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Research Article

Investigation of *In Vitro* Effectiveness of Polymeric Nanoparticles Containing Clinoptilolite on *Cryptosporidium parvum*

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ABSTRACT

Cryptosporidium species, which do not have a definite prevention and treatment protocol, are pathogenic protozoans that cause diarrhoea in humans and many animal species. This study aimed to demonstrate the in vitro efficacy of clinoptilolite polymer nanoparticle, which has known antidiarrheal effects. DNA isolation was made for species identification of *Cryptosporidium* oocysts obtained from faeces samples from naturally infected calves and lambs. RFPL analysis was performed in typing. For this purpose, nested PCR and SrpI, VspI and MboII enzymes were used. The coverslip surface was covered with 80% CaCo-2 cells and infected with $1x10^9$ *C. parvum*. Nanoparticles containing 250, 500, 750 and 1000 µg/mL clinoptilolite were applied to infected cells. Percent of infection rate was calculated by counting under a fluorescent microscope following incubation. While the infection rate was 23.46% in the water-treated control cell group, the percentage infection rates in the clinoptilolite-containing nanoparticle treated group were respectively 15.60%, 8.13%, 10.33% and 13.46%. Inhibition percentages were determined as 33.54%, 65.56%, 55.99% and 42.66%, respectively. As a result, it was observed that the nanoparticle containing clinoptilolite had anticriptocidal activity in infection with *C. parvum* in Caco-2 cells. In addition, it was observed that the efficacy was dose-dependent, and the IC_{50} value was the most appropriate value at 750 and 1000 µg/mL doses.

Keywords: *Cryptosporidium parvum, clinoptilolite, nanoparticle.*

Klinoptilolit İçeren Polimer Nanopartiküllerin *Cryptosporidium Parvum* Üzerine *İn Vitro* Etkinliğinin Araştırılması

ÖZET

Kesin bir korunma ve tedavi protokolü bulunmayan *Crptosporidium* türleri insan ve pek çok hayvan türünde ishale neden olan patojen prozoonlardır. Bu çalışmada antidiyarel etkileri bilinen klinoptilolitin polimer nanopartikülünün in vitro olarak etkinliğinin ortaya konması amaçlanmıştır. Doğal enfekte buzağı ve kuzulardan alınan dışkı örneklerinden elde edilen *Cryptosporidum* ookistlerinin tür tayini için DNA izolasyonu yapılmıştır. Tiplendirmede RFPL analizi gerçekleştirilmiştir. Bu amaçla nestet PCR ve SrpI, VspI ve MboII enzimleri kullanılmıştır. Lamel yüzeyi % 80 CaCo-2 hücresi ile kaplanmış ve $1x10^9$ *C. parvum* ile enfekte edilmiştir. Enfekte hücrelere 250, 500, 750 ve 1000 µg/mL klinoptilolite içeren nanopartikül uygulanmıştır. İnkubasyonu takiben floresan mikroskopta sayım yapılarak yüzde enfeksiyon oranı hesaplanmıştır. Su uygulanan kontrol hücre grubunda enfeksiyon oranı %23,46 iken klinoptilolit içeren nanopartikül uygulanan grupta yüzde enfeksiyon oranları sırasıyla %15,60; %8,13; %10,33 ve %13,46 olarak belirlenmiştir. İnhibisyon yüzdeleriyse sırasıyla %33,54, %65,56, %55,99 ve %42,66 olarak saptanmıştır. Sonuç olarak Caco-2 hücrelerinde *C. parvum* ile enfeksiyonda klinoptilolite içeren nanopartikülün antikriptosidal etkinliğinin olduğu gözlenmiştir. Ayrıca etkinliğin doza bağımlı olduğu ve 750 ve 1000 µg/mL dozlarda IC_{50} değerinin en uygun değerler olduğu görülmüştür.

