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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Molecular Detection Of *Toxoplasma gondii* And *Cryptosporidium parvum* Oocysts Uptake By Carpet Shell Clams (*Ruditapes decussatus* L.) From İzmir Bay (Türkiye) [*]

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Abstract: The research focused on uptake and accumulation of *Toxoplasma gondii* and *Cryptosporidium parvum* oocysts in carpet shell clams (*Ruditapes decussatus* L.) from İzmir Bay. Molecular identification of *T. gondii* and *C. parvum* oocysts was carried out by conventional PCR method. The rate of positiveness of *C. parvum* was 3.33 % and of *T. gondii* 4.16 % in 120 pools examined. Statistical analysis revealed slight positive correlation between the monthly rate of positiveness of *C. parvum* and the clam length. However no statistically significant correlation between clam length and *T. gondii* monthly rate of positiveness was detected. Shellfish farming in non polluted seawater, depuration before consumption and strict food safety control for emerging enteric protozoan parasites such as *T. gondii* and *C. parvum* are recommended.

Keywords: Carpet shell clam, *Cryptosporidium parvum*, food safety, *Toxoplasma gondii*, water pollution.

İzmir Körfezi (Türkiye) Akivadesleri (*Ruditapes decussatus* L.) Tarafından *Toxoplasma gondii* ve *Cryptosporidium parvum* Ookistlerinin Alımının Moleküler Tespiti

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Öz: Araştırma, İzmir Körfezi'ndeki akivadeslerde (*Ruditapes decussatus* L.) *Toxoplasma gondii* ve *Cryptosporidium parvum* ookistlerinin alımına ve birikimine odaklanmıştır. *T. gondii* ve *C. parvum* ookistlerinin moleküler tespiti geleneksel PCR yöntemiyle yapıldı. İncelenen 120 havuzda *C. parvum*'un pozitiflik oranı %3,33, *T. gondii*'nin ise %4,16 olarak bulundu. İstatistiksel analiz, *C.parvum*'un aylık pozitiflik oranı ile akivades uzunluğu arasında hafif pozitif bir ilişki olduğunu ortaya çıkardı. Ancak akivades uzunluğu ile *T. gondii*'nin aylık pozitiflik oranı arasında istatistiksel olarak anlamlı bir korelasyon tespit edilmedi. Temiz deniz suyunda kabuklu deniz ürünleri yetiştiriciliği, tüketimden önce arındırma ve *T. gondii* ve *C. parvum* gibi yeni ortaya çıkan enterik protozoan parazitlere yönelik testlerin eklenmesiyle sıkı gıda güvenliği kontrolü tavsiye edilmektedir.

Anahtar kelimeler: akivades, *Cryptosporidium parvum*, gıda güvenliği, su kirliliği, *Toxoplasma gondii*.

INTRODUCTION

In the recent years, the global shellfish aquaculture production has reached the record total production of 18 911 thousand metric tonnes by 2022 year (FAO, 2024) and Türkiye is not an exception in this global trend. Turkish annual shellfish aquculture production doubled in the recent years and has reached 8 738 tonnes (TÜİK, 2023). This is due to the increased demand for sustainable aquculture products whose nutritional importance is gradually gaining pace (FAO, 2024). Consumption of more sustainably produced marine food is the one of the keys addressing global food security and the preservation of global natural fish resources across the world (Lucas et al., 2019). Although, shellfish production owns its advantages in respect to sustainability and food security, the matter with

[*] *The study presented here was a part of the Esat Çilli`s PhD research project in Aquaculture.*
Burada sunulan çalışma Esat Çilli`nin Su Ürünleri Yetiştiriciliği alanında yürüttüğü doktora araştırma projesinin bir parçasıdır.

the food safety is more intricate (Rousseau et al., 2018). This is due to the feeding mode of bivalves. Filter fedders such as oysters, clams, mussels, cockles and scallops, in their process of filter feeding can acumulate in their tissues zoonotic pathogenic agents suspended in the sea water. There are numerous reports about accumulation of *Toxoplasma gondii, Cryptosporidium parvum, Cyclospora cayetanensis* and *Giardia duodenalis* oocysts in various shellfish representative species, which pose serious health risks to consumers eating undercooked or raw shellfish (Lindsay et al., 2003; Rousseau et al., 2018). Contracting toxoplasmosis, giardiosis or cryptosporidiosis was undoubtfully demonstrated by feeding experiments of rats with contaminated shellfish (Arkush et al., 2003; Fayer et al., 1998).

