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| RESEARCH ARTICLE

Molecular Identification of *Entamoeba histolytica* from Diarrhetic Patients in Baghdad Province, Iraq

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| ABSTRACT
This research used molecular detection tools to identify the amoebiasis-causing Entamoeba histolytica in individuals with diarrhea. We collected stool samples from one hundred individuals exhibiting diarrheal symptoms and categorized them based on their gender and age. Ninety percent of the samples contained entamoeba germs, according to the results of a direct microscope examination. Microscopic examination revealed that females had a much higher rate of Entamoeba infection (86.73%) than males (70.8%), and this difference was significant at the P value threshold of 0.05. Significant infection rates were also found in the age groups of 5–11 years and over 50 years (85.23 and 78.81%), respectively, whereas the age group of more than 2 years had a lower infection rate (43.45%). Using the small subunit gene, the nested multiplex polymerase chain reaction demonstrated that the infection rate for E. histolytica 91 was higher (91 percent). Compared to men (82.35%), females had a much greater prevalence of Entamoeba histolytica infection (89.23%). The infection rate was 100% attributed to E. histolytica in both the younger age group (28–48 years) and the elderly age group (>2). The percentage of individuals with Entamoeba histolytica infection was determined by nested multiplex PCR findings, gender, and age.

| KEYWORDS

Entamoeba histolytica, diarrhea, PCR, Gene Expression

| ARTICLE INFORMATION

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1. Introduction

Entamoeba is a protozoan parasite capable of infecting both vertebrates and animals. Entamoeba may induce illness in people. Entamoeba may infect both animal and plant hosts, resulting in illness (1). Entamoeba species have been detected in the human gut lumen; nevertheless, up to twenty-four Entamoeba species have been recognized globally. Entamoeba histolytica is a protozoan parasite that causes a disease ranked as the third leading cause of mortality globally, behind malaria and schistosomiasis in prevalence. Entamoeba histolytica induces the disorder referred to as Entamoebiasis (2, 3). Over 200 million individuals globally are affected by the illness, with annual fatalities ranging from 50,000 to 120,000 directly attributable to the sickness (4, 5). The fast global dissemination of the illness is mostly attributable to the prevalence of contaminated water and the lack of adequate sanitation systems in underdeveloped countries, both of which are contributing causes. Furthermore, it is becoming prevalent in these regions to overlook the signs of parasite diseases despite their potential indication of a life-threatening condition. Amoebiasis is characterized as a parasitic intestinal disease produced by the bacterium E. histolytica, which is responsible for significant mortality and morbidity. The Baghdad area has been the focus of many experiments on various species of Entamoeba, including one that uses the polymerase chain reaction (PCR) technique to detect and distinguish between Entamoeba species. Additional scholars have also performed

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investigations in this area (8, 9). The aim of this study is to use nested multiplexing to ascertain the infection rate produced by Entamoeba histolytica, considering characteristics such as age and gender.

2. Material and methods

2.1 Sample collection

Patients in the Baghdad province displaying diarrhea symptoms from 'January 4, 2021, to November 1, 2021, were eligible for fecal specimen collection, resulting in a total of one hundred specimens. The patients were examined at many healthcare establishments in the Baghdad province, including medical laboratories, health centers, General Alzahraa, and other clinics and hospitals. These individuals have been evaluated in several medical facilities operated by Waist, located around the province. The samples, collected in Eppendorf tubes, were preserved in cold storage containers after their transfer from the acquisition location.

2.2 Microscopic detection

In the laboratory, the specimens were bisected, and each segment was allocated a weight that closely approximated that of the other half (1.5-3 gm). The original material was placed in an Eppendorf tube and preserved at a temperature of minus twenty degrees Celsius for DNA extraction. This must be accomplished to extract the DNA effectively. Mount smear methods often need sufficient distilled water, generally ranging from 15 to 25 milliliters.

2.3 PCR assay

A Presto TM Stool DNA Extraction Kit, obtained from Geneaid/Korea, was used to extract DNA from the stool samples. The kits often used in this manner are typically more compact than the previously utilized kit. This is a summary of the processes executed according to the manufacturer's directives: Following the addition of 200 milligrams of feces, 900 milliliters of ST1 buffer, and a brief vortex in a bead-beating tube containing ceramic beads, the mixture was incubated for five minutes at -70 degrees Celsius, then vortexed for 10 minutes at room temperature. The sample was thereafter centrifuged for two minutes at a speed of 9,000 x g while maintained at room temperature (RT).