Anahtar Kelimeler: *Cryptosporidium parvum, klinoptilolit, nanopartikül.*

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Introduction

Diarrhoea is an important problem that causes economic loss and death in lambs, goats, and calves. In these species, *Cryptosporidium spp*. is one of the causes of diarrhoea in the first weeks of life worldwide. It is a zoonotic disease that can be affected by animal, environment, and production practices (Adkins, 2022). *C. parvum, C. hominis, C. canis, C. felis, C. meleagridi*s, and *C. muris* have been reported in diarrheal and gastrointestinal diseases in birds, sheep, horses, cattle, camels and rodents (Ranjbar et al., 2018; Haghi et al., 2020). Despite advances in medicine and technology, diarrhoea is the world's main problem in newborn farm animals. In our country, the extent of direct and indirect economic losses due to diarrhoea is increasing due to the breeders' lack of knowledge and financial situation. Treatment of animals with diarrhoea is time-consuming, labour-intensive and expensive. For this reason, it emphasises the prevention or reduction of the occurrence of diarrhoea. Currently, there is no standard preventive and therapeutic protocol for *C. parvum*. Despite the use of many drugs in preventing and treating *C. parvum* infection, complete success has yet to be achieved. Therefore, there is a need for a new treatment protocol for cryptosporidiosis. Natural products significantly impact human and animal health sectors, mainly through drug discovery and chemical biology.

It is known that aluminium silicates, one of the most important components of zeolites containing clinoptilolite, have beneficial biological properties in reducing diarrhoea associated with intestinal diseases, especially in pigs, calves and rats (Sverko et al., 2004; Papaioannou et al., 2005; Deligianis et al. 2005). Various research results show that aluminium silicates, one of the essential components of zeolites containing clinoptilolite, play an indispensable role in regulating the immune system. Clinoptilolite is a crystalline compound with low solubility in the digestive system, non-absorbable, connected by channels, having smooth pores, and contains ions such as sodium (Na), potassium (K), calcium (Ca), magnesium (Mg). It is thought that clinoptilolite can change environmental factors such as moisture and nutrition, which enable it to sporulate and become infective by affecting the hydrostatic pressure of *C. parvum* with its water loss and gain feature. It is predicted that clinoptilolite may play an important role in the prevention and treatment of *C. parvum* infection due to its ability to affect the environmental conditions necessary for the development and survival of the oocyst, as well as its ability to regulate the immune system and be used as an immune stimulant.

It is accepted that nanobiotechnology, which is rapidly entering our lives in fields such as health, food, agriculture and the pharmaceutical industry, will be the most effective technology of our near future and will directly affect our lives and will be a superior technology that can increase our living standards considerably. The nanobiotechnological products to be prepared can radically change human and animal life and economic activities. Therefore, this study aimed to reveal the in vitro efficacy of clinoptilolite-containing polymer nanoparticles on *C. parvum.*

Materials and Methods

Preparation of Cryptosporidium parvum

Cryptosporidium oocysts were collected from naturally infected lambs and calves brought to Adnan Menderes University, Faculty of Veterinary Medicine, Internal Medicine Clinic, as described by Lorenzo et al. (1993). The presence of *E. coli* K99, *Cryptosporidium*, Coronavirus and Rotavirus infections in stool samples taken from animals before the collection was tested using the BoviD-4 Ag test kit (Anigen, Korea). After the faeces collected from lambs and calves with only *Cryptosporidium sp*. infection were diluted with distilled water and passed through a 45 µm perforated strainer, the resulting liquid part was centrifuged at 1000 x g at 4 °C for 5 minutes. The pellet was mixed with 20 mL of distilled water and 20 mL of diethyl ether and centrifuged at 1000 x g at 4°C for 5 minutes, and the upper three layers were removed. This process was repeated until the fat in the stool was wholly removed. The obtained sediment was mixed with 1 mL of distilled water and spread over the Percoll gradient formed from four different densities (1.13, 1.09, 1.05 and 1.01 g/mL, each 2.5 mL). The mixture was centrifuged at 650 x g for 15 minutes at 4°C. The tape containing the oocyst eggs was removed and washed with distilled water by centrifugation at 1000 x g at 4 °C for 5 minutes, and the washing process was repeated three times. The oocysts were stored in 2.5% (w/v) potassium dichromate solution until experimental infection. Figure 1 shows *Cryptosporidium* oocysts collected from feces**.**

