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# Test sensitivity of a commercial serine protease digestion kit for the detection of *Trichinella spiralis* and *Trichinella pseudospiralis* larvae in pig muscle

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ABSTRACT

The reference method for *Trichinella* detection at meat inspection is the magnetic stirrer method (MSM) utilising HCl-pepsin for pooled sample digestion. Due to availability and quality issues with pepsin, alternative digestion methods are being offered, such as the Priocheck *Trichinella* AAD kit (T-AAD), based on serine endopeptidase digestion. In this study the T-AAD kit was compared to the reference method.

Minced pork samples were spiked with *T. spiralis* muscle larvae (ML) with- and without capsule or *T. pseudospiralis* ML, and analysed with both tests. Test results of individually spiked test samples were analysed by generalised linear modelling.

The T-AAD test kit was comparable to the reference method for the qualitative detection of *T. spiralis* in pigs, but not quantitatively. Overall, 94% of spiked *T. spiralis* were recovered using MSM against 75.2% when using T-AAD ($p < 0.0001$). Using the MSM 80.0% of spiked *T. pseudospiralis* were recovered against 20% with the T-AAD ($p < 0.0001$).

Based on our experience with the T-AAD kit, we strongly recommend validating the method on site prior to introduction into routine diagnostic laboratories, but this will not alleviate the poor test sensitivity of the T-AAD for the detection of *T. pseudospiralis*.

## 1. Introduction

Human trichinellosis occurs through consumption of raw or inadequately processed meat or meat products containing larvae of the nematode *Trichinella* spp. Pork represents the most important source of human infection worldwide but horse or game meat also plays a significant role (Murrell and Pozio, 2011). The zoonosis is characterized by a large diversity of symptoms in humans such as fever, headache, periorbital or facial oedema and myalgia (Dupouy-Camet et al., 2002). Depending on the infectious dose, human trichinellosis can be a debilitating and occasionally fatal disease. Currently, nine species and three genotypes are recognized (Pozio et al., 2009; Krivokapich et al., 2012; Korhonen et al., 2016). The encapsulated species T. spiralis and T. britovi are the most widespread etiological agents of Trichinella infection in wild and domestic animals and cause the most human infections worldwide (Pozio and Murrell, 2006). The non-encapsulated species *Trichinella pseudospiralis*, and the encapsulated species *Trichinella nativa* and *Trichinella murelli* play a secondary role as zoonotic agents (Pozio, 2005) and have been implicated in a number of outbreaks (Teunis et al., 2012; Hall et al., 2012); all other *Trichinella* species and genotypes are considered to play a minor role in human infection and are often restricted to specific geographic regions or sylvatic cycles (Pozio and Murrell, 2006).

In most of Europe, trichinellosis cases are rare due to controlled housing conditions in commercial swine herds and/or systematic inspection of meat intended for human consumption (Gottstein et al., 2009). In the European Union the majority of slaughtered domestic swine are systematically sampled and examined for *Trichinella* spp. based on Regulation (EC) No. 2015/1375 (European-Commission, 2015). The internationally accepted reference method for *Trichinella* larvae detection is the HCl-pepsin digestion, which has been extensively validated (ISO, 2015; Gamble et al., 2000; Gajadhar and Forbes, 2002; Pozio and Rossi, 2008; Vallée et al., 2007) and is widely used. The assay is based on the use of pepsin in combination with HCL for an enzymatic

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digestion of striated muscle tissue, followed by the detection and quantification of the *Trichinella* larvae by microscopy. The sensitivity of the magnetic stirred method depends on the larval density in the muscles and the amount of muscle sample tested. For a larval density of 3–5 LPG of muscle tissue, a sensitivity of 100% was reported, but for 1 LPG the sensitivity dropped to 40% based on the 1 g of diaphragm per pig routinely tested at meat inspection (Forbes and Gajadhar, 1999). For spiked samples containing one larva, the probability of detecting a positive sample has been estimated at 0.841, based on proficiency test results of five laboratories (Franssen et al., 2017).

Problems associated with the gold standard method are that a shortage and fluctuating quality of pepsin have occurred several times, resulting in increased prices and uncertain availability. This may threaten routine testing of food production animals and consequently public health and export interests. Hence, alternative tests are being offered that circumvent the use of pepsin and could potentially offer solutions for the above described disadvantages.

