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### Detection of *Cryptosporidium parvum* in locally manufactured yoghurt and Kareish cheese

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#### ABSTRACT

**C***ryptosporidium parvum* is an important zoonotic parasite causing severe diarrhea in humans and young animals. Fifty milk products (25 Baladi yoghurt and 25 Kareish cheese) were tested for detection of *C. parvum* oocysts. The prevalence of *C. parvum* in yoghurt was 44%, 36% and 12% by microscopic examination, experimental infection and PCR, respectively. There is a statistical significant difference of *C. parvum* in yoghurt between microscopic examination and PCR as well as between experimental infection and PCR. While, its prevalence in Kareish cheese was 24%, 44% and 20% by microscopic examination, experimental infection and PCR, respectively. No statistical significant difference of *C. parvum* were detected in Kareish cheese between microscopic examination, experimental infection and PCR. Microscopic examination of prepared yoghurt and Kareish cheese smears and experimentally infected rats fecal smears showed the spherical shape of *C. parvum* oocysts (4-6µm). A banana shape of *C. parvum* sporozoite (3.2x1.4µm) was found in fecal smear of experimentally infected rat by contaminated yoghurt. Meanwhile, the PCR identified the specific Actin gene of *C. parvum* in 3 yoghurt and 5 Kareish cheese samples. From the obtained results, it could be concluded that *C. parvum* oocysts remain viable in locally made milk products.

#### INTRODUCTION

Kareish cheese “skimmed milk soft cheese” is one of Egypt's most popular cheap cheeses, which is high in protein and calcium and low fat content (Zayan 2016). It should be consumed fresh after processing (not pickled) and has a shelf life of no more than 12 days at 5°C (Abou-Dawood and Gomai 1977). While, Yoghurt is nutritional-rich food product as a

result of the bacterial fermentation of milk. It supplies numerous nutrients that can improve health, and considered a good source of calcium, vitamin B6, vitamin B12, riboflavin and essential amino acids (Banerjee et al. 2017).

*Cryptosporidium parvum* is an intracellular protozoan that infects the gastrointestinal epithelium and produce a life-threatening profuse diarrhea in immunocompromised hosts

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(Chen et al. 2002). It is a highly infectious enteric parasite transmitted via the fecal-oral route by ingestion of oocysts that are excreted in the infected host's feces (Santín et al. 2004).

Björkman et al. (2003) reports that *Cryptosporidium parvum* is the most common parasitic infested the newly born calf. Calves are often infected with *C. parvum* oocysts through fecal contaminated milk as a result of poor udder hygiene (Public Health Laboratory Service Study Group, 1990; Harper et al. 2002) resulting in elevated incidence of the parasite in dairy farms (Garber et al. 1994). Furthermore, Hannes et al. (2006) reported that over 50% of dairy farms in Norway had infected calves with *Cryptosporidium*.

Cattle are considered a major *C. parvum* reservoir and contributing zoonotic infection to humans (Xiao and Fayer, 2008). Cryptosporidiosis is a waterborne intestinal infection, but can also account for dietary transmission (through raw milk). Some laboratory confirmed foodborne outbreak reports of cryptosporidiosis (Ursini et al. 2020). High incidence was recorded in developing countries, particularly in children, people suffering from malnutrition and immunodeficiency (AIDS) (Fayer et al. 2000).

Public Health Laboratory Service Study Group (1990) stated that 9% of patients who became infected with *Cryptosporidium* at home, due to drinking raw milk in England and Wales. While, Gelletlie et al. (1997) recorded the largest outbreak involving 50 junior school children in the UK, that linked to the consumption of milk infected with oocysts and confirmed microscopically. Also, Harper et al. (2002) reported an outbreak of cryptosporidiosis associated with unpasteurized cow's milk consumption in Australia. They noticed that drinking of unpasteurized milk for two weeks was sufficient to start of the disease symptoms which was significantly lab-confirmed diagnosis as *Cryptosporidium*.