Beside imposing food safety risks to the consumers, accumulation of various protozoan oocysts in shellfish has another aspect of consideration, which is the fecal contamination of sea water (Kacar, 2011). Despite its measurement by fecal coliform bacteria presence, it can also be measured by presence of accumulated protozoan oocysts in shellfish. There is ample research work on this subject, stressing the critical issue of fecal contamination in shellfish (Ghozzi et al., 2017).

Although there are a few research projects regarding accumulation of protozoan oocysts in carpet shell clams from the globe and Medditerranean sea, none deal with carpet shell clams from Turkish waters and especially urbanized area of İzmir Bay. In spite of the research conducted on mussels collected from İzmir Bay regarding accumulated protozoan oocysts (Aksoy et al., 2014; Erol et al., 2016), there is a need for further verification of the accumulation in different bivalve aquatic species. In this respect, carpet shell clams from İzmir bay are the ideal alternative species for mussels, and can serve as the addiditional confirmatory species for sea water pollution in the afforementioned geographical area (Serdar & Lök, 2005).

The present research was aimed to shed light on uptake and accumulation of *Toxoplasma gondii* and *Cryptosporidium parvum* oocysts in carpet shell clams (*Ruditapes decussatus* L.) from İzmir Bay and further verify this phenomenon in alternative marine bivalve species other than mussels (*Mytilus galloprovincialis*).

MATERIAL AND METHOD

Sample Collection: Since İzmir bay is the only legaly open natural bed for collection of carpet shell clams in Türkiye (Anonymous, 2022), it was chosen as the area of sampling (Üçkuyular/İzmir, coordinates: 38º25'11"N 27º02‘13"E) (Figure 1)

Figure 1. Map image taken from Google Earth program of the point where carpet shell clams were collected.

Each month (12 months, from October 2023 to September 2024), randomly selected samples of 30 carpet shell clams (Figure 2a), total number of 360, provided by local gatherers, were brought to the İzmir/Bornova Veterinary Control Insitute Parasitology Laboratory in styrofoam boxes and during the examination were kept at +4 ºC.

Each clam length was measured by millimetric ruler (Figure 2b).

With the aid of sterile lancet the adductor muscle of the each clam was cut to open the bivalve and to remove the soft part of it. (Figure 2). Then, the clams were pooled in 10 separate groups each consisting of 3 clam soft tissues (120 pools for the total duration of the research, n=120). Individual pools were collected in sterile plastic tubes and pooled tissues were kept at -20 ºC overnight for freezing.

After subsequent thawing, tissues of each pool were mixed with 10 ml sterile phosphate-buffered saline (PBS) and poured in sterile bags for further disintegration of clam tissues via masticator homogenizer device (IUL). Obtained homogenates were collected and transferred to sterile tubes to be kept as 2 ml aliquots at -20 ºC for further DNA extractions.

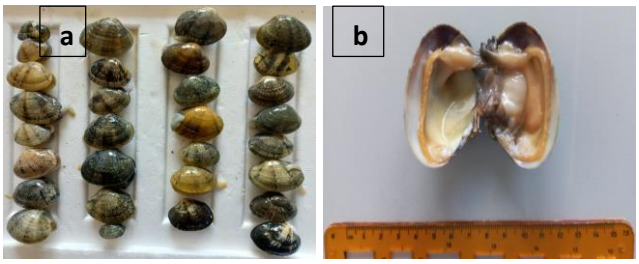

Figure 2. 30 closed(alive) (a) and open(ready) for examination (b) carpet shell clam(s).

Identification of Toxoplasma gondii and Cryptosporidium parvum oocysts by conventional PCR: High Pure PCR Template Preparation Kit (Roche) was used appropriately for obtaining genomic DNA of the protozoan oocysts putatively contained in the clam tissue homogenates kept as 2 ml aliquots at -20 ºC.

