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Seropositivity for toxoplasmosis and other protozoan infections in patients with ocular diseases in Kirkuk Province, Iraq

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ABSTRACT

Background: Toxoplasmosis, a zoonotic disease, has serious consequences for human health, including the development of ocular issues such conjunctivitis and chorioretinitis.

Setting: A cross-sectional study carried on 156 patients with ocular problems (92 men and 64 females).

Methodology: Following the completion of a questionnaire, blood samples were taken for serology (ELISA), two ocular swabs for direct microscopy and culture, and further blood specimens for hematology. Auramine and calcofluor luminous stains were also used .

Results: The overall rate of infectious agents was 46.69%, according to the findings. Toxoplasma gondii, Chlamydia trachomatis, protozoan parasites, yeast, Human Herpes Simplex virus, bacteria, and Syphilis were found in 78.20%, 71.69%, 32.69%, 32.69%, 29.10%, 24.35%, and 23.35% of those who tested positive Seropositive toxoplasmosis was common, especially IgM antibody, which was 26.67% in elderly patients and 11.11 % in one-year-to-15-year olds. unlike Toxo-IgG Abs. The rates of ocular infections caused by other protozoa were 17.68%, 11.10% for Microsporidia and Acanthamoeba, and 3.42 % for Naegleria fowleri. Microsporidia distribution with patient age was statistically significant, particularly for younger patients. Toxo-IgM was linked to a large number of cases of keratitis, but Toxo-IgG was linked to cases of conjunctivitis. The link between patient blood group and toxoplasmosis incidence was considerable; blood group O was dominant when compared to other ABO blood groups.

Conclusions: In Kirkuk Province, the prevalence of toxoplasmosis among individuals with eye problems is high. Soil- and water-dwelling amoebas and microsporidia have been related to eye infections.

Keywords: *Toxoplasma, Chlamydia, Conjunctivitis, Keratitis and ELISA*

INTRODUCTION

Cats are the definitive hosts of Toxoplasma gondii, an obligate intracellular protozoan that belongs to the phylum Apicomplexa. Other mammals, including humans, are intermediate hosts in this complex life cycle [1]. Tachyzoite proliferation characterizes the acute stage of the infection in intermediate hosts. The parasites then develop tissue cysts in many organs, including the brain, and eye, and create a chronic infection [2]. Ocular toxoplasmosis is a disease that can be passed on from mother to child (vertical transmission) or from person to person(acquired). Anyone, anywhere in the world, can get it. Once parasites get into the bloodstream and reach the retina and retinal pigment epithelium, they might hurt or kill cells. Transmission inside leukocytes, free parasites via the paracellular route, and endothelial cell infection are the three ways in which T. gondii gains access to the retina [3 and 4]. Microsporidia are protozoan parasites that can also cause infections of the eye. Soil and water-free amoebas are other potential causes of ocular infections. Primary meningoencephalitis is the most common complication that can be brought on by Naegleria fowleri; however, ocular infections can be brought on by swimming in rivers, using swimming poles, or using swimmers' baths [5 and 6]. whereas the transmission of Acanthamoeba to humans results in persistent granulomatous amoebic encephalitis in addition to ocular keratitis In the early stages, clinical signs of Acanthamoeba keratitis include gray-dirty epithelium, pseudo-dendriform epitheliopathy, perineuritis, multifocal stromal infiltrates, and ring infiltrates. In the later stages, clinical signs include scleritis, iris atrophy, anterior synechiae, secondary glaucoma, mature cataract, and chorio-retinitis [7]. Nosema nosema is a common microsporidian protozoan that dwells in the eyes and causes damage to eye compartments, including retinal pigmentation. It is transmitted from person to person through direct contact with infected eye tissue [8]. Since earlier studies in 1989, the rate of seropositive toxoplasmosis in Kirkuk Province, Iraq, has been high, with an estimated rate of 63.80% in 2005 [9] compared to 84.95% in 2019 by [10]. Toxoplasmosis is properly diagnosed by isolating Toxoplasma gondii stages from aborted tissues. Most studies in this area relied on serology to detect toxoplasmosis [11] because of its speedy and easily obtained results; hence, altering the reliability of serology may have contributed to this variation in infection rates. Researches concerning ocular toxoplasmosis in Iraq were very rare and not given much attention by authors, and even other researches in relation to other infectious agents like bacterial and viral are absent according to available data. Furthermore, due to the severity of ocular toxoplasmosis and the majority of patients visiting ophthalmic clinics suffering from conjunctivitis and ocular keratitis, the current study was designed to evaluate that.