Ursini et al. (2020) stated that milk pasteurization is considered an effective method to destroy *Cryptosporidium* oocysts. Also, Harp et al. (1996) indicated that high-temperature,

short-term pasteurization is enough to destroy the *C. parvum* oocysts infectivity in whole milk. Although chlorine, the most common disinfectant used in water treatment and food processing, it does not significantly affect oocysts viability (Korich et al. 1990).

Di Pinto and Tantillo (2002) observed that *C. parvum* is commonly present in dairy farms and could be transported to humans via contaminated raw milk and milk products and is resistant to many environmental stresses and food processes. As well, Deng and Cliver (1999) detected *C. parvum* oocysts in dairy products with average recoveries of 82.3%, 60.7% and 62.5% of milk, ice-cream and yoghurt, respectively.

Selecting a suitable diagnostic method depends upon the requirements and possibilities available to researchers and diagnostic laboratories (technical expertise, time available, sensitivity and specificity needed and financial resources) (Chalmers and Katzer 2013). Cryptosporidiosis can be diagnosed using milk smears stained by "modified Ziehl-Neelsen technique" (Mosa 2016; Hasan et al. 2018) or by polymerase chain reaction (PCR) in dairy products as reported by Loury et al. (2019).

Shakerian et al. (2015) reported that (5.26%) of raw cow's milk samples of bulk tank were positive for *C. parvum* in Iran using Nested PCR. As well, Mosa (2016) detected *C. parvum* in (5.71%) from markets milk, 2.86% of sheep milk and 5.71% of goat milk samples using PCR in Assiut city. Meanwhile, Hasan et al. (2018) observed that the incidence rate of *Cryptosporidium* oocysts was 32% and 46% of raw ovine and caprine milk samples respectively, in Iraq by microscopic examination.

Loury et al. (2019) documented an outbreak of cryptosporidiosis associated with eating an unpasteurized organic white cheese at "the canteen within a middle school in Loire-Atlantique western France" with more than 150 patients. They detected *C. parvum* using PCR.

This study was conducted to detect *C. parvum* oocysts in yoghurt and Kareish cheese with different diagnostic techniques. In addi-

tion, the examination of oocysts infectivity through experimental infection.

## MATERIALS AND METHODS

### Sample collection

A total of locally made 25 Baladi yoghurt and 25 Kareish cheese samples were taken from dairy stores and street vendors respectively, in Assiut City.

### Sample preparation

Yoghurt or cheese suspension "1 ml" homogenized with an equal volume of T-H<sub>2</sub>O (Distilled water containing 0.05% Tween-20), the samples were mixed with Diethyl ether (Alpha chemika, no. DEP209, India) in 50 ml centrifugal tubes at a ratio of 35/15 (sample/Diethyl ether, v/v). Following a 10-minute centrifugation at 1000×g, the upper layers were removed and the sediment was taken in smear preparation and stored at -20°C for PCR (Deng and Cliver 1999).

### Microscopic examination

Smears were made by taking one drop of sediment from each centrifuged sample on a clean slide and spread into an even thin film and instantly dried by the air. The slide was labeled and fixed by "absolute methyl alcohol", then allow drying and the films stained with "Modified Ziehl-Neelsen stain" according to Rekha et al. (2016). The slides were carefully examined via microscope with an oil immersion lens (1000×) for *C. parvum*.

### Experimental examination

Animal handling and all experimental procedures were carried out in compliance with the recommendations of the "Guide for the Care and Use of Laboratory Animals of the National Research Council" (National Research Council 2011).

Fifty female rats 6 weeks age obtained from Animal Laboratory Unit of Assiut University, Egypt. All animals were housed individually in cages, *Cryptosporidium* pathogen free, and kept on daily 12h cycle of light and dark. Rats received sterilized food and water. Each rat was fed on contaminated yoghurt or cheese samples orally (Del Coco et al. 2012). Then,

fecal samples were collected from each rat in 3, 5, 7 days post infection and its fecal smears were stained by "Modified Ziehl-Neelsen stain" and examined microscopically by an oil immersion lens (1000×) (Del Coco et al. 2012).