Oligonucleotide primers forward primer DS29 (5'-TTGCCGCGCCCACACTGATG) and reverse primer DS30 (5'-CGCGACACAAGCTGCGATAG), which are specific for *Toxoplasma gondii* and targeted the toxoplasma P30 gene, were used for molecular identification (Savva et al., 1990). The presence of a PCR fragment of 914 bp in length as a result of the test was considered as a positive identification.

Similarly, oligonucleotide primers, forward primer AGG GTG AGA GAG CCA TGA CT and reverse primer CAG TTT GGT TGT GCT CGA GC, specific for *Cryptosporidium parvum* and targeting HSP70 gene, were used for molecular identification (Jarad, 2020). The presence of a PCR product of 429 bp in size as a result of the experiment was accepted as positive.

In the study, reference strains of *Toxoplasma gondii* and *Cryptosporidium parvum*, isolated from diseased animals and stocked at İzmir Bornova Veterinary Control Insitute Parasitology Laboratory, were used as positive controls and nuclease-free water (Fermentas) was used as a negative control.

For both parasites, the procedures for PCR amplifications of the targeted DNA fragments were identical.

PCR amplification of the targeted DNA fragments proceeded as follow: 25 µl reaction mixture was prepared by 5,0 µl DNA template, 2 µl of each forward and reverse primers, 3,5 µl nuclease-free water (Fermentas) and 12,5 µl of Xpert Fast Hotstart Mastermix (2X) with dye (GRISP, Portugal).

The amplification was carried out in Techne TC-412 thermal cycler consisting of following steps: 1) an initial denaturation step of 95°C for 5 minutes, 2) denaturation at 95°C for 30 seconds, 3) annealing at 60°C for thirty seconds 4) extension for sixty seconds at 72°C, 5) final extension for 5 minutes at 72°C. Steps 2-3-4 were repeated as a loop 35 times. After the amplification step, the ladder (GRS Universal Ladder GRISP, Portugal) and each PCR product was mixed with stain (Xpert Green DNA Stain Direct, GRISP, Portugal) and subsequently were subjected to electrophoresis at 85 Volts for 1,5 hours (Thermo, model 4000 PECAY LVD) of 10 µl of each mixture in 2% agarose gel within 1 mM TAE buffer. Images of the agarose gels were taken with the aid of ERBiyotek imagining system.

Calculation the rate of positiveness: The rate of positiveness of the sampled pools was calculated as the number of the positively found pools divided by the total number of examined pools and finally multiplied by 100 ((number of positive pools/total number of pools)X100). The rate of positiveness was expressed as a percentage for each parasite separately.

Statistical analysis: Statistical analyses were carried out on Microsoft Excell with aid of statistical concepts explained by Sümbülüoğlu and Sümbülüoğlu (Sümbüloğlu ve Sümbüloğlu, 2016). Each statistical formula was entered manually into Microsoft Excell file. Obtained data from the experimental measurements was initially digitalized and subsequently applied to the formulas. Eventually, the results were recorded electronically. The calculated Pearson correlation coefficients (r) were tested by Student t-test at 95% confidence interval for their statistical significance.

RESULTS

The research showed that at least 9 out of 120 pools of clams collected on monthly basis from İzmir Bay for the time period of an entire year were tested positive for the presence of either *Toxoplasma gondii* or *Cryptosporidium parvum.* The results are shown in Table 1.

Table 1. Morphometric measurements of collected clams along with monthly and annual (overall) positiveness rates of the *Toxoplasma gondii* and *Cryptosporidium parvum.*

Month	Number Of Examined Clams	Average Clam Length (±SD)	Percentage (%) of Positive Pools (n=120)	
			Cryptosporidium parvum	*Toxoplasma gondii*
October	30	42.69 ± 2.75	10.00	0.00
November	30	38.17 ± 3.54	0.00	0.00
December	30	36.43 ± 2.71	0.00	40.00
January	30	37.23 ± 2.34	0.00	0.00
February	30	37.20 ± 1.58	10.00	0.00
March	30	41.73 ± 4.83	0.00	0.00
April	30	39.17 ± 4.65	0.00	0.00
May	30	39.10 ± 3.19	0.00	0.00
June	30	36.93 ± 3.75	0.00	10.00
July	30	37.40 ± 2.98	10.00	0.00
August	30	38.56 ± 3.23	10.00	0.00
September	30	37.23 ± 5.18	0.00	0.00
Overall rate of positiveness			**3.33 (4/120)**	**4.16(5/120)**

Abbreviations: n = number of pools, SD = standart deviation of the mean.