MATERIALS AND METHODS

Period and place

This research was carried out between October 2022 and March 2023 at the Azadi teaching hospital and the Ibn-Nafees medical private lab in Kirkuk, Iraq.

Study design and subjects

Patients aged one year and up to 46 years old were included in the cross-sectional study of ocular diseases.

Exclusion criteria

People who were pregnant or taking anti-Toxoplasma drugs were also ruled out because their blood tested positive for other BOH-causing agents like CMV, rubella, and the human Herpes simplex virus.

Ethical approval

On October 27, 2019, under administrative issue 879 and the responsibilities of the scientific committee at the Kirkuk Health Directorate, the study was permitted to move forward. In a separate questionnaire, each participant's information and permission to take part in the study were written down.

Sample collections and serum separation

Patients who intended to attend ophthalmic clinics were chosen by ophthalmologists, and two sterile bacterial swabs were taken from the congested area of the eye due to suppuration and immediately socked into the second container containing transport media. while corneal scraping was meticulously performed with a scalpel. Five ml of venous blood was drawn from each patient using a sterile technique. The contents of the syringe were drained into a tube that contains a clotting accelerator jell (activator) for clear serum separation. The second After ten minutes, the substance was centrifuged at 3000 RPM for five minutes. Then, non-hemolysis, non-icteric, and non-lipemic blood was transfused into another sterile plan tube and preserved at -20 degrees Celsius until use [12].

Toxo plasma gondii antibodies(Abs)detections

Both IgM Abs and IgG Abs ELISA kits were bought from BioActiva in Germany in different ways by Bio-zek Company (Netherlands) and transported to Kirkuk City. For each serum, both antibodies were tested using the ELISA complete system machine; briefly, 100 microliters of serum were added to ELISA well, agitated, and then left at 37 °C for 30 minutes. The contents of the microwells were discarded and washed with diluted wash solution four times, dried, and 100 microliters of HRP conjugate were added to each well. They were incubated for 30 minutes at 37 °C, then washed, dried, and 100 ml of TMB substrate was added to each ELISA well. The microwell plate was kept in a dark place for 15 to 20 minutes to obtain a blue color, which refers to the positivity of the sample. Finally, 100 microliters of stop solution were added to each ELISA well to stop the reaction by producing a yellow color. The intensity of the yellow was determined using the ELISA reader machine within 20 to 30 minutes. And it was referring to the amount of the antibody present in the tested specimen. For the Toxo-IgG Abs assessment, the procedure was the same, and the difference was in the type of HRP conjugate only. These procedures were done according to the manual instructions of each ELISA kit [13].

Parasites detection from eye swabs

The second swab was used to detect three targeted protozoan parasites mainly Acanthamoeba, Naegleria, and Microsporidia as well as for other parasites that may be found in the specimen. For these purposes, direct microscopy using double wet preparation of 0.85% NaCl and 3 % of Lugols iodine was applied for each swab [14], and for confirmatory flotation technique using 33% of $ZnSO_4$ was applied[15].

Fluorescent staining

Three clean slides were taken for each swab, brushed on the center of slides to obtain a smear, and air dried fixed by gentle flaming then the first slide was stained by a phenol-auramine solution which was spilled over each ocular smear for 15 minutes after they were air dried and cured with moderate flames, as described here for fluorescent staining using phenol-auramine. Distilled water was used to clean the slide. After two or three minutes in the decolorizer (acid alcohol solution), the slide was removed. If pink remained after the initial decolorizing process, the slides were reprocessed. Distilled water was used to thoroughly rinse the slide, and the excess was shaken off. The slide was given 3–4 minutes to absorb the counterstain (potassium permanganate solution). dried on hangers or by blotting with distilled water after cleaning. verified using oil immersion lenses and a fluorescence microscope at low and high magnifications of 400X (UV light source) [16]. The second smear was stained with calcofluor stain as follows "Calcofluor white stain is created by combining Calcofluor White M2R at a concentration of 1 g/l with Evans blue at a concentration of 0.5 g/l. Following the application of one drop of the Calcofluor White stain to each ocular smear, one drop of 10% potassium hydroxide was placed on top of the stain. After waiting for one minute with the sample covered with a fresh cover slip, the cover slip was removed. Under ultraviolet illumination, specimens were examined at magnifications of 100x to 400x [17].