### Polymerase Chain Reaction (PCR)

The prepared samples of 25 yoghurt and 25 Kareish cheese were tested with PCR.

DNA extraction as described by (Qiagen 2016):

Extraction of DNA from concentrated yoghurt and cheese samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturers guidelines. Briefly, "200µl of the sample suspension was incubated with 20µl of proteinase K and 200µl of lysis buffer at 56°C for 10min. After incubation, 200µl of 100% ethanol was added to the lysate". Then, the sample was washed and centrifuged according the manufacturers guidelines. The, nucleic acid was eluted with 100 µl of elution buffer.

### PCR assays

The specific primer was provided from (Metabion international, Germany) and are listed in (Table A). The reaction mixture was used to amplify actin gene which contains a total of 25 µl reaction mixtures containing "12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1µl of each primer 20 pmol concentration, 4.5µl of water, and 6µl of DNA template". The PCR condition used reaction cycles comprised of "an initial denaturing step at 94°C for 5 min followed by 35 cycles at 94°C for 30s, 60°C for 30s and 72°C for 30s with a final extension at 72°C for 7min" using a T3 Biometra thermal cyler

Table A. Primer sequences of *C. parvum*-specifically designed for the PCR .

Target parasite	Target gene	Primer sequences (5'-3')		Amplified segment (bps)	Reference
		Forward	Reverse		
<i>C. parvum</i>	Actin gene	AATCGTGAAA- GAATGACTCAAATA	CTGGTAGTTCAT- ATGTCTTCTCTAA	400	(Santín and Zarlenga 2009)

### PCR Product analysis

The products were separated on 1.5% agarose gel by electrophoresis (Applichem, Germany, GmbH) in "1x TBE buffer" at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. GeneRuler 100bp DNA Ladder (Fermentas, Germany) was used for determination of fragment sizes. The gel was photographed through a gel documentation system (Alpha Innotech, Biometra) and the data were analyzed using software.

### Statistical analysis

The descriptive variance (positive/negative) was conducted using GraphPad Prism (GraphPad 9.3.1 Software Inc., San Diego, CA, USA). Where Chi square test was applied to evaluate if a significant difference between the various diagnostic tests was used, "P value of <0.05" was considered statistically significant.

The lab animal results were used as a gold standard to calculate the sensitivity and specificity of each PCR and microscopic examination using "MedCalc® Statistical Software version 20" (MedCalc Software Ltd, Ostend, Belgium; <https://www.medcalc.org>; 2021) (Bitilinyu-Bangoh et al. 2019).

### Results

A total of 50 milk products (25 yoghurt and 25 Kareish cheese) was tested for *C. parvum* oocysts detection. The prevalence of *C. parvum*

oocyst in yoghurt samples was 44% (11/25), 36% (9/25), 12% (3/25) by microscopic examination, experimental infection and PCR respectively, (Table 1). Yoghurt had statistical significance difference between microscopic examination and PCR; experimental infection and PCR. While, the prevalence in Kareish cheese was 24% (6/25) by microscopic examination, 44% (11/25) by experimental infection and 20% (5/25) by PCR (Table 2). No significance difference was detected in Kareish cheese between microscopic examination, experimental infection and PCR.

Microscopic examination of yoghurt and Kareish cheese smears and experimentally fecal smears of infected rats showed the spherical shape oocysts (4-6 µm) of *C. parvum*. *Cryptosporidium parvum* sporozoite with a banana shape measured (3.2 x 1.4µm) was found in fecal smears of experimentally infected rat with contaminated yoghurt (Figure 1, 2). Meanwhile, the PCR detected the specific Actin gene of *C. parvum* in 3 yoghurt and 5 Kareish cheese samples (Figure 3, 4).