The overall rate of positiveness of *Toxoplasma gondii* was 4.16 %, respectively five positive pools were detected out of 120 pools examined. The presence of a PCR fragment of 914 bp in length in agarose gels was considered as a positive identification (Figure 3). There was no apparent seasonality in detection of *Toxoplasma gondii* (Table 1).

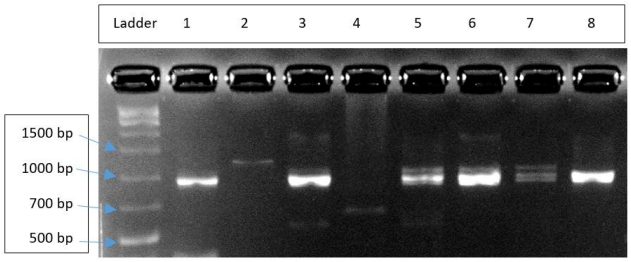

Figure 3. Image of the agarose gel, where DNA of *T. gondii* was detected. Column 1 indicates the positive control, column 2 the negative control. Columnns 3-5-6-8 bear DNA fragments of 914 bp in length and indicate positive identification.

The overall rate of positiveness of *Cryptosporidium parvum* was 3,33 %, in other words four positive pools were detected out of 120 pools examined. The presence of a PCR product of 429 bp in size in agarose gels was accepted as a positive result (Figure 4). There was slight seasonality in detection of *Cryptosporidium parvum*, since positive samples were detected mostly in late summer and early autumn (Table 1).

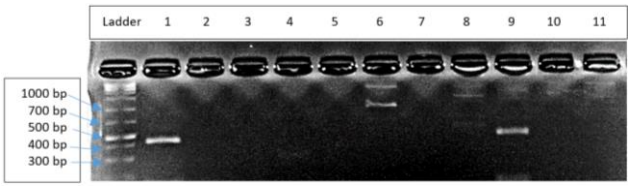

Figure 4. Image of the agarose gel, where DNA of *C. parvum* was detected. Column 1 indicates the positive control, column 2 the negative control. Columnn 9 bears DNA fragment of 429 bp in length and indicates positive identification.

Statistical analysis revealed slight positive correlation between the monthly positiveness of *Cryptosporidium parvum* and the clam length (Pearson correlation coefficient r=0,19). In Student t-test, at 95% confidence interval, the obtained correlation coeficient was regarded as statistically significant, $p<0.05$). No statistically significant correlation between clam length and *Toxoplasma gondii* monthly prevelance was detected ($p>0.05$).

DISCUSSION

The outcome of the present research was molecular detection of *Toxoplasma gondii* and *Cryptosporidium parvum* DNA by conventional PCR method in the carpet shell clams collected from İzmir Bay. This outcome was anticipated in the report of the Turkish Ministry of Agriculture and Forestry, which classified sea water of İzmir Bay as class C (Anonymous, 2022) according to its legislation criteria and the bacteriological research conducted by Kacar and Omuzbuken revealing the high fecal coliforms and streptococci loads in the inner part of the bay (Kacar & Omuzbuken, 2017).

There are many research works shedding light on accumulation, survival and viability of infectious parasitic protozoan oocysts in a number shelfish species (Fayer et al., 1998; Brown et al, 1999; Lindsay et al., 2003; Lindsay & Dubey, 2009; Rousseau et al., 2018). The authors of these research works pointed out that oocysts of *Cryptosporidium parvum*, *Cryptosporidium* spp., *Toxoplasma gondii*, *Giardia intestinalis* and *Giardia muris* can survive long therm exposure to sea water without loosing their infectivity and viability in shellfish species such as oysters and mussels. This fact makes shellfish potential phoretic agents or paratenic hosts for these emerging protozoan diseases (De Venter, 2000) and stresses the role of the shellfish edible part as the complex food matrix to which the protozoan cysts are attached and transferred to our kitchens (Rousseau et al., 2018).