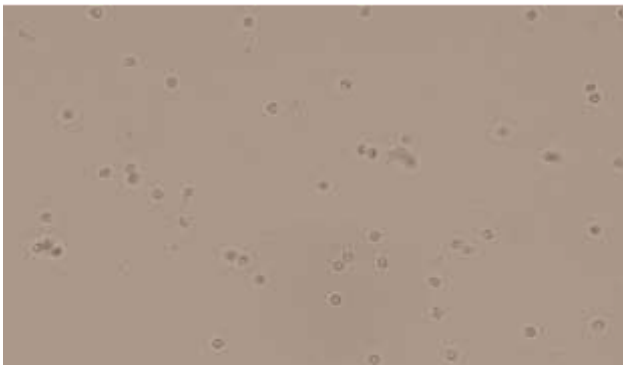

Figure 1. *Cryptosporidium parvum* oocyst (x400)

Determination of the Type of Cryptosporidium Oocysts by PCR and RFLP

DNA isolation was performed in 100 µL suspension using the phenol/chloroform method to determine the type of *Cryptosporidium* oocysts obtained. Nested PCR (Polymerase Chain Reaction) and RFLP (Restriction Fragment Length Polymorphism) analysis were performed to determine the type of oocysts obtained from naturally infected lambs. For this purpose, 100 µL of the obtained oocyst suspension was taken, and DNA was isolated by the classical phenol/chloroform method (Sambrook et al., 1989). The *C. parvum* species was determined by RFLP analysis using Nested PCR and SspI, VspI and MboII enz-

ymes. PCR was performed as previously described using primers of the 18S ribosomal RNA gene specific to the Cryptosporidium strain (Xiao et al., 1994; Aysul, 2009).

The first PCR was performed using primers (forward: 5'-TTC TAG AGC TAA TAC ATG CG-3' and reverse: 5'-CCC TAA TCC TTC GAA ACA GGA-3') amplifying the 1325 bp portion of the 18S ribosomal DNA gene of Cryptosporidium. The second PCR was performed using primers (5'-GGA AGG GTT GTA TTT ATT AGA TAA AG-3' and 5'-AAG GAG TAA GGA ACA ACC TCC A-5') amplifying the 826-864 bp portion of the first PCR product. The PCR mix contained 1 x PCR buffer, 6 mM $MgCl_2$, 200 mM deoxynucleoside triphosphate, 100 nM primers, and 2.0 U of HotStartTaq DNA polymerase. The final PCR volume was 25 µl. 2 µl of DNA sample was used in the first PCR, and 1 µl of the first PCR product was used in the second PCR. Both PCRs were performed in 35 cycles (denaturation at 94°C 45 s, hybridisation at 57°C 45 s, and extension at 72°C 1 min). Both PCRs included cycles of 3 minutes at 94°C initially and 7 minutes at the end of 72°C. Analyses of PCR products were performed with Safeview-stained 1.5% agarose gel electrophoresis.