One such test is the Priocheck *Trichinella* AAD kit (T-AAD), which is based on the digestion of muscle tissue with a serine endopeptidase. The test has been approved and included in Regulation (EU) No. 2015/1375 (European-Commission, 2015; EURL, 2014), but has only been validated for the testing of pork in the EU. The prerequisite for the use of such kits is that test performance parameters such as sensitivity, specificity and robustness are comparable to the gold standard method to ensure consumer health protection.

The objective of the present study was to evaluate robustness of the T-AAD kit on a capsulated and a non-encapsulated representative *Trichinella* species and to investigate its applicability under routine *Trichinella* testing conditions in comparison to the gold standard method.

## 2. Materials and methods

All samples were tested with both the T-AAD kit and the gold standard method (magnetic stirrer method, MSM). The T-AAD kit was performed according to the manufacturer's instructions (version 1.3) and the MSM as described in Regulation EC (No.) 2015/1375, Annex I (Mayer-Scholl et al., 2017). To assess the quality of the digestion process, the amount of remaining indigestible muscle tissue was routinely determined. The digestion process is considered satisfactory if not more than 5% of the starting sample weight remains on the sieve.

### 2.1. Trichinella larvae isolation procedures

Examinations were performed with *T. spiralis*, the most common *Trichinella* species implicated in human infection and *T. pseudospiralis*, which in contrast to *T. spiralis*, does not induce the formation of a capsule in the host muscle tissue.

*T. spiralis* larvae (strain code ISS 003) were isolated from the meat of a domestic pig, which was experimentally infected at the National Reference Laboratory (NRL) for *Trichinella*, Germany (Johne et al., 2018). *T. spiralis* larvae were isolated from tissue with (capsulated) and without (free) intact intramuscular collagen capsule, to evaluate whether the collagen capsule does influence the recovery of larvae. *T. pseudospiralis* (strain code ISS 470) larvae were isolated from a mouse which was kindly provided by the EURL for parasites appointed at the Istituto Superiore di Sanità, Rome (Italy).

The magnetic stirrer method ((EC) No. 2015/1375) was used for the isolation of free *T. spiralis* and non-encapsulated *T. pseudospiralis* larvae (Mayer-Scholl et al., 2017). *T. spiralis* larvae with an intact collagen capsule were isolated using a protocol kindly provided by the EURL in Rome. In short, 10 g *Trichinella* infested, ground meat was added to 250 ml water with 1.25 g pepsin and 1.25 ml HCl, and digested for 5 min at 45 °C. The digestion fluid was transferred to a sedimentation glass funnel, diluted with 250 ml 4 °C water and left to sediment for 10 min. Twenty ml of the sediment was dispensed into a measuring cylinder, left for 10 min to allow larvae to settle and then washed once with 20 ml of water. Only collagen capsules containing a single *Trichinella* larva were collected in water and stored at 4 °C for a maximum of 24h, prior to the spiking experiments. Free *Trichinella* larvae were stored under the same conditions.

### 2.2. Experimentally infected meat samples

One hundred g meat of a pig, experimentally infected with approximately 50,000 T. *spiralis* larvae was blendered using bursts of 2–3 s until no visible pieces of meat remained. The same procedure was performed with 1 g mouse musculature infected with 500 T. *pseudospiralis* larvae (reproductive capacity index 20–30). To establish a proof of principle, one gram of homogenized meat of experimentally infected pig or mouse was added to 9 g *Trichinella* negative minced pork balls and analysed in parallel with both tests. Each analysis was repeated five times.

### 2.3. Spiked pork samples with known numbers of larvae

To compare the limit of detection of both the gold standard method and the T-AAD kit, two series of samples (3 spike levels in 5-fold, 15 samples in total) consisting of 10 g minced pork were spiked with 1, 3 or 10 *T. spiralis* muscle larvae (ML) free of capsule and 1, 3 and 10 encapsulated *T. spiralis* ML. An additional series of 5 samples spiked with 10 free *T. spiralis* ML each, was used as control for the digestion experiments using *T. spiralis* with capsule.

