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Onchocerca volvulus is not detected in the cerebrospinal fluid of persons with onchocerciasis-associated epilepsy

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ABSTRACT

Objectives: Epidemiological evidence links onchocerciasis with the development of epilepsy. The aim of this study was to detect *Onchocerca volvulus* microfilariae or its bacterial endosymbiont, *Wolbachia*, in the cerebrospinal fluid (CSF) of persons with onchocerciasis-associated epilepsy (OAE).
Methods: Thirteen persons with OAE and *O. volvulus* skin snip densities of >80 microfilariae were recruited in Maridi County (South Sudan) and their CSF obtained. Cytospin centrifuged preparations of CSF were examined by light microscopy for the presence of *O. volvulus* microfilariae. DNA was extracted from CSF to detect *O. volvulus* (O–150 repeat) by quantitative real-time PCR, and *Wolbachia* (*FtsZ* gene) by standard PCR. To further investigate whether CSF from onchocerciasis-infected participants could induce seizures, 3- and 7-day old zebrafish larvae were injected with the CSF intracardially and intraperitoneally, respectively. For other zebrafish larvae, CSF was added directly to the larval medium.
Results: No microfilariae, parasite DNA, or *Wolbachia* DNA were detected in any of the CSF samples by light microscopy or PCR. All zebrafish survived the procedures and none developed seizures.
Conclusions: The absence of *O. volvulus* in the CSF suggests that OAE is likely not caused by direct parasite invasion into the central nervous system, but by another phenomenon triggered by *O. volvulus* infection.

Introduction

Currently, there is strong epidemiological evidence that onchocerciasis is associated with epilepsy (onchocerciasis-associated epilepsy, or OAE) (Chesnais et al., 2018; Colebunders et al., 2019). Studies have shown that areas hyperendemic for onchocerciasis also have a high prevalence of epilepsy (Boussinesq et al., 2002; Pion et al., 2009; Levick et al., 2017; Mmbando et al., 2018; Siewe et al., 2018; Mukendi et al., 2019). A prospective study in Cameroon also showed that a high *Onchocerca volvulus* parasitic load in childhood was associated with an increased risk of epilepsy later in life (Chesnais et al., 2018).

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In a household survey conducted in Maridi County, South Sudan in 2018, an overall epilepsy prevalence of 4.4% (range 3.5–11.9%) was documented in onchocerciasis-endemic villages (Colebunders et al., 2018a). These villages have a long history of suboptimal onchocerciasis control, mainly due to political insecurity. Neurocysticercosis could not explain the high epilepsy prevalence, as no pigs are kept in these villages. The 11.9% epilepsy prevalence was observed in a village located close to the Maridi dam, a blackfly breeding site (Colebunders et al., 2018a). Using the recently proposed OAE definition for epidemiological studies on epilepsy in onchocerciasis-endemic areas (Colebunders et al., 2019), it was estimated that about 85% of persons with epilepsy (PWE) in these villages suffered from OAE (Colebunders et al., 2018b).

OAE often starts between the ages of 3 and 18 years in previously healthy children, and cases may present with a wide spectrum of seizures, most commonly generalized tonic–clonic seizures, sometimes combined with nodding seizures (characterized by repetitive forward dropping of the head). It is still unclear how *O. volvulus* is able to trigger seizures. However, in the time before the introduction of community-directed treatment with ivermectin (CDTI), several researchers detected small numbers of *O. volvulus* microfilariae (mf) – the larval stage of the parasite – in the cerebrospinal fluid (CSF) of persons living in onchocerciasis-endemic areas (Duke et al., 1976; Hissette, 1932; Mazotti, 1959).

Hissette et al. were the first to note the presence of *O. volvulus* mf in the CSF of an untreated person (Hissette, 1932), and Mazotti et al. found mf at concentrations of 0.5–1.3 mf/ml in the CSF during the first 3 days of diethylcarbamazine citrate (DEC) treatment in Mexico (Mazotti, 1959). In a study by Duke et al., the CSF of 11 heavily *O. volvulus*-infected individuals with some form of ocular onchocerciasis were investigated before any treatment was given, and mf were found in five individuals (Duke et al., 1976). During DEC treatment, increasing numbers of mf were detected in the CSF of 10/11 patients, a few days after mf levels increased in the blood (Duke et al., 1976); however, none of these patients developed epilepsy, although patients with concentrations of 8–31 mf/ml CSF experienced severe vertigo and one person developed an episode of Parkinson-like symptoms. To exclude contamination of the CSF with mf from the skin, the first 5–6 drops of CSF were discarded (Duke et al. 1976). During treatment with DEC, *O. volvulus* mf were shown to migrate from the skin through the lymphatic system and pass into the blood (Fuglsang and Anderson, 1974). Moreover, mf can pass from blood into urine through the glomerular capillary walls (Duke et al., 1975) and therefore it was hypothesized that they are equally able to pass into the CSF through the capillary walls of the choroid plexus (Duke et al., 1976).

