farms is compared. Although a positive correlation between both parameters is seen, on the majority of farms less than 10% of the pigs had affected livers even though most of these farms had over 50% of pigs testing seropositive (results not shown). These data further underpin the assumption that the use of the percentage of affected livers for the diagnosis of ascariasis can result in a significant underestimation of *A. suum* infection levels in pigs.

More interestingly, initial investigations on several commercial farms also showed an association between serology and different economic parameters (e.g. growth rate, days to market, etc.), suggesting that in the future this serological test could be used as a tool to estimate the economic losses caused by *A. suum* on fattening farms. A similar approach is currently already applied in the dairy industry where ELISA tests on bulk milk tank samples are used to estimate the potential production losses due to the presence of the parasites *Ostertagia ostertagi* and *Fasciola hepatica* (Charlier *et al*. 2012, 2014). Although it has already been shown that *A. suum* infections can reduce farm productivity (Thamsborg *et al*. 2013), many other farm-specific factors will also have a significant influence. Therefore, in order to gain more insight as to what extent infections with *A. suum* affect economic parameters on a fattening farm, further studies need to be performed in which *Ascaris*-positive farms are followed-up for several fattening rounds while administering an optimal treatment strategy. During such experiments, the evolution in serology should be monitored and compared with possible changes in different economic parameters. Eventually, this would provide information on the economic sustainability of the routine deworming of pigs during the fattening phase. Nowadays, it is recommended to treat pigs every 5–6 weeks during the fattening phase in order to prevent the production of fresh *A. suum* eggs, which could otherwise contaminate the environment. A long-term application of a strict deworming protocol has been shown to reduce infection pressure in infected stables and improve performance parameters when applied for a period of at least four fattening rounds (Van Meirhaeghe and Maes, 1996; Jourquin, 2007; Kanora, 2009). However, strictly deworming all pigs every 5–6 weeks comes with a cost. It is estimated that deworming pigs three times during the fattening period, which is approximately 16 weeks, would cost somewhere between 0·24 and 1·16 euro per pig (estimated for currently available pig anthelmintics in Belgium). The actual cost eventually depends upon the type of product used and the selected mode of administration (i.e. feed or water additive). Routine anthelmintic treatments could in some cases be superfluous and not economically sound when parasite infection intensity is low (Roepstorff, 1997; Theodoropoulos *et al*. 2009). Instead, a careful assessment of housing facilities and management factors in combination with routine diagnosis could in these cases be used to monitor and control infections.

Another important aspect in the further development of this serological test is the sampling strategy. The AsHb-ELISA was optimized and evaluated using serum samples from the oldest fatteners, since these have been shown to represent the highest number of seropositive animals and thereby reduce the chance of false negative samples (Vlaminck *et al*. 2012). The possible disadvantage associated with the use of serum is the fact that a skilled veterinarian is needed to obtain the samples. Because of this, the use of additional matrices for antibody testing, such as meat-juice or saliva, which have already proven to be useful for other tests (Vercruysse *et al*. 2006; Wilhelm *et al*. 2007; Prickett *et al*. 2008), should also be evaluated for the future diagnosis of ascariasis in pigs.

DIAGNOSIS OF HUMAN ASCARIASIS

Currently it is possible to detect genetic diversity between different *Ascaris* isolates using molecular techniques such as DNA barcoding, microsatellite DNA profiling (Betson *et al*. 2011, 2012) or even sequencing complete mitochondrial genomes (Liu *et al*. 2012). Despite the nearly identical genetic and antigenic constitution of pig and human *Ascaris* (Abebe *et al*. 2002; Wossene *et al*. 2002; Liu *et al*. 2012) and the reports of cross-infections (Nejsum *et al*. 2005; Bendall *et al*. 2011) it still remains unclear whether *A. suum* and *Ascaris lumbricoides* should be regarded as one or two species (Peng *et al*. 2007; Leles *et al*. 2012; Nejsum *et al*. 2012; Betson *et al*. 2013). It is highly likely, and worth investigating whether the AsHb-ELISA currently used in pigs could have a similar use in the diagnosis of human *Ascaris* infections.

In human helminthology, ascariasis is clustered into the so-called soil-transmitted helminthiasis (STH). This cluster refers to a group of helminthiasis caused by four gastrointestinal nematodes of which infectious stages only develop outside the host in the soil (referring to their common name), including the human *A. lumbricoides, Trichuris trichiura* (whipworm), *Necator americanus* and *Ancylostoma duodenale* (hookworms). In 2010, it was estimated that more than 1·4 billion people are infected worldwide (Pullan *et al*. 2014), causing the highest burden among all neglected tropical diseases (Murray *et al*. 2012), with children and pregnant women being at highest risk of morbidity (Bethony *et al*. 2006). To control the burden of STH on public health the World Health Organization recommends the implementation of preventive chemotherapy (PC) programmes, in which a single oral dose of albendazole (400 mg) or mebendazole (500 mg) are periodically administered to schoolchildren (WHO, 2011). In 2010, the estimated coverage of children at need of PC worldwide was 30% (WHO, 2012*a*), however there is an international and political commitment to upscale these programmes to cover at least 75% of children in need of PC by 2020 (WHO, 2012*b*, NTD Partner Website, 2012). In parallel to these pledges of drugs, there is a need for improved diagnostic tools to monitor the progress of PC programmes and to evaluate their impact on public health, allowing

programme managers, policymakers and donors of the drugs to assess whether the objectives are being met and, if necessary, to correct the implementation strategy (WHO, 2006). Although this topic has indeed been prioritized on the research agenda pertaining to the control and elimination of helminthiases, there has been limited progress in this field for STH (Bergquist *et al*. 2009; McCarthy *et al*. 2012).