The comparison of different diagnostic methods revealed high statistically significant variation between PCR and experimental infection and statistically significant variation between PCR and Microscopic examination for detecting *C. parvum* in milk products (Table 3)

Table 1. Prevalence of *C. parvum* oocyst in yoghurt samples based on microscopic examination, experimental infection and PCR (n=25)

Used test	Results		Chi square	P value
	Positive No.	%		
Microscopic examination <sup>a</sup>	11	44	6.349	0.0117
Experimental infection <sup>b</sup>	9	36	3.947	0.0469
PCR <sup>ab</sup>	3	12		

Significance difference ( $p < 0.05$ ) between tests that have the same superscribed small letters.

Table 2. Prevalence of *C. parvum* oocyst in Kareish cheese samples based on microscopic examination, experimental infection and PCR (n=25).

Used test	Results		Chi square	P value
	Positive No.	%		
Microscopic examination <sup>a</sup>	6	24	0.116	0.7328
Experimental infection <sup>b</sup>	11	44	3.309	0.0689
PCR <sup>c</sup>	5	20		

No statistical Significance difference was detected in Kareish cheese between microscopic examination, experimental infection and PCR.

Table 3. Total prevalence of *C. parvum* in yoghurt and Kareish cheese using different Comparable methods (n=50).

Detection methods	Positive samples	Prevalence (%)	Chi square	P value
Microscopic examination <sup>a</sup>	17	34	4.32	0.0377
Experimental infection <sup>b</sup>	20	40	7.143	0.0075
PCR <sup>ab</sup>	8	16		

Significance variation between PCR and Microscopic examination ( $p < 0.05$ ).

High significance variation between PCR and experimental infection ( $p < 0.01$ ).

Table 4. Sensitivity and specificity of microscopic examination and PCR using experimental infection as the gold standard

	Microscopic examination		PCR	
	Sensitivity	Specificity	Sensitivity	Specificity
Yoghurt	44.44%	56.25%	33.33%	100%
Cheese	36.36%	85.71%	45.45%	100%
Both	40%	70%	40%	100%