However, *O. volvulus* mf or DNA has not been reported in the CSF of PWE in onchocerciasis-endemic regions since the widespread introduction of CDTI (Konig et al., 2010; Winkler et al., 2013). An explanation for the absence of mf in CSF in these more recent studies could be the low mf density in the study participants; for example, in a study in Mahenge, Tanzania, the mean skin mf density was only 1 mf/mg (range 0.2–51.5 mf/mg) (Konig et al., 2010). Furthermore, in one of the early studies by Duke et al., study participants were asked to lie on their back for 30 min prior to lumbar puncture to avoid gravitational settlement of mf in the lower part of the spinal subarachnoid space (Duke et al., 1976). The more recent studies do not mention this procedure and this might also explain why no mf were detected.

Therefore, the aim of this study was to detect *O. volvulus* mf in the CSF of PWE from Maridi County with high skin mf loads using the same procedures described by Duke et al. (1976).

Materials and methods

Study setting and participants

This study was conducted in December 2018 in Maridi County, South Sudan. PWE identified during an epilepsy survey in May 2018 in the Maridi area were asked to participate in the study. After informed consent and assent were obtained from carers and participants, PWE were interviewed and examined clinically by a medical doctor. The degree of disability was assessed using the modified Rankin scale (Zhao et al., 2010); the Rankin score ranges from 1 (no significant disability) to 5 (severe disability). Cognitive disability was investigated by questioning carers about the participant's coherence in speech and whether he/she was able to understand and obey verbal instructions (Colebunders et al., 2018b). OAE was defined as proposed previously (Colebunders et al., 2019). Skin snips were obtained from 318 consenting PWE, and 13 volunteers with OAE and with an mf count of >80 in their skin snips were asked to undergo a lumbar puncture to obtain CSF.

Sample collection and processing

Skin snips were collected at village health centres. Standard operating procedures were used to collect and examine skin snips (Prost and Prod'hon, 1978). Briefly, two skin snips were obtained, one from each posterior iliac crest, using the Holt-type punch. Snips were immediately placed in a 96-well microtitre plate containing 3 drops of normal saline and incubated for 24 h at room temperature to allow mf to emerge into the fluid. After this incubation period, mf in the wells were examined microscopically at 400× magnification and counted by a trained technician. The mf density for each participant was obtained by calculating the arithmetic mean of the number of mf in both skin snips, and expressed as mf per skin snip. Two types of skin punch were used: the corneoscleral punch 2 mm (World Precision Instruments, Hitchin, Hertfordshire, UK) and the AA6052 Holth corneoscleral punch 2 mm (Appasamy Associates and Group of Companies, Chennai, India). One punch was used per participant, and punches were cleaned and sterilized with a steam autoclave between participants.

Lumbar punctures were performed on eligible and consenting individuals at Maridi State Hospital. Contraindications to lumbar puncture that were investigated by the research team included altered state of consciousness, ongoing seizures, focal neurological symptoms, signs of increased intracranial pressure, history of abnormal bleeding, and skin or vertebral abnormalities at the puncture site. Lumbar punctures were performed by an anaesthetist using a rigorous aseptic technique. Before the procedure, participants were asked to lie on their back for 30 min to allow equal distribution of mf in the spinal fluid. To perform the procedure, each subject was placed in the lateral decubitus position. A sterile disposable spinal needle (22 gauge) was inserted into the space between lumbar vertebrae L3 and L4, and 5–10 ml of CSF was collected into two to three sterile plain vacutainer tubes. To avoid contamination of CSF by mf from the skin, the initial few drops were collected separately. A few drops of freshly collected CSF were spotted directly onto glass slides for microscopic examination for the presence of mf, red blood cells, and white blood cells, while the remaining CSF was stored immediately at −20 °C. CSF samples were transferred to the University of Antwerp, Belgium, for further investigations.