2.4 PCR de-inhibition

The material was incubated at four degrees Celsius for five minutes. The material was then centrifuged at 15,000 x g for three minutes at room temperature to exclude any insoluble particles and potential PCR inhibitors. The Inhibitor Removal Column was identified in the clear supernatant remaining following the usage of a 2 ml centrifuge tube. The purple ring representing this column is visible inside the image. The column underwent one minute of centrifugation at 15,000 x g at room temperature before being discarded. The centrifugation continued for one minute. The flow-through obtained from a centrifuge tube containing 2 milliliters of material was preserved for further tests on DNA binding.

2.5 Binding and washings

After incorporating 800 microliters of ST2 buffer into the flow-through, the mixture was exposed to vigorous agitation for five seconds. This technique persisted throughout the investigation. The length of this operation included the whole flow-through. A 1.5 mL collection tube was equipped with a green ring, sometimes known as a green column, and thereafter inserted into the tube. The green column referred to the ring. This procedure was executed a total of three times. The green column contained a combination of samples with a total volume of 800 microliters upon injection. Microliters were used as the measuring unit for the injection volume. The flowthrough was then centrifuged at 18,000 x g for one minute, maintaining a steady room temperature throughout the process. Place the green Column into the Collection Tube, which has a fluid capacity of 1.5 milliliters and is compatible with the Column. Subsequently, the remaining components of the sample combination must be adjusted using the green column. The components were centrifuged for one minute at 18,000 x g, with the temperature maintained at room temperature (RT) during the procedure. The ST2 Buffer, with a total capacity of three hundred and fifty liters, was added to the green column. Subsequently, they each proceeded along their own trajectories for thirty seconds, subjected to an acceleration 16,000 times more than the average gravitational acceleration in RT. Subsequent to the removal and disposal of the flowthrough, the green column was reinserted into the 1.5 ml collection tube to enable a second collecting cycle. This was executed to facilitate the collection of the sample. This was executed to ensure

the complete collection of the sample, and it was successful. A total of 500 microliters of Wash Buffer was determined to be required for the green Column. This was a total amplification of volume. Subsequently, they had an experience equivalent to 18,000 times their body weight in g, followed by a separation at R lasting thirty seconds. The green column must be reinserted into the 1.5 ml collection tube immediately after discarding the flow-through. To dry the column matrix, the dry green column collecting tube was centrifuged at 18,000 x g for three minutes at room temperature. This was conducted while maintaining room temperature.

2.5.1 Qualification of DNA
An examination and assessment of the quality of genomic DNA extracted from fecal samples were conducted using a Nanodrop spectrophotometer. The samples were collected from several persons. The action was executed as intended. The subsequent approaches, detailed below, demonstrate the use of this equipment to investigate and evaluate DNA quality. This is achieved by quantifying the absorbance of the sample over a range of wavelengths (270-280 nm). x Upon activation of the Nanodrop software, choose the application that most closely meets your specifications about nucleic acid, namely DNA. During the cleaning of the measurement pedestals, we found it necessary to use dry wipes on many occasions. To accurately reset the instrument, meticulously pipette two microliters of nuclease-free water over the surface of the lower measurement pedestals. This will guarantee that the gadget is accurately calibrated.