In nested PCR products (approximately 850 bp), RFLP analysis was performed using the enzymes SspI, VspI and MboII as previously described (Feng et al., 2007). These enzymes distinguish *C. parvum, C. bovis, C. andersoni* and *C. range* genotypes. For this purpose, the second PCR product was isolated from agarose gel with the help of the QIAquick gel extraction kit. For each VspI, SspI and MboII enzyme, 15 units of enzyme for 40 µL of the PCR product were mixed with 5 µL of buffer and 2 µL of sterile de-ionized water. It was incubated at 37ºC for 2 hours to complete the cutting process. The mixture was stained with Safeview and visualised by running on a 3% agarose gel. The SspI enzyme cuts *C. parvum* or *C. bovis* at 449, 267 and 108 bp sizes, while it cuts *C. andersoni* at 448 and 397 bp sizes. MboII cuts *C. parvum* at 771 and 76 bp and *C. bovis* at 412, 185, and 162 bp. The VspI restriction enzyme forms 628, 115 and 104 bp fragments in *C. parvum* and 730 and 115 bp in *C. andesroni*. As a result of the RFLP analysis performed in this study, all DNA samples tested were found to be *C. parvum*. Due to the low sensitivity of the agarose gel, products around 100 bp obtained as a result of RFLP analysis were not detected in the gel (Figure 2).

In addition, sequence analysis was performed by cloning the nested PCR product to confirm that the oocysts used in the in vitro experiment were *C. parvum*. DNA samples amplified by nested PCR were visualised under UV light following 2% agarose gel electrophoresis with Safeview. A band of approximately 850 bp was cut from the gel and purified using the QIAquick gel extraction kit. The purified product was then cloned into the pCR4–TOPO plasmid vector and transformed into TOP10 *E. coli* cells, as indicated in the commercial kit. Selected colonies were grown overnight, and plasmids contained in cells were purified using the QIAGEN plasmid purification kit. After cutting with the EcoR1 enzyme, they were electrophoresed in 1.5% agarose gel, and plasmids containing the cloned DNA were determined under UV light. Then, DNA samples of these plasmids were sent to Iontek (Istanbul) company for sequence analysis. The results were compared with sequences stored in GenBank and were found to be 99-100% similar to C. parvum (Figure 3).

Preparation of Polymer Nanoparticle Containing Clinoptilolite

Nanoparticle was prepared using a biopolymer with known biocompatible mucoadhesive properties, and clinoptilolite encapsulation was carried out into the nanoparticle. In this context, syntheses of nanoparticles were carried out in a spray drier device using chitosan (1%), clinoptilolite-containing solution and thiamine pyrophosphate in acetic acid. Optimisation of nanoparticle synthesis was performed by encapsulation at varying flow rates, and syntheses of nanosized particles were performed. In optimising nanoparticle synthesis and characterising nanoparticles prepared under optimum conditions, size measurements were carried out with zetasizers (Malvern Panalytical, Ultra Zetasizer, United Kingdom).

In Vitro Infection

In order to determine the in vitro efficacy of clinoptilolite-containing polymer nanoparticles on *C. parvum* oocysts, the cell culture study was examined in vitro as follows. Human colon adenocarcinoma cells were used to perform experimental in vitro infection. Cells reproduced using 10% Fetal Bovine Serum, 100U/mL penicillin, 100 µg/mL streptomycin added DMEM medium (Sigma D6429) (4500 mg/L Glucose, 110 mg/L sodium pyruvate, L-glutamine) in a 5% CO_2 incubator at 37ºC. In the infection trials, 18 mm sterile round coverslips were placed in 12 well cell culture plates, and Caco-2 cells were seeded on them. After the cells covered 80% of the surface of the coverslips, they were infected with $1x10^6$ C. *parvum oocyst*. The percent inhibition of *C. parvum* for each dilution of the clinoptilolite-containing polymer nanoparticle in vitro culture was calculated according to the following formula.

$$\text{Inhibition (\%)} = \frac{\text{Control oocyst number - Dilution oocyst number}}{\text{Control oocyst number}} \times 100$$

Statistical evaluation of the data mean and standard error values were calculated using the Statistical Package for the Social Sciences (SPSS 19, Chicago, IL, USA) package program.

Results

The per cent effect of polymer nanoparticles containing clinoptilolite on *C. parvum infection* in in vitro tissue culture of the study with Caco-2 cells is shown in Table 1. The appearance of oocysts in the in vitro treatment of clinoptilolite-containing polymer nanoparticles in *Cryptosporidium parvum* infection carried out in Caco-2 cells is shown in Figure 4. The per cent inhibition concentration and IC_{50} values of polymer nanoparticles containing clinoptilolite in *C. parvum* infection carried out in Caco-2 cells are shown in Figures 5 and 6.