Microscopy

CSF was thawed on ice, gently mixed by inverting the tube a few times, and aliquoted for further testing until approximately 1 ml

was left in the tube. The last 1 ml was transferred to a sterile 1.5-ml microcentrifuge tube (Eppendorf, Aarschot, Belgium) and centrifuged for 3 min at 3000 × *g* in a microcentrifuge (Eppendorf, 5417R). The supernatant was transferred to a clean Eppendorf tube and the pelleted material was re-suspended in 200 µl of the remaining CSF by gently pipetting up and down. Superfrost Plus glass slides (VWR, Leuven, Belgium) were assembled to single-use cytofunnels with filter cards for use in a Cytospin 3 centrifuge (Life Sciences International, Cheshire, UK) on which 200 µl of sample was loaded. The samples were centrifuged for 5 min at 2000 × *g*. After centrifugation, the cytofunnel was removed and the glass slides dried at 37 °C for 30 min. The resulting thin cell layer was air-dried and fixed in 4% paraformaldehyde for 20 min and washed in phosphate-buffered saline. The slides were further air-dried and dipped in haematoxylin solution followed by gentle immersion in ultrapure water for staining. The stained thin-layer preparations were examined immediately by light microscopy to visualize mf. As positive controls, centrifuged preparations of mf from skin snips were transferred from a 96-well plate to a clean microcentrifuge tube and fixed in 70% methanol; centrifuged slide preparations were then prepared as described above. Furthermore, the filters were removed entirely from the cytofunnels and the outer edges/rims of the filter that absorbed the excess CSF during centrifugation were excised and processed for DNA extraction.

DNA extraction from CSF supernatant and cytofunnel filters

DNA from CSF and from the cytofunnel filter (see above) was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), essentially following the manufacturer's instructions for the purification of total DNA from blood or cells. For extraction of DNA from cytofunnel filters, the filters were submerged in 500 µl ultrapure water, incubated at room temperature for 2 h with occasional vortexing, and processed for DNA as described for CSF.

O-150 real-time PCR

O. volvulus mf were detected by quantitative real-time PCR targeting the O-150 repeat sequence (GenBank J04659.1) used previously to detect *O. volvulus* in skin snips (Zimmerman et al., 1994). The following primers were used: OvFWD 5′-TGT GGA AAT TCA CCT AAA TAT G-3′ and OvREV 5′-AAT AAC TGA TGA CCT ATG ACC-3′, as described previously (Golden et al., 2016). SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, California, USA) was used with the following volumes and concentrations: OvFWD (10 µM) 1 µl, OvREV (10 µM) 1 µl, template DNA 8 µl to a total reaction volume of 20 µl. DNA extracted from a skin snip and plasmid DNA containing the O-150 gene (10^6 copies/µl) were used as positive controls, and DNA extracted from the CSF of a European control without *O. volvulus* infection was used as a negative control. The lower limit of detection was 10 copies/µl. The following cycling conditions were utilized: initial denaturation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, 49 °C for 15 s, 60 °C for 30 s.

Wolbachia *FtsZ PCR*

DNA of the *Wolbachia* bacterial endosymbiont of *O. volvulus* was detected by standard PCR targeting the *FtsZ* gene (GenBank AJ276501). The following primers were used: FtsZ FW 5′-GCC GAT GCG TTT AGA CTT GC-3′ and FtsZ RV 5′-TAT TCG CCG CAG CAT CAA CT-3′. TaqMan Universal Mastermix 2× (Thermo Fisher Scientific, Warrington, UK) was used with the following volumes and concentrations: FtsZ FW (10 µM) 1 µl, FtsZ RV (10 µM) 1 µl, template DNA 6 µl to a total reaction volume of 20 µl. Reactions were run at 95 °C for 10 min for initial genomic denaturation, followed by 35 cycles with the following cycling conditions: 95 °C for 15 s, 56 °C for 15 s, 68 °C for 1 min. DNA extracted from a skin snip was used as the positive control, and DNA extracted from the CSF of a European control without *O. volvulus* infection was used as the negative control.