2.5.2 PCR reaction
Gene Aid, a company headquartered in Korea, generously supplied the three extraction kits required for the extraction of DNA from human feces and urine. Additionally, forward and reverse primer pairs were used to achieve amplification of the 19S rRNA gene by nested multiplex PCR. This was executed several times to facilitate the gene's replication. This was conducted to ascertain the presence of the gene beyond a reasonable doubt. The first primer pair was designed to facilitate the identification of 900 base pairs (bp) of the 19S rRNA gene in Entamoeba. Primers were used concurrently, and this combination was subsequently employed in the study of (E. histolytica). This study used a one plus primer design and utilized NCBI-Genebank as its main data source. This primer, including 800 base pairs, is applicable in nested PCR studies involving E. histolytica and nested PCR assays for Entamoeba spp. It was generously provided by Macroge Corporation in Korea (532 bp). Primers and probes are two examples of supplementary PCR reaction components that may be included inside a typical Maxime PCR PreMix bottle. These elements are referred to as primers and probes, respectively. The PCR component specified in the table above is included inside this tube. The PCR component detailed in the table is included inside this tube, which includes dNTPs, pH 9.0, KCl, MgCl2, stabilizer, tracking dye, Tris-HCl, and Taq DNA polymerase. Subsequently, a PCR tube was placed in an Exispin vortex centrifuge, which was operated at a speed of 4000 rpm for three minutes. The procedure was executed thrice. This technique was conducted three times in total. Subsequently, the samples were placed into a PCR thermal cycler for processing. The acronym for "polymerase chain reaction" is PCR. The components of the PCR master mix detailed in the preceding table are then included in a standard Maxime PCR PreMix, which exemplifies a formulation that includes all necessary elements for the polymerase chain reaction. Subsequent to this stage, the PCR master mix is prepared for utilization. Once the PCR master mix has been prepared, the following step may be executed. The table above provides a description of the many components of the PCR master mix, including dNTPs, pH 9.0, KCl, MgCl2, stabilizer, tracking dye, Tris-HCl, and Taq DNA polymerase. Subsequently, the PCR tubes were centrifuged for three minutes at a velocity of four thousand revolutions per minute using an Exispin vortex centrifuge. Subsequently, the samples were placed into a PCR thermal cycler and permitted a duration to complete their separate procedures.

2.5.3 Estimation analysis
Upon completion of all procedures, the PCR results were evaluated by agarose gel electrophoresis. Agarose gel with a one percent concentration was prepared by dissolving 2X TBE in a water bath at one hundred degrees Celsius for fifteen minutes, followed by cooling to fifty degrees Celsius. This procedure was executed to produce the gel. This technique was executed to ensure the successful production of the gel. Subsequently, three microliters of ethidium bromide dye were added to the agarose gel solution for coloration. This was executed to facilitate the use of the gel. Upon completing the precise placement of the comb in the tray, the agarose gel solution was then poured into the tray. The operation was performed many times until the tray reached its maximum capacity for stuffing. After

permitting the comb to be set for fifteen minutes at room temperature during the preparation of the agarose gel solution, it was meticulously removed from the tray after the completion of the first setting time. This occurred concurrently with the preparation of the agarose gel solution. The treatment was provided three times during the research. Position 6 on the gel tray inside the electrophoresis chamber was modified to accommodate the addition of a 2X TBE buffer. The chamber houses the gel tray inside its boundaries. The PCR product was dispensed into each well of the comb at a volume of 10 microliters, except for the first well, which received just 4 microliters of the PCR product. Four microliters of the polymerase chain reaction product. The first well functioned as a control for the experiment (120bp Ladder)'. Subsequently, there was an electrical current source that persisted for a whole hour, providing 120 volts and 90 amperes. This source was there subsequent to the incident. A transilluminator emitting ultraviolet (UV) light was used to visualize the PCR results (10).

3. Results

3.1 Microscopical findings

Our investigation revealed that the incidence of infections attributed to Entamoeba spp. was much greater in females (86.73%) than in men (70.8%). Due to natural dynamics, females are more predisposed to engage in food preparation and domestic tasks, hence increasing their exposure to water or food infected with entamoeba cysts. The disparity in infection rates between females and men may be ascribed to several variables, including hormonal influences and the tendency for females to engage more often in food preparation and domestic tasks. The latest revision's data indicated that the biggest number of infectors was seen in the age range of '5-11 years and in those over 49 years old. Entamoeba species were identified as the causative agents of these observations. An infection rate of 85.9 percent was observed in both age groups. The cohort under 2 years had a reduced infection rate compared to other age groups, which recorded a percentage of 63.23%. Microscopic examination revealed the presence of trophozoites of the parasite Entamoeba histolytica in both female and male positive samples (figures 1, 2, 3).