The first swab for each patient proceeded for culture and sensitivity using routine culture media, gram staining, biochemical tests, and antibiotic sensitivity on Muller Hinton agar medium [18]. Results of viral infection and bacterial isolation will be published separately in other manuscripts.

Statistical analysis

All of the data was entered into the appropriate tables in a Microsoft Excel file for statistical analysis. Differences in the prevalence of Toxoplasma gondii antibodies and other protozoan parasites were analyzed using the chi-square and t-student test for imposing variances and significance at the P 0.05 and P 0.01 levels, respectively.

RESULTS

Table 1 summarizes the demographic data of patients enrolled in the trial, including the total number, average age for both genders, residency and enrollment, and symptoms associated with the patients' illness.

TABLE 1: Demographic data of patients enrolled the study.

Parameters	**Values**	**Parameters**	**Values**
Total number	156		
Mean age	38.83 ± 4.78	Artificial lens	3
Mean age for both gender	34.60 ± 4.59	Eye stuffness	67
Residency		Itchy eyes	119
Rural aera	48	Tears	123
Urban aera	108	Blurred vision	3
Conjunctivitis	153	Edema	5
None Conjunctivitis	3	Red eyes	43
Keratitis	8	Eye burning and pain	73
None keratitis	148	suppuration	72

The overall rate of infectious agents was 46.69%, according to the findings. Chlamydia trachomatis, Toxoplasma gondii, protozoan parasites, yeast, Human Herpes Simplex virus, bacteria, and Syphilis were found in 78.20%, 71.69%, 32.69%, 32.69%, 29.10%, 24.35%, and 23.35% of those who tested positive; table.2.

The seropositive toxoplasma rate 71.86 % involved 18.16 % for Toxo-IgM versus to 53.70 % for Toxo-IgG antibody $p<0.05$. According to age, the following high rates were recorded 26.67% and 23.26 % for Toxo-IgM were recorded in patient age over 61 years and 30 to 45 years respectively, versus to 11.11 % in patient aging from 1 to 15 years ,$P<0.05$.Table-3.

TABLE 2: Frequency of Toxoplasma gondii antibodies both IgM and IgG regarding patient age

Type of infectious agents	Total positive		Total Negative	
	No.	%	No.	%
Chlamydia trachomatis	122	78.20	34	25.80
Toxoplasma gondii	113	71.86	43	28.14
Protozoan parasites	51	32.69	105	67.31
Yeast (Candida species)	51	32.69	105	67.31
Human herpes simplex virus	44	29.10	112	70.90
Bacteria	38	24.35	118	75.65
Treponema pallidium	33	23.35	113	76.65

infections = 46.69 %. Sum of

TABLE 3: Positive and negative proportions of various infectious agents among ocular

Age group/ Years	Total No. Exam.	%	Positive *				Total Positive (IgM and IgG)		Mean± s.e IgM	Mean± s.e IgG
			Toxo-IgM		Toxo- IgG					
			No.	%	No.	%	No.	%		
1 to 15	45	28.85	5	11.11	25	55.56	30	66.67	1.03±0.04	1.25±0.06
16 to 30	29	18.59	5	17.24	16	55.17	21	72.41	1.154±0.13	1.598±0.07
31 to 45 *	43	27.56	10	23.26	27	62.79	37	86.05	0.984±0.02	1.521±0.04
46 to 60	24	15.38	3	12.50	10	41.67	13	54.17	1.02±0.09	1.698±0.08
61 >	15	9.62	4	26.67	8	53.33	12	80.00	1.015±0.03	1.65±0.12
Total	156	100	27	18.16	86	53.70	113	71.86	1.04±0.03	1.54± 0.08**

*P<0.05 t=0.423 **P>0.05 F= 0.46

According to patient ages, Table 4 lists additional parasitic causes of ocular conjunctivitis. Microsporidia had the highest rate (17.68%), followed by Acanthamoeba (11.10%), and Naegleria fowleri (3.42%). Patients aged 1 to 15 years had a higher rate of protozoa than Toxoplasma, 10.26%, while patients over 61 years had the lowest rate, 2.56%, P<0.05. Except for Acanthamoeba species, where there was variation between each protozoan detected and patient age, the lowest rate of 6.98% for Acanthamoeba species was found in patients aged 31 to 45 years, whereas Naegleria was not found in patients aged 46 to 60 and 1 to 15 years. Also, when compared to other age groups, a patient over 61 had a low rate of Microsporidia of 6.67%, indicating distributional variability.