In a second experiment, 15 samples consisting of 10 g minced pork were spiked with 1, 3 or 10 *T. pseudospiralis* ML, thus each spike level was tested in 5-fold. The protocol used for the production of proficiency test samples by the NRL for *Trichinella* (Germany) was followed (Johne et al., 2018).

For both methods, 10 g spiked pork samples were added to 90 g *Trichinella* negative pork and digested in 2 L volumes and the amount of residual, undigested meat from the sieve was weighed. The numbers of recovered larvae were determined for both tests.

### 2.4. Statistical analysis

Test results were analysed by descriptive statistics using Microsoft Excel; test results of individually spiked test samples were analysed by generalised linear modelling using software package 'R' version 3.4.0 (R-Team, 2008). Such experimental count data follow a binomial distribution rather than a normal distribution and therefore need statistical analysis that takes this aspect into account. *Trichinella* ML recovery in relation to missed larvae was evaluated as a function of the factors 'method', 'meat residue' ('meat.res') and *Trichinella* species, both for *T. spiralis* with and without capsule, and the non-encapsulated species *T. pseudospiralis*. The model is defined by $count \sim Binomial(p, spike)$, $logit(p) = method + meat.res + (species + capsulated)$. The term $(species + capsulated)$ represents interaction between these variables, since these are not independent. Best model fit was determined by selecting the model with the lowest AIC-value (Akaike's Information Criterion).

## 3. Results

### 3.1. Comparison of methods using infected meat samples

In the experimentally infected pig meat samples, on average 88.0 ± 6.5 (n = 5) *T. spiralis* larvae were recovered from homogenised samples using the MSM, whereas significantly less larvae (61.0 ± 15.3, n = 5) were recovered using the T-AAD kit ($p = 0.0194$, paired *t*-Test). On average 440.2 ± 56.5 (n = 5) *T. pseudospiralis* larvae were recovered using the MSM and significantly less (106.2 ± 49.3, n = 5) were recovered from mouse muscle tissue using the T-AAD kit ($p = 0.0001$, paired *t*-Test).

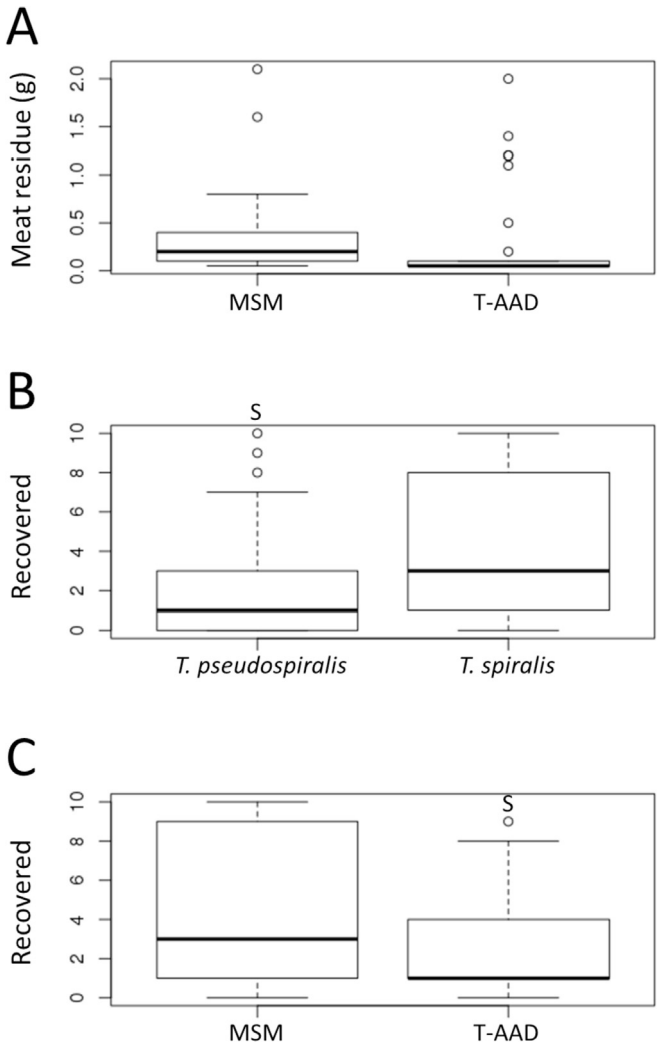


**Fig. 1. Overall results.** A: meat residue per digestion method, B: recovered larvae per *Trichinella* species and C: number of recovered larvae per digestion method. Boxes represent 25th – 75th percentile, thick horizontal line represents median, and whiskers represent all data. Circles outside whiskers represent outlier data. S: significant difference. MSM: magnetic stirrer method. T-AAD: Priocheck *Trichinella* AAD kit.

