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Oseltamivir resistance mutation N294S in human influenza A(H5N1) virus in Egypt☆

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Summary In December 2006, three human specimens were received that were suspected positive for influenza A(H5N1). The specimens were tested using real time PCR. And the presence of A(H5N1) virus was confirmed in 2 patients (16F and 26M), The NA sequence from A(H5N1) positive specimens collected before and after antiviral therapy revealed a mutation (N294S) (N295S according to N1 numbering), previously associated with resistance to oseltamivir. When tested with NA inhibition assays, the two N294S viruses from Egypt exhibited from 57 to 138-fold reduction in susceptibility to oseltamivir, depending on the assay. To our knowledge, this is the first time oseltamivir resistance has been detected in A(H5N1) infecting a human prior to treatment.

Introduction

Avian influenza A(H5N1) virus is by far one of the most important public health concerns world-

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wide. It has sparked fears based on its potential to serve as the source of the next pandemic strain of influenza. A(H5N1) has been evident in South East Asia since 2003. Since late 2005, the virus has spread to many countries in Europe, Asia and Africa [1]. At present, there is no commercial vaccine for A(H5N1) for humans. Oseltamivir is the most recommended antiviral for treatment of confirmed or strongly suspected infections of A(H5N1) [2]. As with any antiviral drug, emergence of a pandemic strain or a strain that is resistant to oseltamivir is a concern. Previously, oseltamivir resistance was detected in persons undergoing treatment for influenza A and B infections (Reviewed by Aoki et al. Antiviral Therapy, 12:603-16, 2007) [3], with the highest frequency of resistance seen in young children after initiation of oseltamivir treatment. A number of amino acid changes in the neuraminidase (NA) were responsible for variable degrees of resistance, when assessed in NA inhibition assays. In the 2007—2008 season, the emergence of wide spread oseltamivir-resistant A(H1N1) viruses carrying H274Y mutation was detected in several countries, with the highest frequency of resistance (up to 67%) seen in Europe [4]. A lack of apparent correlation between the prevalence of resistance in A(H1N1) viruses and in-country use of oseltamivir raises concerns regarding uncompromised fitness of oseltamivir-resistant viruses. In the United States during the 2007—2008 season 12.3% of H1N1 influenza viruses demonstrated resistance to oseltamivir and early surveillance during the 2008—2009 season reports 98.5% resistance of H1N1 isolates with no clinical or demographic difference between cases [5]. In 2008, 100% oseltamivir resistance was reported from 92 viruses isolated in South Africa [6]. As of 18 March 2009 the World Health Organization reports widespread oseltamivir resistance in 30 countries from all regions [7]. Emergence of resistant A(H5N1) variants with H274Y mutation in the NA was detected in patients in Vietnam who were treated with oseltamivir for A(H5N1) infection [8,9]. These recent findings highlight the importance of close monitoring of neuraminidase inhibitor (NI) susceptibility among A(H5N1) viruses.

The first report of A(H5N1) virus introduction in Egypt occurred in poultry and was confirmed in February 2006. Poultry in Egypt was infected with the virus similar to the Qinghai strain first detected in western China in 2005 [1]. Human influenza A(H5N1) infection was first identified in Egypt in March and April 2006 and was mainly due to exposure to/or handling sick household poultry. No further cases of human infections were identified until October 2006. From March 2006 to February 2007 Egypt reported 22 human cases of influenza A(H5N1) with 13 deaths. All cases were treated with oseltamivir using WHO guidelines.

In December 2006, three deadly human cases of A(H5N1) were detected in a family in the Gharbiya governorate (Nile Delta region, Lower Egypt). The cases included 2 females (age 35 and 16) and 1 male (age 26) from an extended family living in the Nile Delta. All cases participated in the slaughter of household ducks on December 13 and developed influenza like illness between December 15 and 19. On December 21, a throat swab was collected from each case before beginning oseltamivir treatment. A second throat swab was collected on December 23rd. All 3 cases died from pneumonia complicated by adult respiratory distress syndrome. The viruses isolated from two of those cases are the subject of the present study. As a WHO reference laboratory for avian influenza, NAMRU-3 confirms human cases of avian influenza. Monitoring genetic changes in the HA and NA genes of viruses isolated in Egypt is important to detect emerging possible pandemic strains or antiviral resistance. In Egypt, A total of 59 human cases with 23 fatalities have been reported up to March 2009 (WHO update; 23 March 2009).