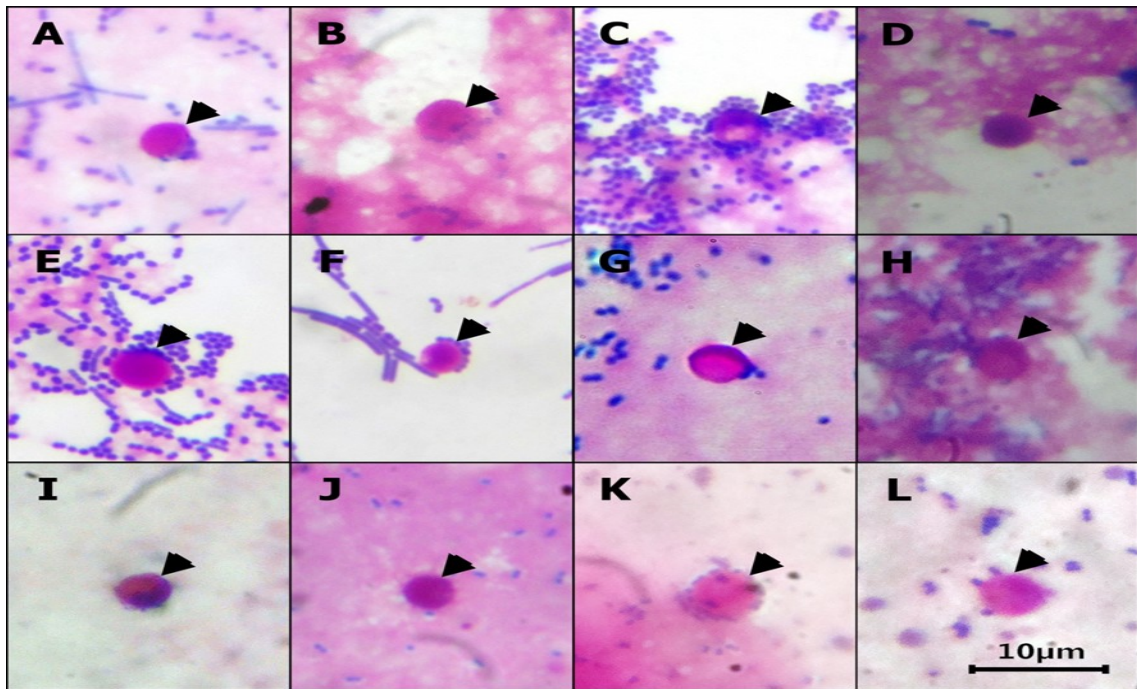


Figure 1. Showing *C. parvum* oocysts in smears stained by modified Zeihl- Neelsen stain X1000 in: A-H: of yoghurt and I-L: of Kareish cheese (arrowhead; Scale bar=10µm).

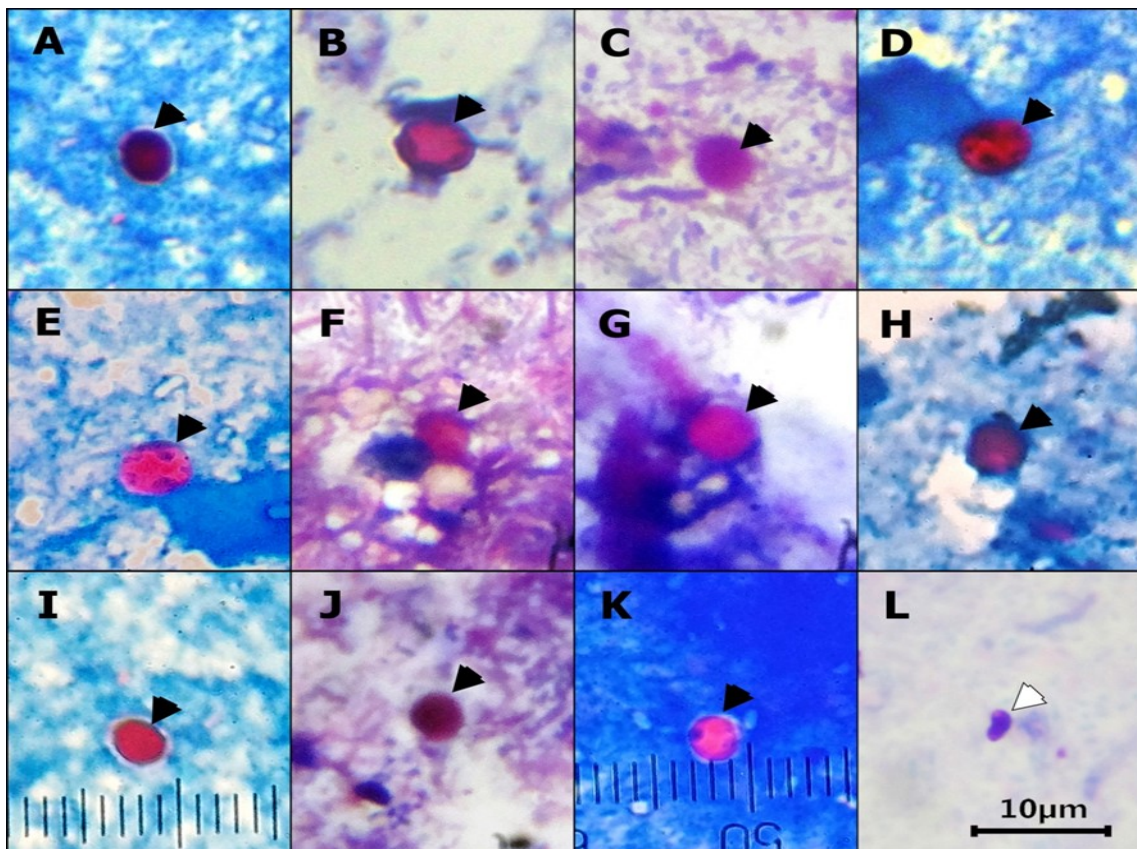


Figure 2. Showing *Cryptosporidium parvum* oocysts in fecal smears of infected rats stained by modified Zeihl-Neelsen stain (X1000): A-E: infected by yoghurt and F-K: infected by Kareish cheese. L: showing sporozoite in fecal smears of infected rat by yoghurt (arrowhead; Scale bar=10 µm).