Zebrafish model for epilepsy

To explore the presence of potential seizure-inducing compounds in the CSF, a zebrafish model was utilized (Liu et al., 2019; Pham et al., 2016; Purdie et al., 2009). This model has been used in the past as a high throughput screening method for potential neurotoxic or seizure-inducing properties of chemical compounds or toxins (Pham et al., 2016; Purdie et al., 2009), as well as to investigate novel anti-epileptic compounds (Liu et al., 2019). Briefly, 2 nl of CSF from a person with recent onset of nodding seizures (case 6, Table 1), 2 nl from a person with more advanced disease without nodding seizures (case 13, Table 1), and 2 nl from a European control without epilepsy were injected into the heart of 10 larvae at 3 days post-fertilization (dpf) and into the peritoneum of 10 larvae at 7 dpf. The CSF from the selected cases was diluted 1:10 v/v in larval medium (Danieau's solution, 1.5 mM HEPES, 17.4 mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4$, and 0.18 mM $Ca(NO_3)_2$ and 0.6 µM methylene blue) and three zebrafish larvae at 3 dpf were kept in this solution in a 96-well plate. All larvae were kept at 28 °C in an incubator in dark conditions and monitored microscopically for 1 min every hour on the first day and once every day in the week following exposure for the potential

Table 1
Summary of the clinical characteristics of study participants.

No.	Sex	Age (years)	Mf density (per skin snip)	Seizure frequency (monthly)	Duration of epilepsy (years)	Nodding seizures	Generalized tonic–clonic seizures	Rankin score	Cognitive impairment	Onchocercal skin lesions	Muscle wasting	Ivermectin use in 2018
1	F	17	100	1	3	No	Yes	1	No	No	No	No
2	M	11	122	150	6	Yes	Yes	3	Yes	Yes	Yes	No
3	M	18	117	120	12	Yes	Yes	3	Yes	Yes	Yes	No
4	F	9	127	90	4	Yes	No	2	Yes	Yes	Yes	No
5	M	14	120	8	1	Yes	Yes	1	No	Yes	No	No
6	M	9	84	120	2	Yes	Yes	2	Yes	Yes	Yes	Yes
7	M	16	86	90	12	Yes	Yes	3	Yes	Yes	Yes	No
8	F	28	119	<1	22	No	No	1	No	No	No	No
9	M	12	102	4	2	Yes	No	1	No	No	No	No
10	F	15	85	24	8	Yes	Yes	1	No	Yes	No	No
11	F	19	110	12	4	Yes	Yes	1	No	No	No	No
12	F	11	105	8	6	Yes	Yes	1	No	No	No	No
13	M	24	108	90	10	No	No	2	No	No	No	Yes

Mf, microfilariae; F, female; M, male.

development of seizures, abnormal swimming behaviour, or signs of toxicity, such as abnormal heart rate, unresponsiveness to touch, or death.

Results

Thirteen persons with OAE and high skin mf loads agreed to participate in the study, seven male and five female (Table 1). All participants met the criteria of the OAE definition. The average age (± standard deviation) was 15.7 ± 5.9 years. Ten (77%) PWE had a history of nodding seizures, while nine (69%) reported generalized tonic–clonic seizures and two (15%) reported myoclonic seizures. No PWE experienced absence seizures based on the carers' reports. The individuals had a mean seizure frequency of 57.8 seizures per month (range 1–150), and the average skin mf density was 106.5 mf/skin snip (range 84–127). Rankin disability scores of the PWE ranged from 1 to 3, with a mean score of 1.8. Only two PWE (15.4%) had received ivermectin in 2018.

In three individuals, mobile mf and red blood cells were observed during direct examination of the first drops of CSF. However, on further examination of all subsequent CSF samples, no mf could be detected in the cytospin preparation by light microscopy or in the CSF supernatant and cytofunnel filter by PCR. No *Wolbachia* DNA could be detected in the CSF supernatant and cytofunnel filter. No white blood cells were observed in the first drops of CSF of any individual. All zebrafish larvae survived the CSF exposure and no toxicity or development of seizures was observed.

Discussion

Despite performing lumbar punctures as described by Duke et al. and all the study participants having high skin mf loads, we found no evidence of *O. volvulus* passage into the CSF. The detection of mobile mf and red blood cells during direct examination of the first drops of CSF in this study was most likely a result of contamination from the skin rather than mf in the CSF. Indeed, it has been suggested that detection of mf in the CSF in some of the earlier studies was caused by contamination from skin mf during the lumbar puncture procedure (Winkler et al., 2013). Interestingly, Duke et al. also did not use the initial 5–6 drops of CSF and observed mf in CSF; however, in their study the CSF collection immediately followed DEC treatment and an increase in CSF mf was preceded by an increase in blood mf (Duke et al., 1976). Thus, it is possible that *O. volvulus* mf may have been present in the CSF of persons with OAE at a certain stage of the disease but subsequently disappeared.