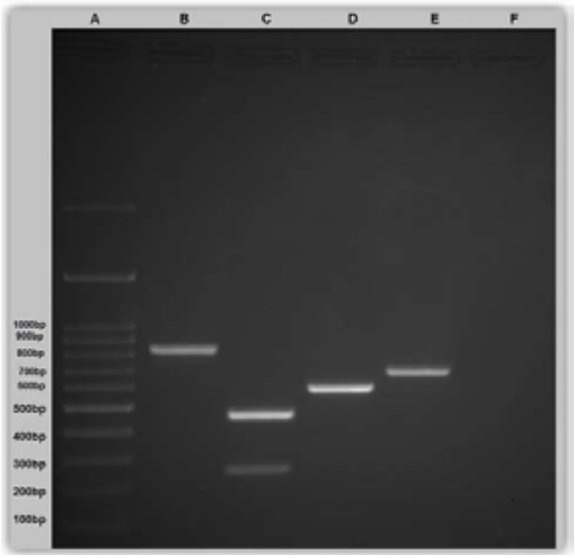

Figure 2. RFLP analysis of the nested PCR product of *Cryptosporidium parvum* using cutting enzymes SspI, VspI, and MboII. A; Molecular Marker (ABM 100bp plus), B; *C. parvum* nested PCR product (847 bp), C; The nested PCR product (449 and 267 bp) cut with SspI, D; The nested PCR product (628 bp) cut with VspI, E; The nested PCR product cut with MboII (771 bp), F; negative control. 3% agarose gel stained with SafeView (ABM).

Figure 3. Sequence analyses of *Cryptosporidium parvum*

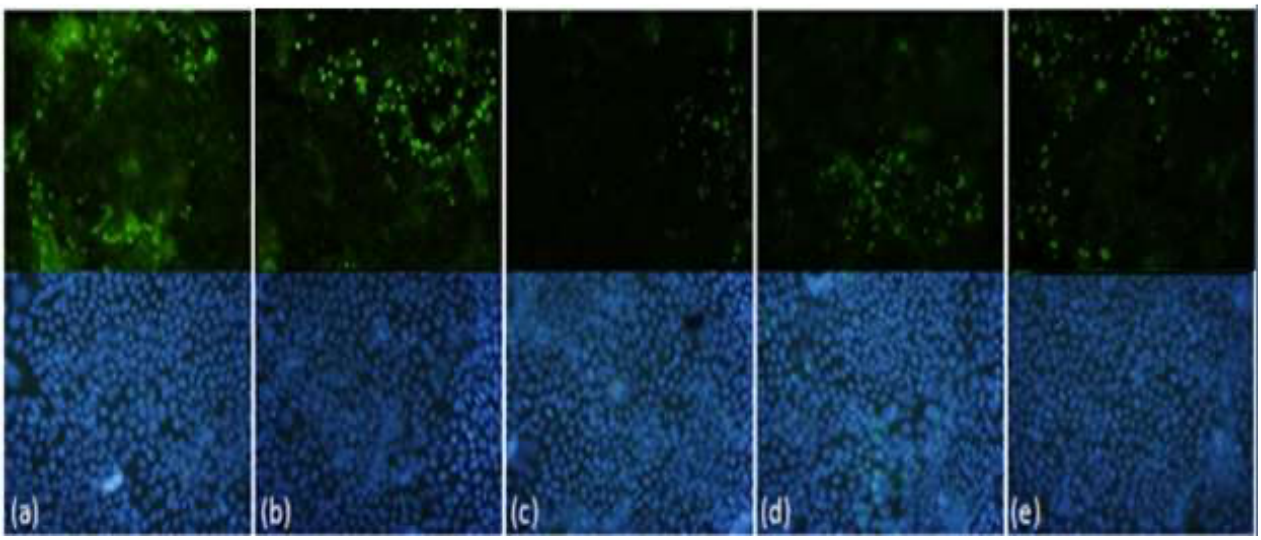

Figure 4. In vitro appearance of *C. parvum* oocysts of Caco-2 cells treated with nanoparticle-containing clinoptilolite. Real-time cell nucleus staining with DAPI(400X)