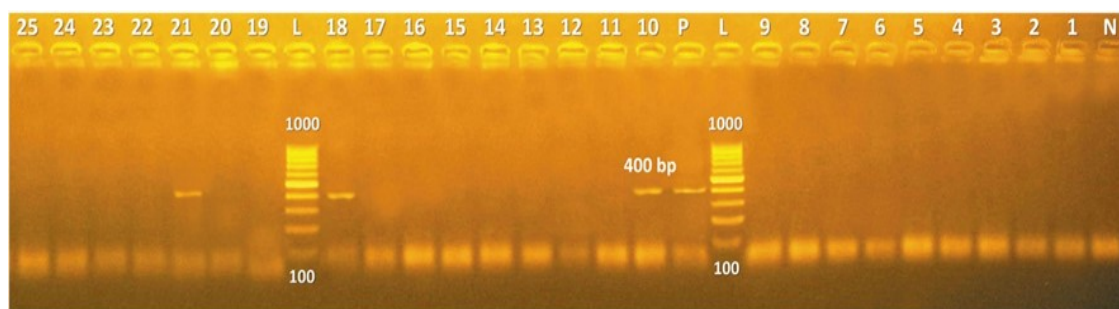


Figure 3. Electrophoresis pattern of PCR amplicon on yoghurt samples using Actin gene *C. parvum*-specific primer (400bp PCR product). L: 100bp DNA ladder; P: control positive; 10, 18 and 21: Positive samples; 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16, 17, 19, 20, 22, 23, 24 and 25: Negative samples

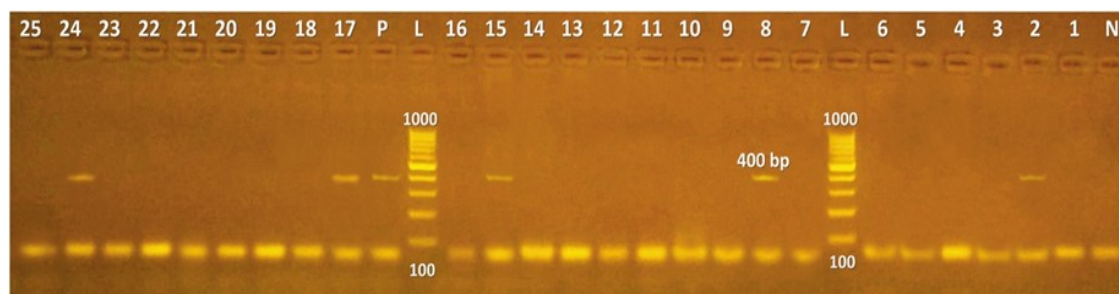


Figure 4. Electrophoresis pattern of PCR amplicon on Kareish cheese samples using Actin gene of *C. parvum*-specific primer (400bp PCR product). L: 100bp DNA ladder; 2, 8, 15, 17 and 24: Positive samples; 1, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 16, 18, 19, 20, 21, 22, 23 and 25: Negative samples.

## DISCUSSION

*Cryptosporidium parvum* is a protozoan parasite that is deemed as a major reason of young ruminants neonatal diarrhea leading to economic losses. Furthermore, cryptosporidiosis has zoonotic importance and constitute a serious public health problem (Hameed 2020). It is generally characterized by the sudden onset of watery diarrhea in human. The incubation period, typically varies from 2–3 days to 2 weeks depends on the dose. Symptoms appear within 5–7days and can persist up to a month (Chen et al. 2002).

