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Morphological diversity of *Trichuris* spp. eggs observed during an anthelminthic drug trial in Yunnan, China, and relative performance of parasitologic diagnostic tools

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ABSTRACT

The presence of large *Trichuris* spp. eggs in human faecal samples is occasionally reported. Such eggs have been described as variant *Trichuris trichiura* or *Trichuris vulpis* eggs. Within the frame of a randomised controlled trial, faecal samples collected from 115 Bulang individuals from Yunnan, People's Republic of China were subjected to the Kato–Katz technique (fresh stool samples) and the FLOTAC and ether-concentration techniques (sodium acetate–acetic acid–formalin (SAF)-fixed stool samples). Large *Trichuris* spp. eggs were noted in faecal samples with a prevalence of 6.1% before and 21.7% after anthelminthic drug administration. The observed prevalence of standard-sized *T. trichiura* eggs was reduced from 93.0% to 87.0% after treatment. Considerably more cases of large *Trichuris* spp. eggs and slightly more cases with normal-sized *T. trichiura* eggs were identified by FLOTAC compared to the ether-concentration technique. No large *Trichuris* spp. eggs were observed on the Kato–Katz thick smears.

1. Introduction

Eggs of *Trichuris trichiura* are amongst the most common and readily diagnosed parasitic elements in human faecal samples. Indeed, more than 460 million people are currently infected with this soil-transmitted helminth species (Pullan et al., 2014). The disease burden due to trichuriasis has been estimated at 638,000 disability-adjusted life years (DALYs) in 2010 (Murray et al., 2012). The Kato–Katz thick smear technique (Katz et al., 1972) is the current standard approach to diagnose human intestinal helminth infections, including soil-transmitted helminths (WHO, 2006). Numerous other *Trichuris* species parasitise a range of wild and domestic mammals, but the identity of many described species is debated (Bundy and Cooper, 1989). Human infections with such veterinary species are commonly assumed to be relatively rare. One of the species for which human infections have been reported is *Trichuris vulpis*, a common parasite of canids, namely dogs, foxes and wolves (Hendrix et al., 1987; Rinaldi et al., 2006; Traversa, 2011; Ugbomoiko et al., 2008). According to the Centers for Disease Control and Prevention (CDC), *T. trichiura* eggs typically measure 49–65 μm × 20–29 μm, whereas *T. vulpis* ova are considerably larger (72–89 μm × 37–40 μm) (Dunn et al., 2002).

Most *T. vulpis* diagnoses were based on egg size, and some authors have questioned the real nature of these infections as none of them was backed up by unequivocal morphological and/or molecular evidence (Yoshikawa et al., 1989). To our knowledge, no systematic review of alleged human *T. vulpis* infections, including underlying supporting evidence, has been published thus far but Traversa (2011) provided a useful overview. Individual human infections with *T. vulpis* have sporadically been described since the first case report dating back to 1956 (Hall and Sonnenberg, 1956). The most recent report is from Mexico (Márquez-Navarro et al., 2012). Reported cases appear to be concentrated among supposed high-risk populations such as dog owners and kennel workers (Dunn et al., 2002; Kagei et al., 1986), as well as children and institutionalised individuals (Kagei et al., 1986; Yoshikawa et al., 1989).

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A few small epidemiological studies also described the presence of 'large *Trichuris* spp. eggs' that were subsequently identified as *T. vulpis* infections based on morphological and epidemiological considerations. They have mainly been reported from the Indian sub-continent (Mirdha et al., 1998; Singh et al., 1993) and Vietnam (cited in Kagei et al., 1986).

The observation of large *Trichuris* spp. eggs in sodium acetate–acetic acid–formalin (SAF)-fixed faecal samples obtained from a rural community in Yunnan province, People's Republic of China (P.R. China), prompted us to note the frequency at which such large-sized eggs could be identified in samples collected before or after anthelminthic drug administration using two diagnostic techniques. We ponder the possibility that these eggs might represent *T. vulpis* infections and discuss the importance of microscopists remaining attentive to details, and immediately reporting uncommon observations so that they can be followed up by molecular techniques.