Materials and methods

Viruses and RNA isolation

Pre-treatment throat swab samples were received from the Central Public Health Laboratory (CPHL) of the Egyptian Ministry of Health in viral transport medium. RNA extracts were received from samples collected both before and after initiation of treatment with oseltamivir. Viral RNA extraction was performed using the Qiagen Viral RNA mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer procedure. CPHL initial positive results were confirmed at NAMRU-3 using the specific real time PCR method for the detection of influenza A matrix and H5 gene according to Spackman et al. [10]. N1-gene specific real time PCR was performed according to Payungyoung et al., 2005 [11]. Virus was isolated from both pre-treatment samples.

RT-PCR and sequencing

PCR amplification of overlapping fragments from each HA and NA gene was performed using in-house designed specific primers (primers available upon request). Sequencing of the PCR

amplicons was performed using the BigDye Terminator cycle sequencing reaction mix Version 3.1 (Applied Biosystems, Foster city, CA). Sequences were edited using the Sequencher software Version 4.6 (Gene Codes Corporation, MI, USA) and analyzed using Bioedit [12] and MEGA3.1 software [13].

Neuraminidase inhibition (NAI) assays

Compounds

Zanamivir (GG167) was provided, courtesy of GlaxoWellcome Research and Development (Stevenage, United Kingdom); oseltamivir carboxylate (GS4071) was provided by Roche Laboratories, Inc. (Nutley, NJ); peramivir was provided by BioCryst, Inc. (Birmingham, AL).

Phenotypic drug susceptibility testing

Viruses isolated (without further passage) and RNA extracts from pre-treatment collected samples were shipped to the U.S. Centers for Disease Control and Prevention (CDC) for further testing. Phenotypic testing was performed on both isolates using the chemiluminescent assay and on A/Egypt/14725-NAMRU3/2006 using fluorescent and colorimetric assays in the BSL-3 laboratory of the Influenza Division at CDC, Atlanta, Georgia, USA, as previously described [14—16].

Phylogenetic analysis was performed using the Molecular Evolutionary Genetics Analysis (MEGA 3.1) [13].

Results

Two of the three Gharbiya cases were confirmed by A(H5N1)-specific real time PCR at NAMRU-3. Alignment of their NA amino acid sequences revealed a mutation N294S (numbering according to N2) previously reported to be associated with oseltamivir resistance in N1 and N2 subtypes of influenza A viruses [9,17]. The N294S mutation was observed in samples collected pre and post treatment in both confirmed cases as well as in the viruses isolated from the pre-treatment sample. Prior to initiating phenotypic testing CDC confirmed presence of N294S mutation in the submitted samples. Initially, the oseltamivir-susceptibility of the two influenza A(H5N1) viruses was assessed using the chemiluminescent NA inhibition assay. The IC_{50} values were similar for both viruses and exhibited a 57-fold increase (17 nM vs. 0.3 nM) compared to those of the A/Turkey/15/2006 (H5N1) virus, a sensitive control from the same genetic clade (2.2). To confirm the decreased oseltamivir-susceptibility, A/Egypt/14725-NAMRU3/2006 virus was then tested using the fluorescent and colorimetric NA inhibition assays which utilize different enzyme substrates. The results of these two assays indicated even greater reduction (93-fold and 138-fold, respectively) in oseltamivir susceptibility (Table 1).

The IC_{50} value for the wild type virus, A/Turkey/15/2006 (H5N1), varied from 0.3—3.1 nM, depending on the enzyme substrate used in the test, which is a known phenomenon [15]. The difference was greater for the A/Egypt/14725-NAMRU3/2006 virus, ranging from 17.0—426.6 nM (Table 1).