### 3.2. Comparison of methods using spiked pork samples

#### 3.2.1. Quality of digestion process

Using the MSM, on average 0.29 ± 0.36 g (median 0.20 g, n = 50) of undigested meat residue was left. Using the T-AAD kit, on average 0.21 ± 0.41 g (median 0.05 g, n = 45) undigested meat remained of 100 g meat samples (Fig. 1A). The amount of meat residue did not differ significantly between digestion methods (p = 0.201, Fig. 1A).

#### 3.2.2. Recovered Trichinella larvae

Overall, significantly more *T. spiralis* than *T. pseudospiralis* larvae were recovered ($p = 2.70 \times 10^{-12}$, Fig. 1B) and more larvae were recovered using MSM compared to T-AAD ($p = 2.42 \times 10^{-14}$, Fig. 1C).

The exact spike level was determined significantly more often in samples that had been analysed using the MSM (34 out of 50) than in samples that had been analysed using T-AAD kit (15 out of 45, Table 1) (p = 0.0007, Fisher's Exact test). The same holds true for samples that were spiked with either *T. spiralis* (p < 0.0001, Fisher's Exact test) or *T. pseudospiralis* (p = 0.0007, Fisher's Exact test).

Using the MSM, 94.2% (95%CI 89.9–96.7) of spiked *T. spiralis* larvae were recovered, whereas this was significantly lower at 76.4% (95%CI 69.9–82.9) using the T-AAD (p < 0.0001, Fisher's Exact test) (Table 1).

When meat samples were spiked with *T. pseudospiralis*, significantly more samples were classified false negative when using the T-AAD kit (all 5 samples spiked with 1 larva, 3 out of 5 samples spiked with 3 larvae, and 1 out of 5 spiked with 10 larvae), compared to the MSM (1 out of 5 samples spiked with 1 larva and 1 out of 5 samples spiked with 3 muscle larvae,Table 1) (p = 0.023, Fisher's Exact test).

Significantly more (p < 0.0001) *T. pseudospiralis* spiked larvae were recovered using the MSM (80.0% recovery, 95%CI 69.2–87.7) than with the T-AAD (20% recovery, 95%CI 12.3–30.8) (Table 1). Generalised linear model analysis revealed that factor 'capsule' did not significantly contribute to the model (p = 0.739). Factors 'method' and 'species' contributed highly significantly to the model (P-values $4.19 \times 10^{-14}$ and $6.60 \times 10^{-12}$, respectively).

Consequently, it was decided to compare the recovery of *T. pseudospiralis* larvae with *T. spiralis* larvae that were liberated from their capsule. In formula: *count ~ Binomial (p, spike), logit (p) = method + meat.res + species*. Factor 'meat.res' did not contribute significantly to the model (p = 0.0645); factors 'method' and ' species' contributed highly significantly (p-values $1.29 \times 10^{-11}$ and $9.54 \times 10^{-12}$, respectively). Finally, the model was used to evaluate the influence of digestion method per species: *count ~ Binomial (p, spike), logit(p) = method* for each spike level of both species.

Fig. 2 shows the larval recovery results for all spike levels. For *T. spiralis*, the difference between MSM and T-AAD was significant at 10 spiked larvae (p = 0.0387). For samples spiked with 3 or 1 larvae, differences were not significant (p > 0.346). For *T. pseudospiralis*, differences between MSM and the T-AAD kit were significant at all three spike levels (p-values $6.82 \times 10^{-8}$, 0.0024 and 0.0039, respectively).

## 4. Discussion

National proficiency test results obtained from routine laboratories at the NRL for *Trichinella* in Germany between 2016 and 2018 raised concerns regarding the suitability of the T-AAD kit for the correct detection of *Trichinella* in pork. Proficiency test participants using the T-AAD kit found approximately half the number of spiked ML in comparison to laboratories using the gold standard method (38.5% vs 70% of larvae, spike levels between 3 and 18 larvae, unpublished data). These results prompted us to initiate the described validation study.