2. Materials and methods

2.1. Ethics statement, study area and population

The study was conducted within the frame of a randomised controlled trial to assess the efficacy and safety of a single oral dose of albendazole and tribendimidine for treating soil-transmitted helminths, *Strongyloides stercoralis* and *Taenia* spp. (Steinmann et al., 2008b). Ethical clearance was obtained from the ethics committee of Basel, Switzerland (EKBB, reference no. 149/07) and the academic board of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention in Shanghai, P.R. China. The trial is registered with controlled-trials.com (ISRCTN01779485).

Details of the study area and population participating in this trial have been presented elsewhere (Steinmann et al., 2008b). In brief, the trial was conducted between May and July 2007 in the village of Nanweng in Menghai county, Yunnan province, P.R. China. The sanitation infrastructure in Nanweng is poor and open defecation is common. Soil-transmitted helminths and other intestinal parasites are rampant, and rates of intestinal multiparasitism and reinfection after treatment are high (Steinmann et al., 2007, 2008a; Yap et al., 2013).

2.2. Faecal sample collection and diagnostic work-up

Study participants were invited to provide 2–3 stool samples before and again 2–3 weeks after directly-observed intake of randomly assigned anthelminthic drugs (dosage of both albendazole and tribendimidine: 200 mg for children aged 5–14 years, and 400 mg for individuals aged ≥15 years). For soil-transmitted helminth diagnosis, a single Kato–Katz thick smear (Katz et al., 1972) was prepared, and approximately 2–3 g of stool of one sample collected before treatment and another one submitted after treatment were fixed in 10 ml SAF for subsequent analysis by an ether-concentration method (Utzinger et al., 2010) and the FLOTAC technique (Cringoli et al., 2010).

The SAF-fixed samples were forwarded to a specialised laboratory in Naples, Italy, where the standard FLOTAC analysis was performed using two different flotation solutions (FS), namely (i) FS4 (sodium nitrate; specific density = 1.20), and (ii) FS13 (zinc sulphate plus mercury II iodide and potassium iodide; specific density = 1.45). The stool samples were first screened through a mesh (width: 250 μm) to remove debris, and an additional 10 ml SAF was used to rinse the holdover. The suspension was then vigorously stirred and equally distributed into three tubes, two of which were randomly selected and centrifuged for 3 min at 120 × *g*. After discarding the supernatant, each pellet was re-suspended in 6 ml of either FS4 or FS13, and 5 ml of each suspension was pipetted into the flotation chambers 1 (FS13) and 2 (FS4) of a FLOTAC apparatus, respectively. The apparatus was centrifuged for 5 min at 120 × *g* and the apical portion of each flotation column "translated" for microscopic examination of the entire graded observation grids at 100× magnification.

The third tube was forwarded to the Swiss Tropical and Public Health Institute, and subjected to an ether-concentration method, adhering to a standard protocol. In brief, samples were filtered, centrifuged for 1 min at 500 × *g*, the sediment re-suspended in 7 ml 0.9% saline solution and 3 ml ether, centrifuged for 5 min at 500 × *g*, and the resulting sediment screened for helminth eggs, larvae and intestinal protozoa (Utzinger et al., 2010). The frequency of parasitic elements was semi-quantitatively noted on a per-species basis.

Trichuris spp. eggs of uncommon size were first noted during the FLOTAC analysis, and their occurrence reported separately. The laboratory technicians performing the ether-concentration test were alerted of the possible presence of large *Trichuris* spp. eggs, but were unaware in which samples such eggs had been encountered during the preceding FLOTAC analysis.

2.3. Statistical analysis

Statistical analysis was done using STATA version 10.1 (StataCorp; College Station, USA). Pearson's χ^2-test and Fisher's exact test, as appropriate, were employed for investigating possible associations between the occurrence of *Trichuris* eggs of a particular size and epidemiological risk factors as well as albendazole or tribendimidine treatment. Binomial 95% confidence intervals (CIs) were calculated for prevalence and sensitivity estimates, and the agreement between different diagnostic approaches was examined using Kappa statistics (Landis and Koch, 1977).