Because of uncertainty regarding the predictive value of in vitro NI assays, we compared the IC_{50} values to those of the human reference virus, A/Texas/36/1991 (H1N1) wild type and with the oseltamivir resistance-conferring mutation H274Y [18]. The wild type A/Texas/36/1991 (H1N1) virus exhibited IC_{50}s similar to those of the sensitive control A(H5N1) virus from clade 2.2., A/Turkey/15/2006 (Table 1). The Egypt/14725-NAMRU3/2006 showed IC_{50}s approximately 10-fold lower compared to those of the oseltamivir-resistant A/Texas/36/1991 using the chemiluminescent and fluorescent assays. Of note, the difference was less than 2-fold (426.6 nM vs. 719.6 nM) using the colorimetric assay, which utilizes a large and natural substrate (fetuin).

Furthermore, the susceptibilities of the A/Egypt/14725-NAMRU3/2006 virus and the control viruses were also assessed against other NIs, zanamivir and peramivir. Based on the IC_{50} values obtained using chemiluminescent and fluorescent assays, the susceptibility of the A/Egypt/14725-NAMRU3/2006 to either zanamivir or peramivir was not substantially altered (2—4-fold increase). However, a 27- and 130-fold reduction in susceptibility to these drugs, respectively, was detected using the colorimetric assay (Table 1).

The HA gene from the two positive cases revealed newly acquired amino acid changes that were not seen in March—April 2006 cases, mainly M230I and V223I. M230I is a mammalian polymorphism adjacent to a receptor binding site. Phylogenetic analysis of the HA and NA genes (Figs. 1 and 2) revealed that these two viruses belong to the 'Qinghai-like' group of viruses and cluster with the previous A(H5N1) strains from human or avian species from Egypt. Furthermore, the phylogeny, supported with high bootstrap values, suggests at least two main subclusters of A(H5N1) viruses co-circulating in Egypt, one in Upper Egypt and another in the Delta region. The

Table 1 The IC_{50} values (nM) against 3 NA inhibitors and fold difference in 3 different NA inhibition assays. Comparison between H5N1 virus isolated from human case prior to treatment, oseltamivir sensitive H5N1, and oseltamivir sensitive and resistant H1N1 viruses.

Virus	NA	Chemiluminescent			Fluorescent			Colorimetric		
		Osel	Zan	Per	Osel	Zan	Per	Osel	Zan	Per
A/Egypt/14725-NAMRU3/2006 (H5N1)	N294S	17.0 (57)	0.9 (2)	0.7 (3)	138.9 (93)	1.5 (3)	1.7 (4)	426.6 (138)	33.7 (27)	25.9 (130)
A/Turkey/15/2006 (H5N1)	Wt	0.3	0.4	0.2	1.5	0.5	0.46	3.16	1.2	0.2
A/Texas/36/91 (H1N1)	Wt	0.42	0.53	0.19	2.40	3.17	1.39	3.10	2.08	0.47
A/Texas/36/91 (H1N1)	H274Y control	169 (402)	0.8 (1)	19.52 (103)	598 (666)	3.7 (1)	83.74 (60)	719.6 (232)	1.94 (1)	51.4 (110)

Wt, wild type; Osel, oseltamivir; Zan, zanamivir; Per, peramivir.

resistant strains belong to the subcluster from Lower Egypt (Nile Delta region) (Fig. 2).