Hamnes et al. (2006) noted that *Cryptosporidium* spp. does not proliferate in milk, but infected oocysts may survive in it. In addition, Rosenthal et al. (2015) documented 2 cases of cryptosporidiosis in siblings under 3 years old who drunk raw (unpasteurized) goat milk in “Idaho 2014”. They reported that 4 raw goat milk samples from the household and dairy milk containers were positive for *Cryptosporidium* by real-time PCR.

Freidank and Kist (1987) found *Cryptosporidium* infection in 4 patients stool who drunk raw milk prior to the onset of the disease in Germany. Also, Ursini et al. (2020) assessed the clinical features of children diagnosed with Cryptosporidiosis and found that 2 of 46 cases were associated with the drinking of unpasteurized milk in France.

In this study, we found that the risk of human infections with *C. parvum* through consumption of contaminated unpasteurized dairy products, which consistent with (Smith 1993; Laberge and Griffiths 1996). The risk of human infections may be due to the cross-species transmission of *C. parvum* oocysts (Fayer and Ungar 1986), the environmental survival of oocysts (Chauret et al. 1995), and oocysts resistance to most sanitizers (Korich et al. 1990). In the current study, the prevalence of *C. parvum* in yoghurt was 44% by microscopic examination, 36% via experimental infection and 12% by PCR (Table 1). The difference between the prevalence of *C. parvum* by microscopic examination and experimental infection may be due to reduction of the oocyst viability.

Similar results were recorded by **(Deng and Cliver 1999)** who noticed that the oocysts viability decreased from 83% to about 60% after 48h at 37°C and to around 58% following storage by 8 days and added that contaminated yoghurt can lead to consumers infections.

The experimental infection revealed that *C. parvum* oocysts remain viable in yoghurt samples which agree with **Romanova et al. (1992)** who recorded 13 cases with cryptosporidiosis in infants fed on fermented milk product (kefir) and assumed that milk contamination with oocysts took place on a milk farm. They found an oocyst in milk sediments from a dairy factory tank without oocysts detection of the kitchen workers in Russia.

The current research revealed that the prevalence of *C. parvum* in Kareish cheese was 24%, 44% and 20% by microscopic examination, experimental infection and PCR, respectively (Table 2). Similar result recorded by **Shahrokhi et al. (2022)** who showed that 18% of cheese samples were contaminated with *Cryptosporidium* species using the nested PCR technique in Iran. This agrees with **Loury et al. (2019)** who reported an outbreak caused by *C. parvum* associated with organic unpasteurized white cheese consumption in France by using PCR.

The high prevalence in this research may be caused by using contaminated milk with *C. parvum* oocysts in yoghurt and cheese manufacturing, which agreed with **(FSANZ 2009)** who mentioned that the anus of all dairy animals is positioned above the udders, leading to fecal contamination during milking, even in the most hygienic operations. Also, Cattle are considered a major source of *C. parvum* due to their large fecal output, high herd prevalence and year-round calving patterns, particularly in dairy herds **(Hammes et al. 2006)**. **Chalmers and Giles (2010)**; **Hammes et al. (2006)** reported large numbers of oocysts are excreted by the infected hosts (30 billion oocysts from a single infected calf over a 2-week period) and the oocysts are environmentally robust and can survive well long outside the host, especially in wet environments.

**Ursini et al. (2020)** mentioned that milk borne outbreaks of cryptosporidiosis can be either due to ingestion of unpasteurized/inadequately pasteurized milk products or to post-pasteurization contamination, that can be related to both human and zoonotic transmissions. These results are matched with our results, that *C. parvum* infection of yoghurt and Kareish cheese may be due to traditional milking and processing without any pasteurization. This is in agreement with **(Crawford 1990)**.

Low pH of yoghurt and Kareish cheese can be appropriate for the survival of *Cryptosporidium* oocysts. This is in accordance with **Deng and Cliver (1999)** who stated that oocysts can survive slightly better at a relatively low pH (better at a pH of 4.8 than at a pH of 6.8).