The first experiences using the T-AAD test kit showed serious difficulties in both the Dutch and German National Reference Laboratories; both laboratories with ample experience in the MSM. Preliminary results analysing spiked proficiency samples with the T-AAD kit showed a larval recovery rate below 40% in both NRLs (data not shown). Reasons for this could be that the critical control points (CCPs) for the T-AAD kit differ substantially from those for the MSM, e.g. digestion time is critical when using the T-AAD, whereas it is more flexible using MSM.

For *T. spiralis* in the present study, independent of the encapsulation status of the larvae, both tests were comparable at low spike levels (1 and 3 ML), yet the MSM performed significantly better at spike level 10. Overall, 94% of spiked larvae were recovered using MSM and 75.2% when using T-AAD. Recent estimates indicate that a 15% test sensitivity decrease at meat inspection may lead to an eleven fold increase of human trichinellosis cases (Franssen et al., 2018). Another study reported 38% lower larval recovery for the T-AAD using horsemeat samples spiked with 5 *Trichinella* larvae, compared to MSM (Konecsni et al., 2017). In the same study, in pig diaphragm samples spiked with 5 *Trichinella* larvae, on average 74 ± 10% of larvae were recovered using T-AAD, whereas 90% ± 11% of spiked *Trichinella* larvae were recovered using MSM. Gajadhar et al. (2018) validated the T-AAD using 100 g samples of different pork muscle types at a spike range of 3, 4, 5 or 25 *T. spiralis*, in comparison with the MSM. At a spike level of 3 *T.*

**Table 1**
Performance of MSM and T-AAD using spiked pork samples.

| Table 1. | test | n (samples) | Spiked[a] | Recovered[a] | Exact count | False neg. | % recovery | 95%CI |
|---|---|---|---|---|---|---|---|---|
| Overall | MSM | 50 | 260 | 235 | 34 | 2 | 90.4% | 86.2–93.4 |
| *T. spiralis* overall | MSM | 35 | 190 | 179 | 25 | 0 | 94.2% | 89.9–96.7 |
| *T. spiralis* with capsule | MSM | 15 | 70 | 67 | 12 | 0 | 95.7% | 88.1–98.5 |
| *T. spiralis* free larvae | MSM | 20[b] | 120 | 112 | 13 | 0 | 93.3% | 87.4–96.6 |
| *T. pseudospiralis* | MSM | 15 | 70 | 56 | 9 | 2 | 80.0% | 69.2–87.7 |
| Overall | T-AAD | 45 | 210 | 121 | 15 | 10 | 57.6% | 50.9–64.1 |
| *T. spiralis* overall | T-AAD | 30 | 140 | 107 | 15 | 1 | 76.4% | 68.8–82.7 |
| *T. spiralis* with capsule | T-AAD | 15 | 70 | 51 | 8 | 0 | 72.9% | 61.5–81.9 |
| *T. spiralis* free larvae | T-AAD | 15 | 70 | 56 | 7 | 1 | 80.0% | 69.2–87.7 |
| *T. pseudospiralis* | T-AAD | 15 | 70 | 14 | 0 | 9 | 20.0% | 12.3–30.8 |

Exact count: number of samples for which reported larval count was equal to spike. False neg.: number of samples that were reported false negative.
[a] Larval counts.
[b] One extra series of 5 samples spiked with 10 free *T. spiralis* ML each, was used as control for the digestion experiments using *T. spiralis* with capsule. Average and median for both series of 5 samples spiked with 10 free *Trichinella* larvae were equal at 9.2 and 9 larvae respectively.