3. Results

3.1. Frequency and distribution of large Trichuris spp. eggs

Complete results from the FLOTAC and the ether-concentration analyses were available from 115 individuals out of the 123 otherwise fully compliant participants (Steinmann et al., 2008b). Standard-sized *T. trichiura* eggs (Fig. 1A) were diagnosed in 89.5–100% (mean: 93.0%, 95% CI: 86.8–96.9%) of the faecal samples before treatment and in 80.7–100% (mean: 87.0%, 95% CI: 79.4–92.5%) of the samples after anthelminthic drug administration, depending on the participants' age group (Table 1). No clear relationship between age and prevalence was observed ($P > 0.05$).

Large *Trichuris* spp. eggs (Fig. 1B) were noted in stool samples submitted by 30 individuals belonging to 23 households. Three members of one household submitted faecal samples containing large *Trichuris* spp. eggs and another five families were represented with two individuals each. In two individuals, large *Trichuris* spp. eggs were found both before and after anthelminthic treatment. In the remaining 28 individuals, large *Trichuris* spp. eggs were found only before ($n = 5$) or after treatment ($n = 23$). Thus, the observed prevalence of large-sized *Trichuris* eggs was 6.1% (95% CI: 2.5–12.1%) before treatment and 21.7% (95% CI: 14.6–30.4%) after treatment (Table 1). The observed prevalence was similar among males and females both before and after treatment. Regarding age, the peak prevalence was found among school-aged children (5–14 years) with an observed prevalence of 15.4% (95% CI: 1.9–45.4%) before and 46.2% (95% CI: 19.2–74.9%) after treatment. A second peak was observed among the elderly (Table 1).

At baseline, large *Trichuris* spp. eggs were equally common in the two treatment arms. After drug administration, 72% of the samples

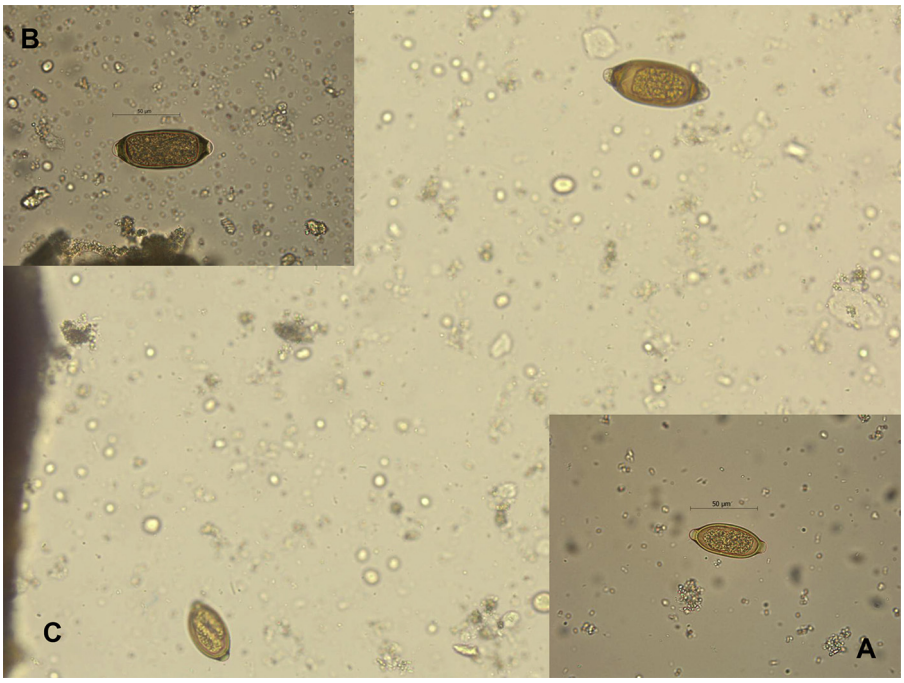

Fig. 1. *Trichuris* spp. eggs of different size: standard *Trichuris* spp. egg (A); large *Trichuris* spp. egg (B), and both standard *T. trichiura* and large *Trichuris* spp. eggs in the same view (C). A Leica DFC420C camera was used to take these pictures of eggs identified with an ether-concentration technique.

with large *Trichuris* spp. eggs were from individuals treated with albendazole ($P = 0.019$; Table 2).