Currently, the detection and quantification of helminth eggs excreted by adult worms in stool remains the only diagnostic tool for the detection of STH infections and hence for the evaluation of treatment efficacy. However, this tool has some important limitations in terms of both application and interpretation, which are similar to those in the veterinary field.

For decades the Kato-Katz thick smear has been the standard mean of diagnosing helminth eggs in stool (Katz *et al*. 1972), but its diagnostic performance is complicated by variations in day-to-day egg excretion, the heterogeneous distribution of the eggs within stool samples, and the relatively low diagnostic sensitivity of the Kato-Katz thick smear due to the limited amount of stool examined (41·7 mg) (Sinniah, 1982; Engels *et al*. 1996, 1997; Ye *et al*. 1997; Krauth *et al*. 2012). As a response to this it has been recommended to increase both sampling (examination of multiple stool samples from consecutive stool collections) and diagnostic effort (multiple stool examinations on the same stool or combination of different methods or usage of more sensitive methods) (Booth *et al*. 2003; Knopp *et al*. 2008, 2009; Cringoli *et al*. 2010; Glinz *et al*. 2010; Jeandron *et al*. 2010). Although this increased effort has clearly improved the diagnostic performance of stool examination, it also increases technical, financial and human resources requirements, potentially leading to a non-optimal use of funds allocated for PC programmes (Levecke *et al*. 2009; Speich *et al*. 2010), and hence making them less feasible to implement in the resource-constraint settings in which PC programmes usually operate. Moreover, any diagnostic based on the demonstration of eggs in stool is bound to fail to give a complete insight into the epidemiology and morbidity of STH, and this for four reasons. First, eggs cannot be demonstrated in stool before the worms have grown to adulthood, which takes several weeks in the human body, and hence the prevalence of STH is highly underestimated (Bethony *et al*. 2006). Second, not all immature worms will eventually grow into adult worms, and hence the number of adults in the intestine will not be representative for the initial exposure. Third, egg production by adult worms may vary largely due to density dependent factors (the number of eggs excreted per female drops when the population density in the intestine increases), male/female worm ratio (in the absence of either male or female worms no eggs will be found in the stool) and immunity development (Maizels *et al*. 1993; Jungersen *et al*. 1997; Hall and Holland, 2000; Kotze and Kopp, 2008; Walker *et al*. 2009). Hence the correlation between number of eggs in stool samples and worm-counts is low. Finally, the morbidity caused by STH remains poorly explored because immature stages are migrating through organs other than the intestine (*A. lumbricoides*: liver and lungs, hookworms: lungs) without ever producing a patent infection with adult worms (Bethony *et al*. 2006). Hence, despite the parasitological importance of egg prevalence data, they are of limited value for analysing interactions between helminth infections and other diseases such as HIV/AIDS, tuberculosis or malaria because many egg-negative people in endemic areas will have been immunologically activated by previous, abrogated or non-patent helminth infections (Adams *et al*. 2006; Fincham *et al*. 2007).

Based on the findings in animal experiments described in the previous sections and the limitations of stool examination for human helminthology highlighted above, immunology-based assays may provide additional insights in both epidemiology and morbidity of human STH. This is particularly so for assessing morbidity. With the exception of the blood-sucking hookworms for which we can use anaemia, we are currently lacking clear parameters to measure morbidity for the remaining STH (Bethony *et al*. 2006). It would therefore be interesting to verify whether results from veterinary helminthology can be repeated in human helminthology. As a start, the results of the analysis of human sera with the ELISA test based on the detection of antibody levels produced against the AsHb molecule from pig *Ascaris* could be compared with those of stool examination in school children. On top of that, it would be interesting to check for associations between the outcome of the serology and other parameters such as growth and cognitive development in these children.

CONCLUSION

Overall, current diagnostic techniques for the detection of ascariasis in both pigs and humans fall short in their ability to provide information on the actual infection pressure to which pigs and humans are being exposed. They rather indicate the presence or absence of adult parasites by detecting parasite eggs in the stool, which is not at all representative of the number of larvae that have migrated through the body. Yet, recent developments in the serological diagnosis of *Ascaris* in pigs have shown that serology could provide an improved way to estimate parasite presence (not only adults) and their impact on farm productivity. Work is in progress to determine if the new serological test could be used to monitor morbidity of human ascariasis.

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