The issue of public health related to consumption of bivalve mollusc harvested from the Gulf of İzmir and contaminated with parasitic protozoan cysts and viral pathogens was thoroughly investigated by Aksoy et al. (2014) and Erol et al. (2016). Aksoy et al. (2024) found significant part of the mussels (*Mytilus galloprovincialis*) collected from the Gulf of İzmir to be contaminated with *Toxoplasma gondii*. Respectively they found 9.4% overall positiveness for this protozoan parasites which is very close to the result of the present research. However, the method of choice was different and they preffered RealTime PCR for molecular detection and no investigation for the presence of the intestinal zoonotic pathogen *Cryptosporidium parvum* was carried out.

Another aspect of the present research was the correlation of the findings with the sea water pollution in İzmir Bay. According to the Ghozzi et al., (2017), water fecal contamination of the sea coasts can be measured indirectly by using carpet shell clams (*Ruditapes decussatus* L.) as biological indicator. They found at least one of the following parasites *Giardia duodenalis*, *Cryptosporidium* spp., *Toxoplasma gondii* and *Cyclospora cayetanensis* in 6.9% of the examined clams. *Toxoplasma gondii* was found at the positiveness rate of 6.6% but no *Cryptosporidium spp*. was encountered. The present research reached similar results with detection of *Toxoplasma gondii* at the overall positiveness of 4.16 %, with the addition of *Cryptosporidium parvum* detection. Although in both research works the number of examined clams in each pool was different, 9-18 specimen versus 3 specimens in ours and main method of molecular detection qPCR versus conventional PCR in the present study, both research works revealed that mollusk bivalves such as carpet shell clams can acumulate oocysts of protozoan parasites. Unfortunately, pooling of bivalves tissues might have reduced the sensitivity of the applied molecular identification methods. Higher positivity rates could be expected if bivalves are analyzed individually.

Discharged waste domestic and industrial waters from municipility and industrial waste water treatment plants around the Gulf of İzmir, presence of family cattle farms near İnciraltı coast and unusually high number of stray animals in province İzmir might contribute to the parasitic protozoan load in İzmir Bay waters. The problem with stray animals was analysed in the report of Turkish Academy of Sciences (TUBA) stressing the ever increasing risks of zoonotic diseses in Türkiye (Yardımcı, 2024). In this respect, rain water may directly wash the oocysts from open animal farms in İnciraltı district and city streets directly to the Gulf of İzmir.

Statistical analysis revealed slight positive correlation between the monthly positiveness of *Cryptosporidium parvum* and the clam length. This is due to increased capacity of larger clams to filtrate larger volume of sea water and accumulate more oocysts in their gills and digestive tracts. The same phenomenon was observed in mussels from Black sea coast, where Özer and Güneydağ found the positive correlation between mussel size and parasite numbers, respectively larger mussels harboured higher parasite loads (Özer & Güneydağ, 2015). Similar results were obtained by Villalba et al., (2005) during investigation of another protozoan parasite of carpet shell clams - *Perkinsus*. They also found that the number of protozoans is positively correlated with the clam size or age (Villalba et al., 2005). This is important finding from the point of consumption, because table sized clams are the larger ones and can harbour more oocysts of *Toxoplasma gondii* and *Cryptosporidium parvum,* increasing the risk of contracting the diseases cryptosporidiasis and toxoplasmosis. This problem can be avoided by cleansing the shellfish before marketing.

Cleansing of shellfish can be performed by the method of depuration. Indeed, Da Fonseca et al. (2006) demonstrated that carpet shell clams contaminated with relatively high dose of *C. parvum* were depurated after one day of depuration application. Thus, it is evident that carpet shell clams collected from The Gulf of İzmir and destined for the domestic or foreign markets have to be thoroughly depurated.

Generally, for molecular detection of enteric zoonotic pathogens in shellfish, conventional PCR has not been chosen as the method of choice. Real Time PCR and HRM assay (Aksoy et al. 2014), RT-nested PCR (Erol et al., 2016), qPCR (Ghozzi et al., 2017) and TaqMan PCR (Arkush et al., 2003) were prefered for detection of *Toxoplasma gondii, Cryptosporidium parvum, Cyclospora cayetanensis* and *Giardia duodenalis* oocysts in various shellfish representative species. However, the present method of choice, respectively utilisation of conventional PCR has expanded the portfolio of molecular detection methods of enteric zoonotic pathogens in shellfish. Indeed, conventional PCR has been succesfully used for detection and differentiation of toxigenic cyanobacteria accumulated in various bivalve molluscs destined for human consumption (Dittmann et al., 2017).