Discussion

Since 1997, avian influenza A(H5N1) virus caused over 412 human infections with more than half of these cases being fatal ($n = 256$). In Egypt, the number of human infections caused by A(H5N1) virus reached 59 with 23 deaths (WHO updated report of 23 March, 2009; http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_03_23a/en/index.html). Currently, there are four FDA approved drugs for influenza belonging to two classes, M2 blockers and NA inhibitors. The usefulness of M2 blockers (amantadine and rimantadine) has diminished in recent years due to emergence of cross-resistant influenza A viruses of H3N2, H1N1 and H5N1 antigenic subtypes [19—24]. For reasons that are not yet known, oseltamivir resistant H1N1 now appears to be widely circulating during the 2008—2009 season. The WHO recommends using oseltamivir at 75 mg twice daily [2] to treat A(H5N1) infections and supports stockpiling in advance of a pandemic. The necessity of inhaled delivery hinders the use of zanamivir. Another NI, peramivir, is undergoing clinical evaluations at this time [25]. Because treatment options of influenza infections are limited, monitoring of susceptibility to existing antiviral drugs is critical, especially against oseltamivir, which is the current first line of defense against highly pathogenic A(H5N1) viruses. Data on molecular markers of oseltamivir-resistance (acquired and spontaneous) in A(H5N1) viruses is insufficient, which emphasizes the need for investigating the changes in drug-targeted molecule (NA) of viruses recovered from treated as well as untreated patients. Emergence of virus variants carrying H274Y mutation that confers a high level of oseltamivir-resistance was previously detected in viruses recovered from three patients in Vietnam, treated with oseltamivir for A(H5N1) virus infections (clade 1 genetic group) [8,9]. Moreover, virus carrying mutation N294S was also detected in one of the patients mentioned above. In clade 1 genetic background, the N294S mutation conferred a moderate oseltamivir-resistance (7.1—12.5 nM), when assessment was done using the fluorescent NA inhibition assay [6].

In the present study the mutation N294S was detected in the A(H5N1) viruses recovered from two patients in Egypt who did not survive the infection. It is uncertain whether N294S mutation by itself was responsible for treatment failure. To be effective, treatment with oseltamivir needs to be