Microscopic examination of prepared yoghurt and cheese smears and experimentally infected rats fecal smears stained with modified Ziehl–Neelsen stain showed spherical shape oocysts 4-6 µm of the diameter of *Cryptosporidium parvum*. Fecal smears of experimentally infected rat with yoghurt showed *Cryptosporidium parvum* sporozoite with banana shape 3.2 x 1.4µm in diameter (Figure 1, 2). This coincided with **Hameed (2020)** who found a small protozoan parasite (4-6 µm) of *Cryptosporidium parvum* in neonatal ruminants. Furthermore, **Aldeyartbi and Karanis (2016)** observed banana-shaped extracellular sporozoites, 2.2 × 1.6 µm in cell-free cultures. Also, **Edwinson et al. (2016)** detected banana-shaped sporozoites (mean length to width ratio: 2.96 ± 0.54 µm) of *C. parvum*.

Although, traditional staining techniques of *Cryptosporidium* oocysts by acid-fast staining have been reported to be less specific and sensitive and time consuming **(Clark 1999; Morgan et al. 1998; Quilez et al. 1996; Chalmers and Katzer 2013; Silverlås et al. 2013)**. The present study showed that examination of concentrated samples following Modified Ziehl–Neelsen stain was more specific in cheese than yoghurt. The low specificity in yoghurt may be due to the presence of more fat globules than cheese which may be confused *Cryptosporidium* **(Casemore 1991)**. These findings agreed with **Mirhashemi et al. (2015)**.



Extracting high-quality DNA is an important step in PCR detection. However, inhibition of the PCR by various substances commonly found in environmental samples is a well-documented disadvantage. This is mainly for samples with low levels of parasites, typical of surface water samples and samples from animals with chronic low-level infections (**Bakheit et al. 2008**).

Our finding revealed that PCR of *C. parvum* in milk products showed very high specificity and low sensitivity (Table 4), which conflict with **Santín and Trout (2008)** and **Xiao (2010)** who stated that the only available method for identifying *Cryptosporidium* species is PCR. **Chalmers and Katzer (2013)** recorded that PCR assay give high sensitivity and specificity. In this work, the low sensitivity may be due to the amount of extracting DNA, which was too low to be detected by PCR as a result of the low number of oocysts in comparison with microscope and experimental infection. These results matching with **Leetz et al. (2007)** and **Bakheit et al. (2008)** who concluded that PCR detection protocols for *Cryptosporidium* are unable to detect all isolates especially with low oocysts number in contaminated samples. Also, **Mirhashemi et al. (2015)** and **Leetz et al. (2007)** highlight challenges in dealing with low oocyst numbers and/or low *Cryptosporidium* DNA when working with fecal and/or environmental samples.

In the present investigation, the use of different diagnostic methods revealed high statistically significant variation between PCR, experimental infection and statistically significance difference was recorded between PCR and microscopic examination for detecting *C. parvum* in some locally made milk products (Table 3). Comparing sensitivity and specificity among different diagnostic methods can be performed by designating a method as the gold-standard (**De Waele et al. 2011**). In this study, it was established that experimental infection is a more exact diagnostic method and applied it as gold-standard, which agree with **Deng and Cliver (1999)** who recorded that the most dependable and direct method is to inoculate an animal model and observe shedding of oocysts, since it measures the oocysts capability to complete its life cycle.

## CONCLUSION

This is a first work on the detection of *C. parvum* in dairy products by different diagnostic methods in Egypt. The results revealed the presence of viable *C. parvum* oocysts in locally made yoghurt and Kareish cheese due to traditional milking and processing without pasteurization. Experimental infection is a more accurate diagnostic method than other techniques. So, pasteurization of milk is recommended before the manufacture of dairy products. Also, it is necessary presence of appropriate health education programs to improve dairy production.

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