To the best of the authors' knowledge, there has not been previous molecular detection of *Cryptosporidium parvum* in shellfish from the Gulf of İzmir. The works of Aksoy et al. (2014) and Erol et al. (2016) were concentrated on molecular detection of *Toxoplasma gondii, Cyclospora cayetanensis, Microsporidia* spp., hepatitis A virus (HAV) and norovirus (NoV) and that of Kacar (2011) focused on bacterial pathogens such as fecal coliforms and *Salmonella* spp. in mussels (*Mytilus galloprovincialis*) harvested from the Gulf of İzmir. Thus this is the first report of accumulation of *Cryptosporidium parvum* in bivalve mollusc species in İzmir Bay.

In this research work, the oocysts of the protozoan parasites were not recovered by classical staining methods such as Ziehl-Neelsen Carbol Fuchsin staining for *Cryptosporidium parvum* (Jarad, 2020) or Kato Katz variation of Kinyoun staining for *Toxoplasma gondii* (Meireles et al., 2008). This was due to the role of shellfish as phoretic agents or paratenic hosts for these emerging protozoan diseases, which are not supposed to harbour relatively high concentration of the parasite oocysts as in the case of the final hosts. Instead only detection of the parasite DNA in the sample tissues was performed. However, to ensure food safety and the public health is at the minimum risk, we should accept the worst case scenario approach (Rousseau et al., 2018). In other words, detection of *Cryptosporidium parvum* and *Toxoplasma gondii* DNA in the carpet shell clams should imply the presence of *Cryptosporidium parvum* and *Toxoplasma gondii* viable oocysts and overestimating the risk of contracting the diseases cryptosporidiasis and toxoplasmosis by eating raw or undercooked shellfish.

This research should be accepted as preliminary investigation and in the future, experiments dealing with infection in animal models contracting the diseases cryptosporidiasis and toxoplasmosis by eating raw clams with molecularly confirmed contamination of *Cryptosporidium parvum* and *Toxoplasma gondii* DNA must be performed. In future experiments, instead of pooling the samples, clams must be examined individually to improve the sensitivity of the molecular identification method.

CONCLUSION

The present research shed light on uptake and accumulation of *Toxoplasma gondii* and *Cryptosporidium parvum* oocysts in carpet shell clams from İzmir Bay and further verified this phenomenon in alternative marine bivalve species other than the mussel (*Mytilus*

galloprovincialis). The overall positiveness of *Cryptosporidium parvum* was 3,33 % and that of *Toxoplasma gondii* was 4,16 % in 120 pools examined.

This reinforced the already existing concerns about the growing Turkish shellfish industry and parallel increase of sea water pollution in seas around Türkiye, possing food safety risks for domestic and foreign consumer eating raw or undercooked shellfish.

Shellfish farming in non polluted seawater, depuration before consumption and strict food safety control for emerging enteric protozoan parasites such as *T. gondii* and *C. parvum* are recommended.

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Author's Contributions: Esat Çilli conceptualised, performed the experiments and wrote the manuscript, Aynur Lök conceptualised and reviewed the manuscript.

Conflict Of Interest: The authors declared that there are no conflicts of interest regarding the publication of this article.

Views and opinions expressed are however those of the authors only and do not necessarily reflect those of the Republic of Türkiye Ministry of Agriculture and Forestry. Neither the Republic of Türkiye Ministry of Agriculture and Forestry can be held responsible for them.

Ethical Approval: Experimental Animals Ethical Commitee of İzmir Bornova Veterinary Control Institute, upon application and evaluation of Esat Çilli's PhD proposal "İzmir Körfezinde Akivadeslerde (*Ruditapes decussatus* (Linnaeus, 1758)) Parazit Faunasının Araştırılması/ Investigation of the Parasite fauna in Grooved Carpet Shell (*Ruditapes decussatus* (Linnaeus, 1758)) in Izmir Bay", made the decision on 5th September 2023 that there is no need for ethical approval.

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Data Availability: All relevant data are in the article.

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