Another difference between our study and that of Duke et al. is that the latter study investigated CSF from individuals with ocular lesions. It is possible that mf could pass from the eye into the CSF through the glymphatic system, where transport occurs between the eye and the central nervous system (Jessen et al., 2015). While *O. volvulus* mf have also been observed previously in the optic nerve of an infected individual (Rodger, 1959), only a minority of people with OAE have ocular lesions (Colebunders et al., 2018b). This observation, and the absence of *O. volvulus* mf in the CSF of persons with OAE, suggests that it is unlikely that the persistent invasion of *O. volvulus* mf into the CSF is responsible for OAE or its progression. Moreover, a post-mortem study investigating the brains of people who died with OAE showed signs of neuro-inflammation and neuronal loss but no evidence of worms in the brain (Hotterbeekx et al., 2019). However, it is possible that a soluble product/toxins derived from *O. volvulus* or from *Wolbachia* generate a systemic (auto)immune response (Johnson et al., 2017), or cross the blood–brain barrier to cause a direct insult to neurons or neuro-inflammation leading to OAE.

A normal CSF white blood cell count was observed in all individuals and is an argument against a direct infection of the central nervous system. Signs of central nervous system inflammation have generally also been absent in the CSF obtained from patients with nodding syndrome in previous studies (Foltz et al., 2013; Konig et al., 2010; Tumwine et al., 2012). Although pleocytosis is often observed in autoimmune encephalitis, normal white blood cell counts do not exclude an autoimmune mechanism (Graus et al., 2016).

The absence of an epileptic reaction in the zebrafish larvae cannot exclude an epileptogenic effect of *O. volvulus* products in the CSF, due to the differences in zebrafish physiology or low concentration in the collected samples, which were injected undiluted in the maximum volume for this model. More research is needed to confirm or exclude the presence of *O. volvulus* products that cross the blood–brain barrier and have a neuromodulatory effect and to explore the role of (auto)immune reactions in the development of disease.

This study has several limitations. No blood test was performed and only a limited number of tests on the CSF of participants were conducted. No other para-clinical workup (such as brain imaging, EEG) was performed on the study participants to investigate other causes of epilepsy. However, a detailed clinical history was recorded to exclude common causes of epilepsy including perinatal insult, febrile illness with seizures, head trauma, or prior infection of the central nervous system. Also, the sample size was small and the seizures had started many years before the study in most participants (mean duration 7.5 years, range 1–27 years). Moreover, in contrast to Duke et al., we did not perform lumbar punctures in the days following onchocerciasis treatment.

Conclusions

This study showed that *O. volvulus* mf or its DNA was absent from the CSF of 13 persons with OAE, indicating that OAE is likely not caused by a persistent invasion of *O. volvulus* mf into the central nervous system. Other mechanisms causing OAE should be explored, like *O. volvulus* excretory/secretory products crossing the blood–brain barrier or an indirect effect triggered by *O. volvulus* infection, such as an autoimmune reaction or a neuro-inflammatory reaction.

Author contributions

AH, RC, and SK-S: conception; AH, RC, GA-E, JYC, JS, MYL, and PCO: design and study preparation; GA-E, SR, WS, AS, and KPB: performed the study in Maridi; AH, PD, and SK-S: laboratory work; AH, SK-S, and RC: wrote the first draft; all authors critically reviewed, corrected, and approved the final version of the manuscript.

Ethical approval and consent to participate

Ethical approval was obtained from the ethics committees of the Ministry of Health of the Republic of South Sudan and the University of Antwerp, Belgium. The purpose and procedures of the study were explained to all study participants in their local language. All participants were asked to sign an informed consent form, by fingerprint in the case of illiteracy, and only consenting individuals were enrolled. Minors >12 years and <18 years of age signed an assent form in addition, while parents or legal guardians consented for younger participants. All individual data were encoded and treated confidentially.

Consent for publication

Consent was obtained from the Ministry of Health of the Republic of South Sudan.

Availability of data and material

All collected data are stored confidentially at the Global Health Institute, University of Antwerp, Belgium. The datasets are available from the corresponding author on reasonable request.

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Conflict of interest

None.

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