In table 5, Toxoplasma gondii-associated ocular infection symptoms and frequencies are displayed; the following rates were recorded: 98.07%, 77.56%, 75.64%, 44.23%, 42.30%, 29.48%, 23.07%, and 4.48% for conjunctivitis, an excess of tears, itchy eyes, pain, eye stuffiness, red eyes, and burning. Considering Toxo-IgG Abs, the rate of keratitis was 85.71 percent, while the rate of burning sensation was 52.71 percent. In contrast to Toxo-IgM, which demonstrated high rates of 28.57 percent for keratitis and ocular congestion compared to pain, the rate was 10.26 percent.

TABLE 4: Age-related parasite prevalence (including Acanthamoeba, Naegleria, and Microsporidia)

Age groups	Total		Acanthamoeba		Naegleria		Microsporidia		Total	
	No. exam	%	No. exam	%	No. exam	%	No.	exam%	No. exam	%
1 to 15	45	28.85	4	8.89	0	0	12	26.67	16	10.26
16 to 30	29	18.59	4	13.79	1	3.45	4	13.79	9	5.76
31 to 45	43	27.56	3	6.98	3	6.98	7	16.28	13	8.33
46 to 60	24	15.38	3	12.5	0	0	6	25.00	9	5.76
61 >	15	9.62	2	13.33	1	6.67	1	6.67	4	2.56
Total	156	100	16	11.1	5	3.42	30	17.68	51	32.69 *

* involve Blastocystis hominis (1) and two cases of ocular Myiasis

Table 6 lists the symptoms of an eye infection and the frequency of other protozoan parasites. For example, conjunctivitis was very common (18.95%) with Microsporidia and 10.46% with Acanthamoeba species, but keratitis was very common (28.57%) with Naegleria fowleri. On the other hand, 23.08% of people who took Microsporidia had pain, and 21.2% had stuffy eyes.

TABLE 5: Symptoms associated with ocular infections in relation to Toxoplasma and human

Symptoms	Total No. exam. %		positive			
			Toxo-IgM No. exam.	%	Toxo-IgG No. exam	%
Eye stuffiness in the morning	66	42.30	14	21.21	39	59.09
Itchy eyes	118	75.64	17	14.41	70	59.32
An excess tear	121	77.56	22	18.18	72	59.50
Conjunctivitis	153	98.07	27	17.65	92	60.13
Keratitis	7	4.48	2	28.57	5	71.43
Suppurated	69	44.23	10	14.49	37	53.62
Pain	78	50.00	8	10.26	45	57.69
Red eye	46	29.48	5	10.87	26	56.52
Burning	36	23.07	4	11.11	19	52.78

TABLE 6: Symptoms associated with ocular infections regarding to another protozoan parasites.

Symptoms	Total No. exam%		Acanthamoeba No. exam%		Naegleria No. exam%		Microsporida No. exam%	
Eye stuffiness in the morning	66	42.30	9	13.64	4	6.06	14	21.21
Itchy eyes	118	75.64	12	10.17	5	4.24	23	19.49
An excess tear	121	77.56	14	11.57	5	4.13	23	19.00
Conjunctivitis	153	98.07	16	10.46	4	2.61	29	18.95
Keratitis	7	4.48	1	14.29	2	28.57	1	14.29
Suppurated	69	44.23	9	13.04	4	5.80	9	13.04
Pain	78	50.00	10	12.82	4	5.13	18	23.08
Red eye	46	29.48	1	2.17	1	2.17	4	8.70
Burning	36	23.07	5	13.89	0	0	9	25.00

Table 7 shows that the ABO blood group of patients with ocular issues is associated with their rate of Toxoplasma gondii seropositivity, with the highest rate30.12 % documented in the O blood group, followed by 14.74% in the A blood group, and 9.61% in the B blood group. whereas the AB blood group had the lowest rate of all, at only 4.48%. $p<0.05$. By comparing O to other blood groups, Toxo-IgM antibodies were statistically significant, while Toxo-IgG antibodies were not.

TABLE 7: Seropositive distribution Toxoplasma gondii in ABO-related ocular disorders.