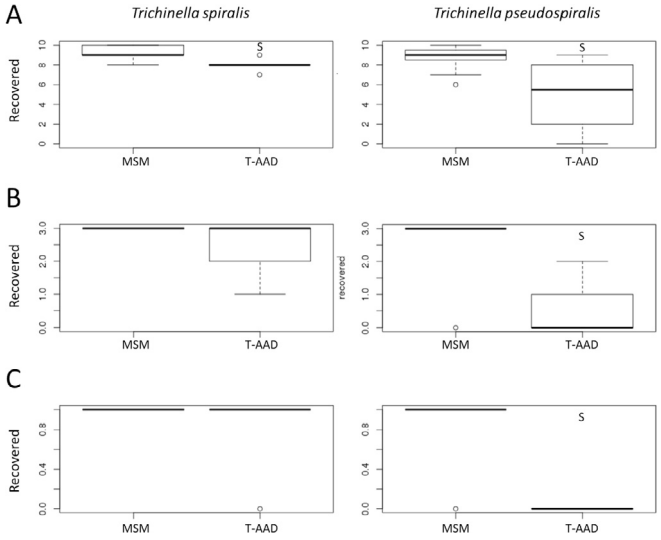


**Fig. 2. Box-plots of recovered larvae per spike level for *T. spiralis* and *T. pseudospiralis* using both the MSM and the T-AAD kit.** A: 10 larvae, B: 3 larvae and C: 1 larva. Boxes represent 25th – 75th percentile, thick horizontal line represents median, and whiskers represent all data. Data outside of whisker range represent outlier data points. S: significant difference ($p < 0.05$). MSM: magnetic stirrer method. T-AAD: Priocheck *Trichinella* AAD kit.

*spiralis* larvae, ≥86% of *Trichinella* larvae were recovered using the T-AAD and > 80% when using the MSM, but mean recoveries using the MSM were higher for all other tested sample sites and spike levels in that study. Interestingly, the reported rinse volume used in that study did not comply with the instructions given by the T-AAD kit; it was increased from 300 ml to 1 litre (Gajadhar et al., 2018), although Konecsni et al. (2017) reported earlier that increasing the rinse volume did not yield more larvae from spiked horse tongue samples. For *T. spiralis*, the present study as well as the studies conducted by Konecsni et al. (2017) and Gajadhar et al. (2018) report recovery of larvae at varying efficacy for all spike levels and no false negative results using either digestion method.

The performance of the T-AAD kit was much lower for *T. pseudospiralis* compared to *T. spiralis* in the present study. At all spike levels, significantly less ML were recovered with the T-AAD in comparison to the MSM. A major concern here are the high numbers of false negative results, making the test kit unsuitable for the detection of *T. pseudospiralis*, and possibly other *Trichinella* species. Teunis et al. (2012) showed that the human infection risk with *T. pseudospiralis* is not as high as for *T. spiralis* or *T. britovi*, but still considerable (> 20%), after ingestion of 10–100 larvae. This is based on an average 0.1–1 larva per gram of ingested meat, when considering one portion to be 100 g (Teunis et al., 2012). It is unknown what caused the lower retrieval of *T. pseudospiralis* larvae using the T-AAD in the present study and more studies are needed, since only few *Trichinella* species have been used to spike experimental samples for digestion. Konecsni et al. (2017) reported comparable yields using MSM and T-AAD for naturally *T. nativa* or *Trichinella* T6 infected masseter from bear and wolf. Additionally, Konecsni et al. (2017) reported comparable larval *T. spiralis* recoveries from spiked pork with either method, but a 38% lower yield for *T. spiralis* larvae spiked in horse tongue using the T-AAD, compared to MSM.

In summary, the T-AAD test kit is comparable to the gold standard method for the qualitative detection of *T. spiralis*, but not for *T. pseudospiralis* in pigs. Moreover, accurate determination of the number of *Trichinella* larvae is not possible using the T-AAD test, which hampers on site quality control. Depending on the pass criteria of the proficiency test provider, the participants are at risk of failing due to shortcomings in the quantitative results.

## 5. Conclusions

Based on our experience with the T-AAD kit, we strongly recommend validating the method on site prior to introduction into routine diagnostic laboratories. Usually, there is no prerequisite to validate a standard method which has not been modified and which is used within the intended scope (ISO, 2017); but the handling and relevant CCPs differ to such a great extend from the CCPs of the MSM, that validation is strongly advisable. However, this will not alleviate the poor test sensitivity of the T-AAD for the detection of *T. pseudospiralis*.

## Ethics statement

All animal work to obtain *T. spiralis* larvae was conducted according to the guidelines of the German Protection of Animals Act (Anonimous, 2006) The State Office of Health and Social Affairs Berlin approved the described animal work (approval No. H 0078/00).

## Declaration of interests

The authors declare that they have no conflict of interests.

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