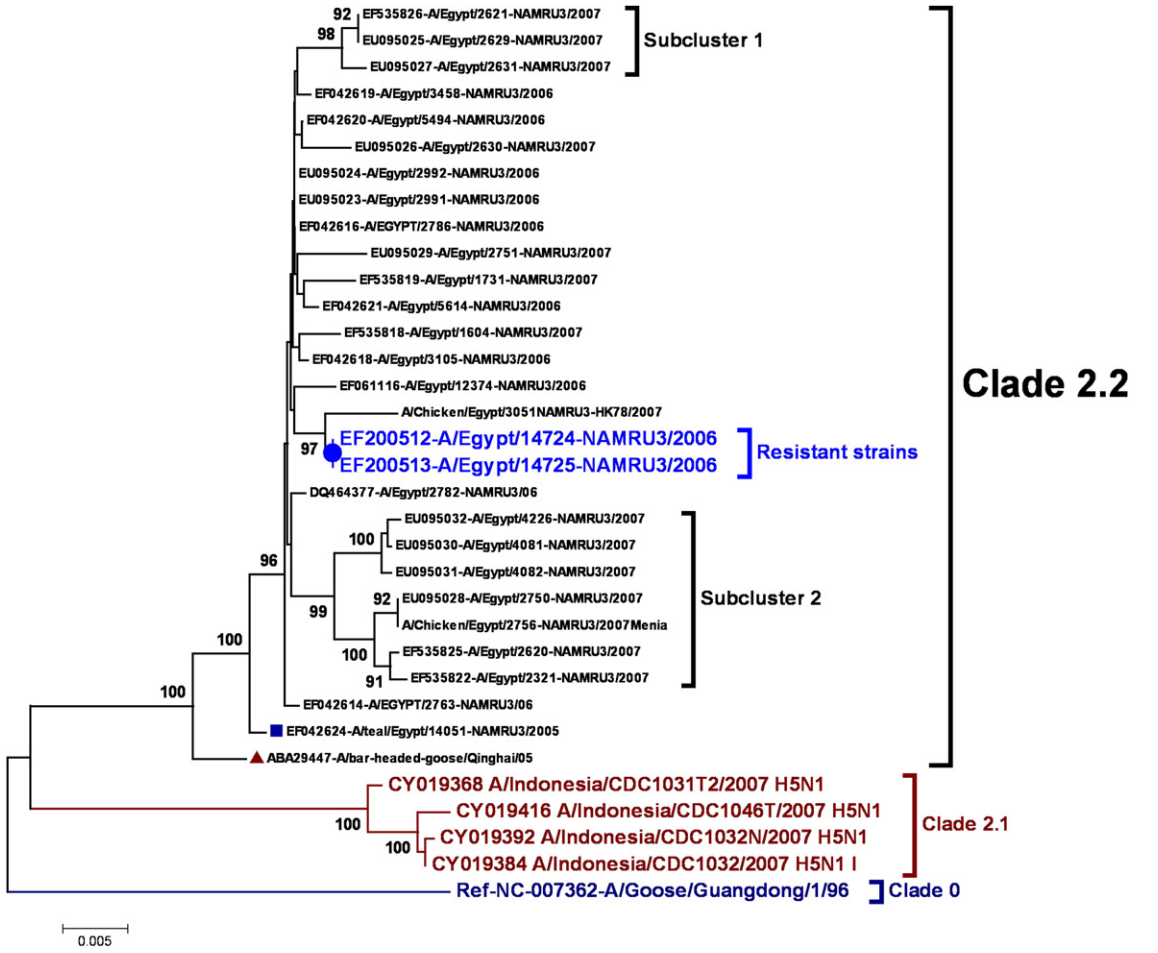

Figure 1 Neighbor-joining phylogenetic tree of the influenza A virus (H5N1) HA gene of strains from Egypt with strains from clade 2.1 (Indonesia) and Clade 0 (Guangdong 1996 strain). Two distinct phylogenetic subclusters of A(H5N1) viruses are clearly co-circulating in Egypt, one in Upper Egypt and another in the Delta region.

initiated during the first two days after symptoms onset, which is not usually possible [26]. In contrast to the previous reports from the Vietnamese cases, the resistance-conferring mutation was detected before and after oseltamivir-treatment, indicating its spontaneous nature. There is significant divergence in the NA sequences and structures among avian A(H5N1) viruses [27] which may have significant implications for antiviral susceptibility, especially for oseltamivir [28]. This is the first report demonstrating the effects of N294S mutation on susceptibility of A(H5N1) viruses from clade 2.2 to oseltamivir and other NA inhibitors.

Testing with all three NA assays yielded increased IC_{50} values against oseltamivir. Noteworthy, the IC_{50} value for the A/Egypt/14725-NAMRU3/2006 virus assessed using the colorimetric assay was greater than those obtained in the small-substrate assays (427 nM equals ~142 ng/ml) and was close to observed plasma concentration of oseltamivir carboxylate (median C_{min} = 167 nM; C_{max} = 332 ng/ml) [29] achieved using the recommended oseltamivir treatment regimen [2]. The IC_{50} value (719.6 nM equals ~240 ng/mL) of the oseltamivir-selected H274Y mutant assessed using the fetuin-based assay was within the range of plasma concentrations of oseltamivir carboxylate (167–332 ng/mL); whereas those plasma concentrations substantially (~140-fold) exceeded the IC_{50} values of both wild type viruses. Regardless of the substrate utilized, the IC_{50} values determined against zanamivir and peramivir for the A/Egypt/14725-NAMRU3/2006 (N294S) virus were much lower than those against oseltamivir.

Although no poultry were available for testing from the house of the studied cases, existence of resistance mutation prior to treatment in both cases strongly suggests its spontaneous nature and may have existed in other viruses infecting poultry in Gharbiya governorate in Egypt. Since oseltamivir is the most widely stockpiled drug for a future possible influenza virus pandemic, based on the findings