Blood groups	Examined No.	%	IgM Abs No	%	IgG Abs No.	%	Total Toxoplasma +ve No.	%
A	38	24.35	6	12.76	17	36.17	23	14.74
B	28	17.95	4	14.28	11	39.28	15	9.61
AB	10	6.41	1	10 .0	6	60	7	4.48
O	80	51.29	16	34.04	31	65.96	47	30.12
Total	156	100	27	17.30	65	41.66	92	58.98

Twenty-one of IgM +IgG equivocal were not excluded

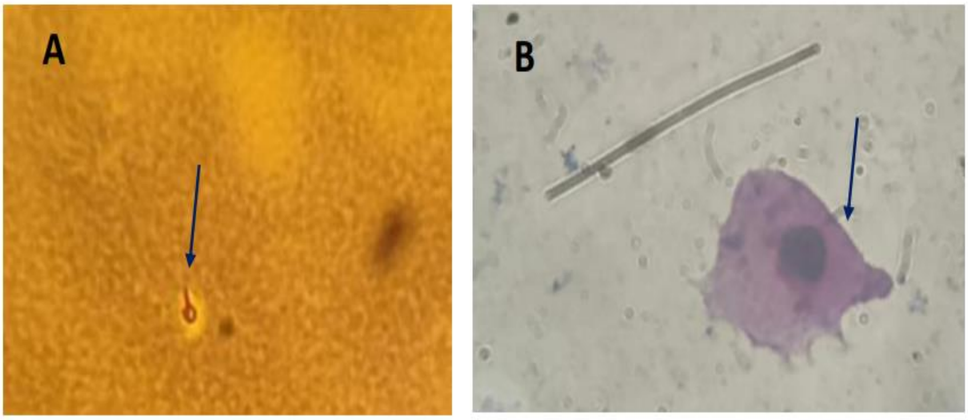

FIGURE A: representing Microsporidia parasite with extrusion of endo flagellate (pellicle) fluorescent phenol-auramine stain 40X. **FIGURE** B. Acanthamoeba spp. trophozoite Calcofluor white stain showing massive nucleus and multiple spike like pseudopodia 100 X visualizing by normal microscope.

FIGURE C: An ocular smear stained with Giemsa shows the trophozoite of Naegleria fowleri with a large nucleus and two flagella at a magnification of 40X.

DISCUSSION

When the protozoan parasite "Toxoplasma gondii" gets into the eye, it deposits itself as a pigment on the retina. This makes the retina less effective and can eventually lead to acute macular retinitis associated with primary acquired toxoplasmosis, requiring immediate systemic therapy. and peripapillary scars secondary to toxoplasmosis. [19]. Other protozoan infections, like those caused by Acanthamoeba species, Microsporidia, and Naegleria fowleri in contaminated water, can also cause conjunctivitis and other squeals. Nematoda also lives in the eye socket like Loa Loa, which can cause conjunctivitis, vision loss, or even bleeding into the eye socket. Animal ascariasis, caused by parasites like Toxocara cati, can cause blindness and other symptoms due to the aberrant migration of their larvae, including damage to the eyes [20]. As a result, the overall rate of microbial infection in this province was high at 46.69%, which highlighted the fact that people were exposed to multiple sources of contamination. The primary source of contamination was the oocysts of Toxoplasma gondii, which contaminated the soil and water. This was because cats live on the thresholds of houses and spread the infective stage throughout the environment, particularly in gardens and water sources[9]. Another theory is that the lives of people in Iraq changed after 2003, especially their habit of eating frozen chicken meat imported by local markets and the fact that the infective stage of the protozoa could get through home water filters. All of these factors contributed to 53.70% of patients having positive Toxo-IgG antibodies which indicate previous exposure to the parasite[21]. In the meantime, 18.16 percent of cases, or 27, tested positive for the Toxo-IgM antibody. This result is more significant to take into account because a positive result of this type indicates a recent primary infection and an acute infection after IgG antibodies have been ruled out. This conclusion is reflective of the fact that people's exposure to events that invade the tachyzoites of the retina leads to scarring in the macular and peripapillary regions of the retina. In the meantime, 18.16 percent of cases, or 27, tested positive for the Toxo-IgM antibody. This result is more significant to take into account because a positive result of this type indicates a recent primary infection and an acute infection after IgG antibodies have been ruled out. This conclusion is reflective of the fact that people's exposure to events that invade the tachyzoites of the retina leads to scarring in the macular and peripapillary regions of the retina. [22]. The high proportion of 26.67% of seropositive Toxo-IgM among older patients suggests that this age group has a weak immune system, which is particularly concerning in light of the COVID-19 outbreak that has been going on around the world. This finding was consistent with those that were recorded in the same province as those that were reported by [23].and disagree with that recorded by [24] who found only Toxo-IgG antibodies.