The number of large *Trichuris* spp. eggs observed in individual samples was very low, usually 1–3 eggs were found by the FLOTAC and ether-concentration techniques, whereas considerably higher counts of standard *T. trichiura* eggs were common (Steinmann et al., 2008b). Without exception, large *Trichuris* spp. eggs were found in individuals also excreting standard-sized eggs in their stools (Fig. 1C). The size of the observed *Trichuris* spp. eggs was not routinely determined but did not appear to vary considerably; the size of the large *Trichuris* spp. egg depicted in Fig. 1B is 76×34 μm.

Further epidemiological analyses proved unrewarding due to the homogeneous character of the study population with regard to education, occupation, socioeconomic status and near–universal dog ownership (92.6% of the families with at least one dog) (data not shown).

3.2. *Detection of* Trichuris *spp. eggs by different diagnostic method*

Table 3 details the performance of the FLOTAC and the ether-concentration techniques with regard to diagnosing differently sized *Trichuris* spp. eggs. The FLOTAC technique was superior

Table 1
Prevalence of standard and large sized *Trichuris* spp. eggs, stratified by sex and age group. Number and prevalence (binomial 95% confidence interval [CI]) of large *Trichuris* spp. and standard *T. trichiura* eggs among 115 Bulang ethnic minority individuals from upper Nanweng village, Yunnan province, P.R. China, before and after anthelminthic treatment.

		Pre-treatment				Post-treatment			
		Positive	Prevalence (95% CI)	χ^2	*P*-value	Positive	Prevalence (95% CI)	χ^2	*P*-value
Large *Trichuris* spp. eggs									
Total		7	6.1 (2.5–12.1)	–	–	25	21.7 (14.6–30.4)	–	–
Sex	Female	3	5.3 (1.1–14.6)			13	22.8 (12.7–35.8)		
	Male	4	6.9 (1.9–16.7)	NA	1.00[a]	12	20.7 (11.2–33.4)	0.08	0.783
Age	5–14 years	2	15.4 (1.9–45.4)			6	46.2 (19.2–74.9)		
	15–24 years	0	0 (0–26.5)[b]			2	16.7 (2.1–48.4)		
	25–39 years	0	0 (0–10.6)[b]			7	21.2 (9.0–38.9)		
	≥40 years	5	8.8 (2.9–19.3)	NA	0.122[a]	10	17.5 (8.7–29.9)	NA	0.189[a]
Standard *T. trichiura* eggs									
Total		107	93.0 (86.8–96.9)	–	–	100	87.0 (79.4–92.5)	–	–
Sex	Female	55	96.5 (87.9–99.6)			50	87.7 (76.3–94.9)		
	Male	52	89.7 (78.8–96.1)	NA	0.141[a]	50	86.2 (74.6–93.9)	0.06	0.810
Age	5–14 years	13	100 (75.3–100)[b]			13	100 (75.3–100)[b]		
	15–24 years	11	91.7 (61.5–99.8)			11	91.7 (61.5–99.8)		
	25-39 years	32	97.0 (84.2–99.9)			30	90.9 (75.7–98.1)		
	≥40 years	51	89.5 (78.5–96.0)	NA	0.479[a]	46	80.7 (68.1–90.0)	NA	0.244[a]

NA: not applicable.
[a] Fisher's exact test.
[b] One-sided, 97.5% CI.

Table 2
Prevalence of standard and large-sized *Trichuris* spp. eggs before and after treatment. Occurrence of standard and large sized *Trichuris* spp. eggs among 115 Bulang ethnic minority individuals from upper Nanweng village, Yunnan province, P.R. China, before and after anthelminthic treatment. Data are stratified by drug.

	Pre-treatment		Post-treatment	
	No or only standard *T. trichiura* eggs	Large *Trichuris* spp. eggs	No or only standard *T. trichiura* eggs	Large *Trichuris* spp. eggs
Albendazole	55	4	41	18
Tribendimidine	53	3	49	7
χ^2; *P* value	NA; 1.00[a]		5.48; 0.019	

NA: not applicable.
[a] Fisher's exact test.

in detecting both small and large *Trichuris* spp. eggs. Indeed, not a single case of large eggs was only diagnosed by the ether-concentration method among the samples collected before treatment. After treatment, three cases of large *Trichuris* spp. eggs were only found by the ether-concentration method, four cases were positive according to both methods and 18 were positive by FLOTAC only. The FLOTAC method was also more sensitive in diagnosing small *Trichuris* spp. eggs: it identified 98.9% (95% CI: 94.2–100%) of the cases identified using the ether-concentration method before treatment and 92.7% (95% CI: 84.8–97.3%) after treatment. Of all standard-sized *T. trichiura* eggs diagnosed with FLOTAC, the ether-concentration technique only identified 86.8% (95% CI: 78.8–92.6%) at baseline and 80.9% (95% CI: 71.4–88.2%) after anthelminthic drug administration.