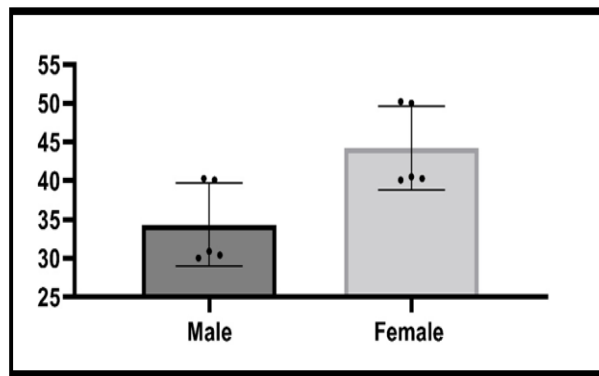

Figure 1: Microscopic detection *Entamoeba histolytica* could be seen in both female and male positive samples

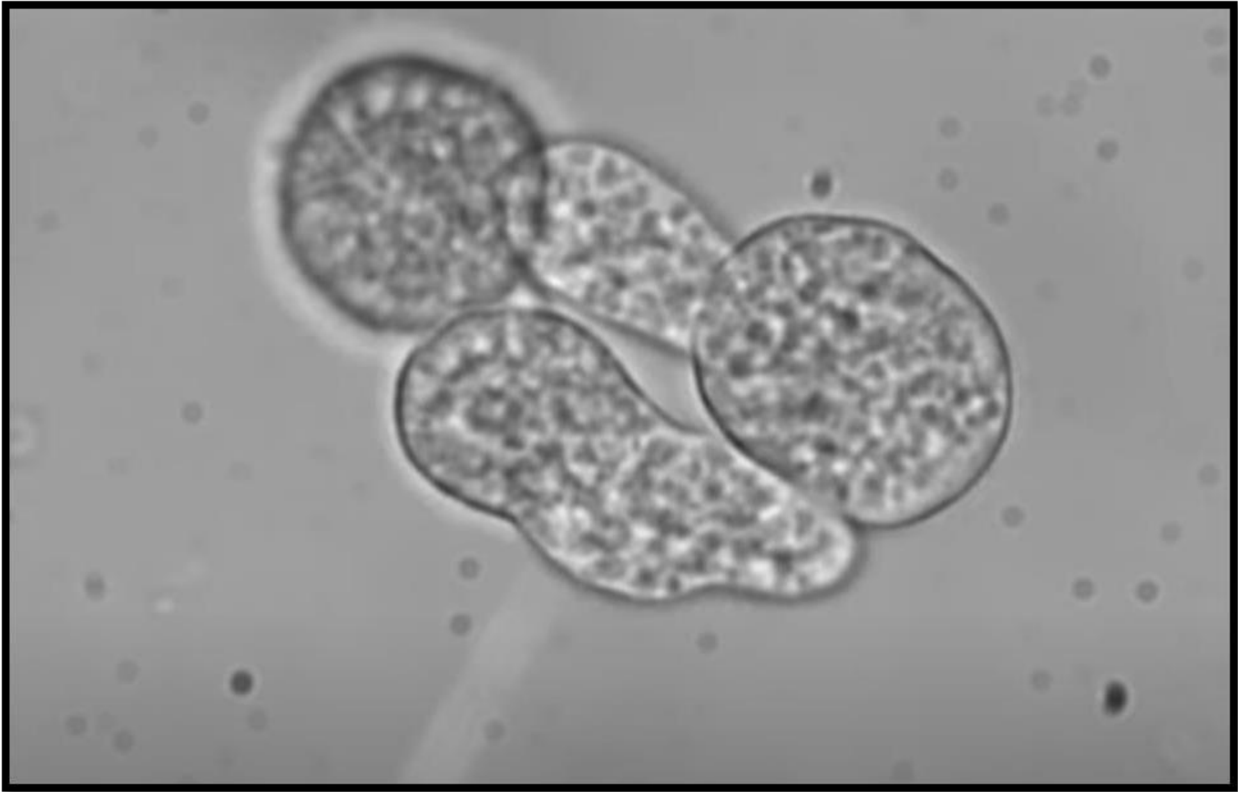

Figure 2: Microscopic detection *Entamoeba histolytica* could be seen in male positive samples