For a different protozoan ocular infection, the rate of 32.69% was also high. However, when the rate was compared to the patient's age, it became clear that the younger the patient, the higher the rate was. This difference may be due to the younger patients' higher levels of motivation and activity, which may expose them to these parasites more frequently than older patients. Together with their daily routines, they also acquire contaminants via swimming, eating improperly cooked meat (pork), and wearing contact lenses [25]. These facts, along with the fact that these parasites are neglected in Iraq and that research is scarce or dispersed, can help explain why the rate of individuals with ocular disorders was so high. Due to the lack of prior research on our destination, we made recommendations based on our observations. On this particular point, it was decided that discovered in the Chicago metropolitan area by [26]. This is the first time Naegleria fowleri has been found in Kirkuk, Iraq. Five people between the ages of 16 and 45 were affected. Most likely, the infection was spread by coming into contact with contaminated water or by using contact lenses that had been stored in solutions with the protozoan.

Toxoplasma incidence correlated with the ABO blood group; 30.12% of the infection was found in women with the O blood group, which was higher than in other blood groups. This can be explained by the fact that the surface of this type of blood group is free of any antigens, which means that it won't cause any antibody crosses compared to other blood groups. Also, the O blood group was found to be more common among people around the world [27]. On the other hand, there are 4.42% of records of people with blood group AB. This is most likely because this blood group isn't very common [28].

Toxoplasmosis shows up clinically in an intermediate host, like a human, when oocysts that have been eaten burst and release the tachyzoites. The tachyzoites then travel through the whole blood and reach different organs, like the eyes, over time. As this trophozoite reaches the retina, it will immediately enter a parasitophorous vacuole and encyst [31]. Ocular toxoplasmosis has both specific and general symptoms, like excessive tears and itchy eyes, but it eventually leads to chorioretinitis [32]. The very high reported rate of conjunctivitis (98.05%) could be caused by other infectious agents. Moreover, the rupture of tissue cysts found within previous lesions has been linked to the development of reactivation of retinitis in 17.65% of conjunctivitis cases associated with acute or recent instances (IgM positive). It is possible, however, for new lesions to appear in unrelated areas to older scars [33]. The underlying cause of these observations in patients with ocular toxoplasmosis is still unclear. Despite the presence of toxoplasmas' retinochoroiditis, T. gondii has been recently reported to be present in the peripheral blood of acutely and chronically infected patients by Silveira et al. [34]. Hence, parasitemia may be associated with the reactivation of the ocular disease [34, 35], suggesting that the parasite may circulate in the blood of immunocompetent individuals. But the microfilament and polar tubule of this parasite can irritate the conjunctiva and other front surfaces of the eye [31]. This can cause a high rate of conjunctivitis (18.95%) and other symptoms, like a burning sensation and pain in the eye. The high rate of ocular keratitis in the current study is explained by the fact, that using a non-sterile lens solution or swimming in unchlorinated pools and lakes might cause Acanthamoeba keratitis. Contact lenses pose the greatest risk of AK. Pathogens can grow on the cornea due to contact lenses or corneal abrasions. Contact lens wearers make up nearly 85% of AK cases [36]. Overnight contact lens wear or storage in non-sanitized solutions such as tap water or unpreserved saline solution enhances the risk of AK [37].

CONCLUSIONS

Those with ocular problems in Kirkuk province have a high rate of infection with Toxoplasma gondii and other protozoan parasites. Certain symptoms, like pain, itchy eyes, conjunctivitis, and keratitis, can be traced back to prolonged medication use, particularly polyethylene biguanide. Pathogens like Microsporidia species and Naegleria, in addition to bacteria, viruses, and fungi, also played a role in the development of eye disorders. This was a small-sized study, and future research will use larger patient populations to illuminate the whole picture of these results.

CONFLICTS OF INTEREST

According to the authors of this study, they have no interests that conflict with the work they are presenting.

Author's Contribution

Salman and Hamad designed the study. Hassan collected 156 blood and eye swabs. Salman guided all lab processes, solutions, and stains. Hassan performed the lab analysis. Hamad analyzed the data, and Salman and Hassan prepared the paper. Hamad assessed the manuscript. The corresponding author has been informed that all research authors approve of the manuscript and any further amendments.

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