FS13 employed for the FLOTAC analysis performed slightly better than FS4 for diagnosing *Trichuris* spp. eggs, revealing four out of seven cases with large eggs before treatment (FS4: 5/7; Kappa: 0.42, indicating moderate agreement) and 17 of 22 cases after treatment (FS4: 13/22; Kappa: 0.47, moderate agreement). Using FS13, all but one case of *T. trichiura* infection with standard-sized eggs were detected (Kappa before and after treatment: 0.79 and 0.78, both indicating substantial agreement; data not shown).

4. Discussion

Veterinary parasitologists examining the SAF-fixed human faecal samples from an ethnic minority in Yunnan using the FLOTAC technique were the first to note the presence of large *Trichuris* spp. eggs. The tentative identification of these eggs as *T. vulpis* was then – again tentatively – put forth by medical parasitologists using an ether-concentration method. Due to the retrospective nature of the study and the conservation of the samples in SAF solution, no molecular data could be obtained. No large *Trichuris* spp. eggs were observed during subsequent studies in the same population and including deworming with albendazole. In general, *T. trichiura* eggs are readily identified based on their characteristic shape. However, the correct identification of eggs sharing some key characteristics of *T. trichiura* eggs is controversial (Brustoloni et al., 2009; Fugassa, 2010; Núñez, 2010). Larger-than-standard *T. trichiura* eggs have been reported to co-exist with normal-sized eggs, sometimes in the same worm (Yoshikawa et al., 1989). Others dispute the common occurrence of oversized *T. trichiura* eggs (Kenney and Yermakov, 1980). It has also been reported that *T. trichiura* eggs of abnormal size and shape can be found following the administration of anthelminthic drugs (Wagner and Chavarria, 1974). A study in Thailand, employing both traditional parasitological methods and molecular techniques (i.e. polymerase chain reaction (PCR)), called the entire concept of egg size and species-specificity of *Trichuris* spp. into question (Areekul et al., 2010). It found not only considerable variability in *T. trichiura* egg size, but also in the dimensions of *T. vulpis* eggs and, even more importantly, the presence of *T. vulpis* in humans and that of *T. trichiura* in dogs, both with no relationship to measured egg size distributions. We tentatively identified these large *Trichuris* spp. eggs as those of *T. vulpis*, the whipworm of dogs and other canids, based on several considerations and knowing that if confirmed, the findings of Areekul et al. (2010) would render all subsequent deliberations obsolete. Crucially, we suggest they represent spurious infections, i.e. *T. vulpis* eggs ingested and excreted again. This view is supported by the very low egg counts (typically 1–3 large *Trichuris* spp. eggs in ~0.8 g stool sediment). Adult *T. vulpis* usually produce about 2000 eggs per day (Hendrix et al., 1987). Of note, the potential of *T. vulpis* to establish overt infections and reproduce in humans is currently unknown. Definitive identification of the observed eggs would have required molecular diagnostic tools, such as PCR (Cutillas et al., 2007). Isolation of adult worms from individuals excreting such eggs would have proved true human infections (Yoshikawa et al., 1989).

Our tentative identification of these large *Trichuris* spp. eggs as *T. vulpis* is justified as follows. Firstly, the eggs in question were unanimously identified as *T. vulpis* based on their size, shape and appearance first by veterinary and later by medical parasitologists, employing two diagnostic methods. Secondly, during our previous work in the study area, no indications were found of varying *T. trichiura* egg sizes suggesting they fell into two distinct groups,

Table 3
Comparison of the FLOTAC and ether-concentration method for diagnosis of standard and large-sized *Trichuris* spp. eggs. Comparison of the diagnostic accuracy of the FLOTAC technique (pooled data from flotation solutions FS4 and FS13) and an ether-concentration method for detecting standard and large-sized *Trichuris* spp. eggs among 115 Bulang ethnic minority individuals from upper Nanweng village, Yunnan province, P.R. China, before and after anthelminthic treatment.