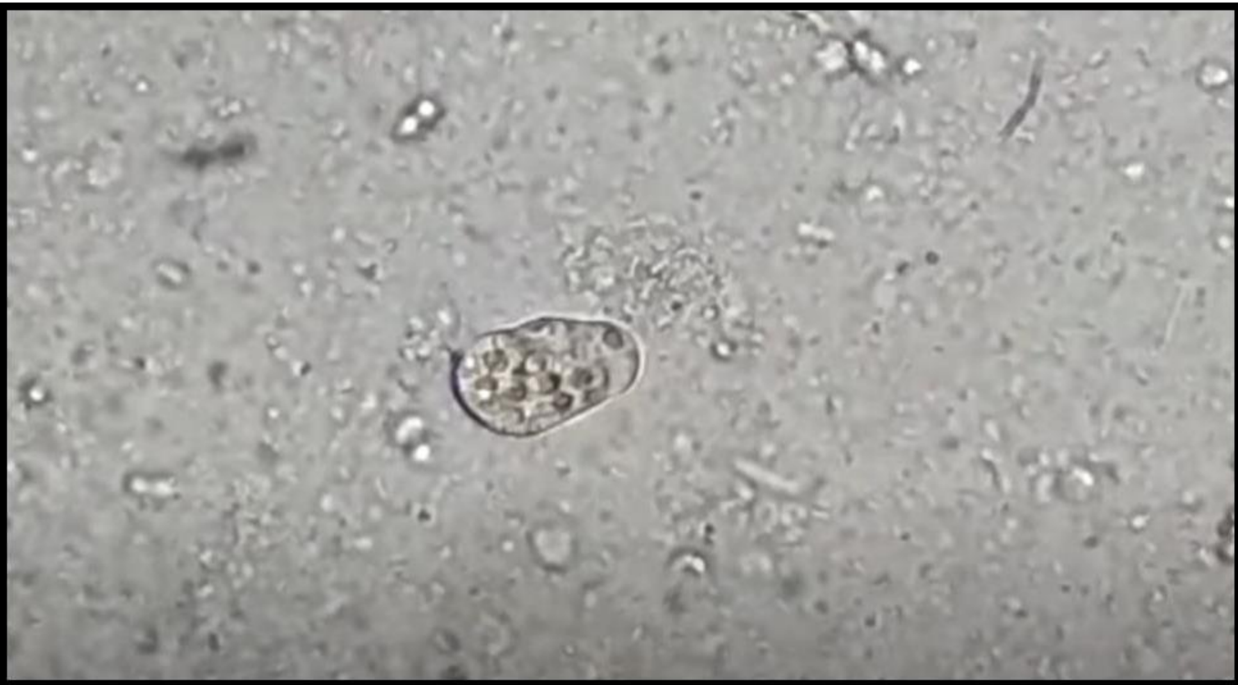

Figure 3: Microscopic detection *Entamoeba histolytica* could be seen in female positive samples

3.2 Molecular finding

The first round of PCR for DNA samples indicated that 79 of 100 feces samples effectively amplified the 19sRNA gene via nested multiplex PCR. This may be computed to have a success rate of 79 percent. Although Entamoeba infections are often moderate, some strains may adhere to the gut wall, potentially resulting in severe extraintestinal

pathogens and amoebic colitis. Notwithstanding this, amoebic dysentery is evident in clinical environments (Table 1, Figure 4)'.

Table 1: Molecular detection *Entamoeba histolytica* could be seen in both female and male positive samples

Gender	Samples of study	Infected results (+)	Total Percentage
Male	54	59.2	70.8%
Female	46	64.23	86.73%
Significancy	P. value = 0.002		

P. value <0.005

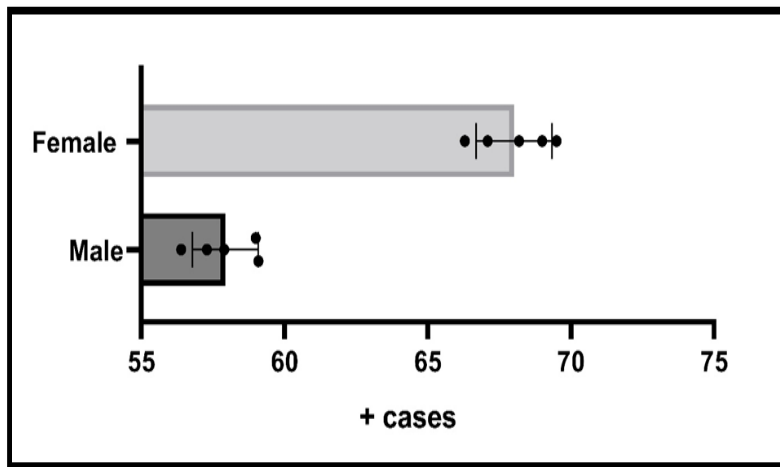

Figure 4: Molecular detection *Entamoeba histolytica* could be seen in both female and male positive samples