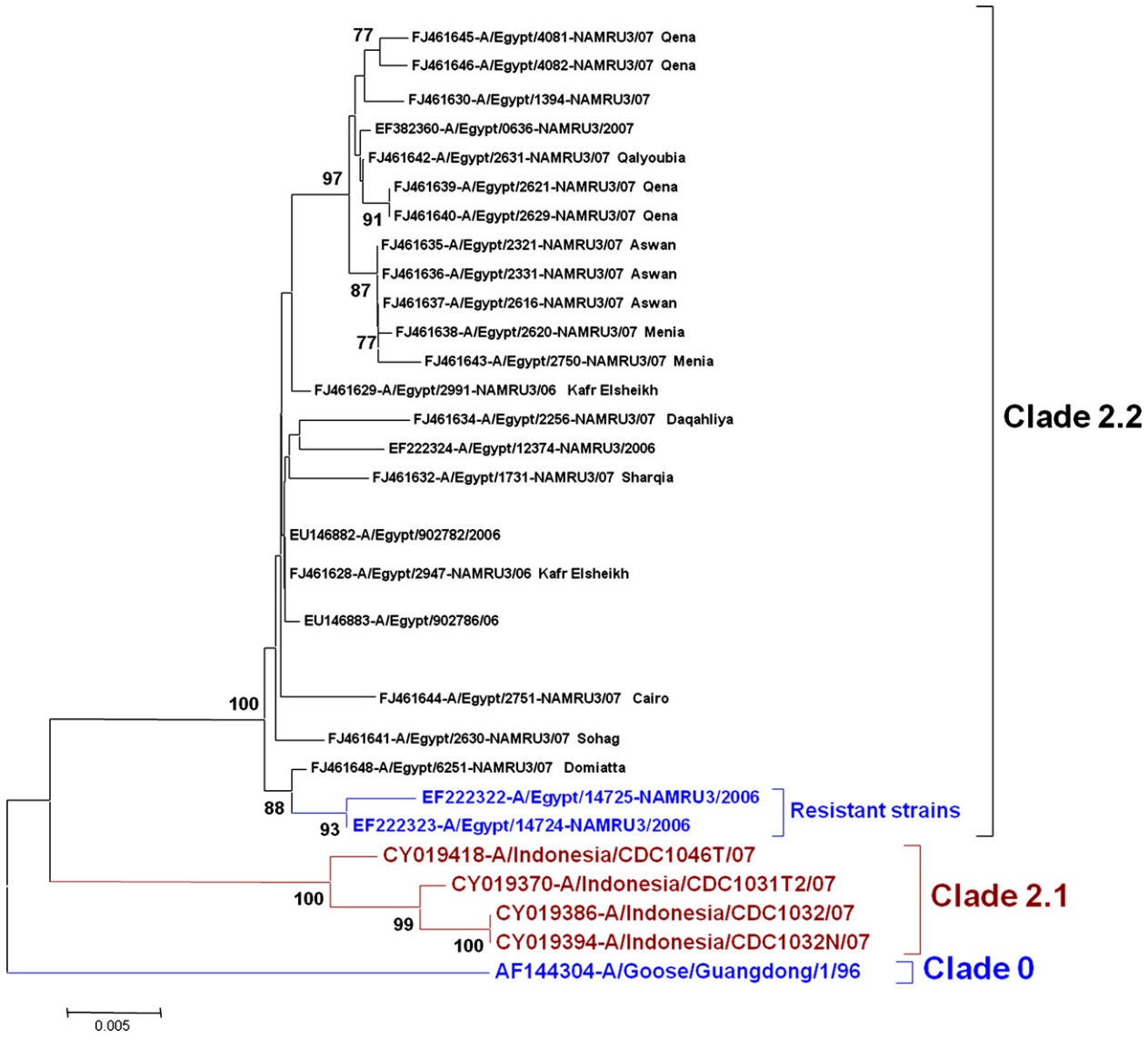

Figure 2 Neighbor joining phylogenetic tree of the influenza A (H5N1) NA gene of strains from Egypt. Value at nodes represent bootstrap support values expressed as percentage of 1000 replicates. Tree was generated by MEGA software version 4.0. Strain name starts with GenBank accession number. Tree was rooted on Indonesia clade 2.1 strains as outliers.

of this study, the development and inclusion of alternative influenza A virus antiviral drugs in the treatment strategy need to be considered.

To our knowledge, this is the first report where oseltamivir resistant A(H5N1) viruses were recovered from humans prior to initiation of treatment. No further oseltamivir resistant isolates have been identified, however, A(H5N1) continues to circulate in Egypt and other countries. Oseltamivir resistance in H1N1 resulting from H274Y mutation is now widespread. Although the clinical relevance of oseltamivir resistance has not been determined, our findings emphasize the necessity of close monitoring of A(H5N1) viruses for the emergence of resistance.

Conflict of interest

Funding: No funding sources.
Competing interests: None declared.
Ethical approval: Not required.

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