		Pre-treatment; ether-concentration			Post-treatment; ether-concentration		
		Positive	Negative	Agreement	Positive	Negative	Agreement
Large *Trichuris* spp. eggs							
FLOTAC	Positive	0	7	Kappa: 0.00[a]	4	18	Kappa: 0.20[b]
	Negative	0	108	*P* = NA	3	90	*P* = 0.004
Standard *T. trichiura* eggs							
FLOTAC	Positive	92	14	Kappa: 0.46[c]	76	18	Kappa: 0.43[c]
	Negative	1	8	*P* < 0.001	6	15	*P* < 0.001

NA: not applicable.
[a] Poor agreement.
[b] Fair agreement.
[c] Moderate agreement.

one standard-sized and the second of a larger size which might be confounded with *T. vulpis* recovered from dogs as reported by Yoshikawa et al. (1989). The evidence from our routine diagnostic laboratory, our extensive fieldwork in various parts of the world and from the general scientific literature also does not support the notion of routinely variable *T. trichiura* egg size. Thirdly, the observation of large *Trichuris* spp. eggs already before anthelminthic drug administration, their normal appearance except for their size including intact polar plugs and the absence of otherwise deformed, abnormal or incomplete eggs (Wagner and Chavarria, 1974) makes it unlikely that treatment was the sole factor. In numerous drug trials conducted by the authors in different parts of the world, no change in *Trichuris* spp. egg sizes and shapes following treatment has been observed. However, we found a disproportionately high number of large *Trichuris* spp. eggs among those treated with albendazole 2–3 weeks before, suggesting treatment might still influence the frequency at which such large eggs occur. Further, the presence of polar plugs and the absence of deformed eggs render it unlikely that the eggs expanded during laboratory manipulation, and there were no signs of advanced embryonation. Fourthly, the epidemiological conditions in the study village are permissive for human infections with different parasites.

The low number of large *Trichuris* spp. eggs in the samples also means that there was a high probability that they were absent from some sub-samples rather than missed by the respective diagnostic method. This might also explain the poor-to-moderate agreement between the FLOTAC and ether-concentration techniques, and the failure of the Kato–Katz method – in which only 41.7 mg faecal material are analysed – to detect these eggs. In this respect it must also be remembered that the amount of faeces examined by the FLOTAC and the ether-concentration method, respectively, was 2:1. Even after taking into account the larger amount of stool screened by FLOTAC compared to the ether-concentration method, the FLOTAC still appears to be superior in diagnosing *Trichuris* eggs, both large and standard-sized. FLOTAC has already been shown to out-perform other diagnostic techniques for the diagnosis of non-human primate *Trichuris* spp. infections (Levecke et al., 2009) and several other veterinary parasites (Rinaldi et al., 2007a,b). It was also more sensitive than both the ether-concentration method and the Kato–Katz technique (single or multiple thick smears) for the diagnosis of human soil-transmitted helminth infections (Barda et al., 2013; Glinz et al., 2010; Knopp et al., 2009, 2011; Utzinger et al., 2008). Another source of confusion and under-reporting of large *Trichuris* spp. eggs may be simple neglect due to the overall similar shape and appearance of both large and standard *Trichuris* eggs, especially when large numbers of normal *T. trichiura* eggs potentially distract the microscopists' attention.

We conclude that the observed large *Trichuris* spp. eggs might represent spurious infections with *T. vulpis* but that conclusive evidence is currently lacking. This reminds us how important it is that microscopists always remain attentive to details, and report uncommon observations so that they can be followed up with advanced diagnostic techniques and in-depth epidemiological and molecular investigations. Our observations also underscore the need for improved diagnostic tools, and highlight the potential of interdisciplinary collaboration.

Authors' contributions

PS, GC, HM, XNZ and JU conceived and designed the study. PS, LR, ZWD, JYJ and HZ supervised and helped implement the fieldwork, including the parasitological diagnosis. PS, LR, GC, HM, XNZ and JU analysed and interpreted the data. PS and JU wrote the manuscript. All authors read and approved the final manuscript.

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