4. Discussion

The results of our research align with previously reported data, indicating that the waist region in Iraq has a larger female-to-male ratio (11). Conversely, the data from Baghdad indicated that females exhibited a much greater infection rate than men (86.73 percent), in contrast to the infection rate seen in males (70.8%) (12). However, a recent study indicated that the prevalence of Entamoeba spp. infections were higher in females '(44.1%) than in men, the researchers determined that males were equally vulnerable to the parasite (22.3%). Women are more susceptible to exposure to water or food contaminated by Entamoeba cysts during cooking or cleaning activities (14). The disparity in infection rates between females and males may be attributed to several factors, including hormonal influences or inherent biological differences, which render females more susceptible to infection upon exposure to water or food contaminated with Entamoeba cysts (15). Entamoeba species identified in The latest study results indicate that both the age group of 5-11 years and the age group over 49 years exhibited a 65% prevalence of infectors within their respective populations (16). The cohort under 2 years of age had a reduced overall infection rate, recorded at 45.3 percent. A greater incidence of infection was seen in children aged 5 to 14 (31%), but a lower frequency was noted in children aged 0 to 2 years. This aligns with our prior knowledge (11%) (17). Similarly, numerous researchers observed a higher prevalence of Entamoeba spp. infections in young children in Iraq and Yemen, noting a significant infection rate of 50.2% among those under ten years of age while reporting a markedly lower infection rate of 7.7% in individuals over 45 years (18). This occurred despite the observation of a reduced infection incidence among those over 45 years of age (19). The impact on youngsters will be characterized by numerous factors stemming from their activities, which emerge from their diminished concern for personal hygiene (20). These activities stem from the youngsters dedicating less time to self-cleaning (21). Furthermore, the regions of their bodies that are exposed possess the largest amounts of nutrients derived from diverse sources (22). Some individuals may exhibit resistance to infectious organisms owing to the use of medications that target certain pathogens (23). The elevated infection incidence in older adults may be attributed to factors such as compromised

immune systems; however, this does not apply universally to all senior persons (24). Although a high infection incidence in older age may be the underlying source of this detail, it remains valid (10, 25). In the first round of PCR for DNA samples, the 19sRNA gene was effectively amplified in 86.8 percent of the stool samples via nested multiplex PCR, as shown by the findings of that testing phase (26). Although Entamoeba infections are generally not hazardous in most instances, some strains may adhere to the gut wall, leading to severe extraintestinal pathogens and amoebic colitis (27). These circumstances may be induced by certain strains of Entamoeba. Amoebic dysentery is consistently evident in the clinical presentation. Through nested PCR, the researchers determined that 55% of the 80 samples collected in Baquabah City were positive (28). These results align with those seen in Iraq, which reported a lower proportion than that documented in this city (29). However, the nested multiplex PCR technique achieved an even greater proportion (88%) (30).

5. Conclusion

The latest data indicated that individuals experiencing diarrhea had an elevated incidence of infection with Entamoeba histolytica. This information was obtained in alignment with the results of a prior investigation. The study results indicated that female participants exhibited a markedly greater infection rate of E. histolytica compared to male participants; however, when analyzed using the P0.05 level, no significant difference was seen between the two groups. Although microscopic examination is a dependable approach for detecting Entamoeba spp. in diarrheal patients, there exists a significant likelihood of misdiagnosing amoebic dysentery as being attributable to Entamoeba histolytica. This was attributable to the microscopic inspection method's dependence on the observation of a limited number of organisms'. This was due to the germs being too tiny to be seen by the naked eye, even under microscopic examination. This study's results indicate a high frequency of E. histolytica infection among those aged under 2 years and those between 25 and 49 years old. The median age of the research participants was 25 years.

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