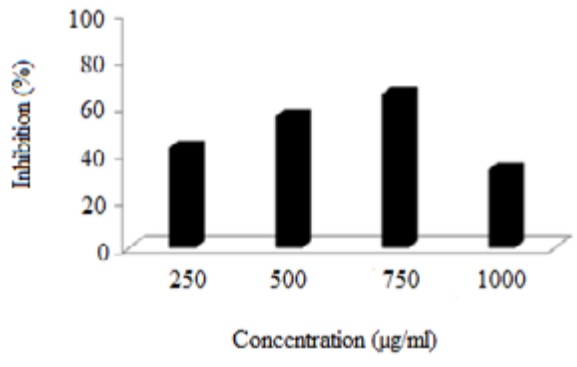

Figure 5. Percentage of infection of nanoparticles containing clinoptilolite

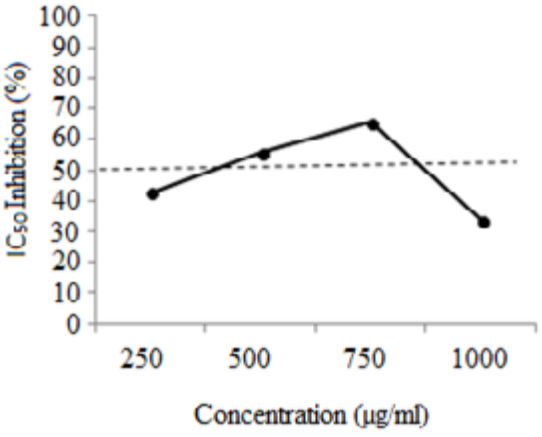

Figure 6. IC_{50} values of polymer nanoparticles containing clinoptilolite

Table 1. Effect of polymer nanoparticle containing clinoptilolite on *C. parvum* in invitro cell culture ($(\bar{X})\pm S_{\bar{x}}$)

Groups	Control	Clinoptilolite dilution			
		1000 µg/mL	750 µg/mL	500 µg/mL	250 µg/mL
Percentage of infection (%)	23.46±5.50	15.60±2.54	8.13±1.60	10.33±1.70	13.46±2.06

Discussion

Diarrhoea in farm animals is more common, especially in the neonatal period, with severe consequences. In addition to *E. coli*, Rotavirus and Coronavirus in the neonatal period in ruminants, another factor that causes diarrhoea and whose importance is increasing today is *C. parvum*, which has a zoonotic character. The number of oocysts required to establish or establish clinical infection with *Cryptosporidium* varies considerably depending on individual susceptibility and host resistance. Some researchers (Viel et al., 2007; Schnyder et al., 2009; Al-Mathal & Alsalem, 2012; Zambriski et al., 2013) have revealed experimental clinical infection in different animal species by administration of *Cryptosporidium parvum* oocyst between $1x10^3$ and $1x10^7$. The factor that facilitates the rapid spread of the infection and becomes a herd problem is that an infected animal sheds millions of oocysts with 1 gram of feces (de Graaf et al., 1999; Hamnes et al., 2006; Divers & Peek, 2008). In vitro studies create infection by applying oocysts at a rate similar to in vivo studies (Santın et al., 2004).

Various ionophore polyether antibiotics such as Lasalocid Na, paromomycin, azithromycin, halofuginone, decoquinate, and Nitazoxanide (NTZ) have been tried in the treatment of *Cryptosporidium parvum* infection. However, there are certain limitations in their use due to their toxic or side effects. In addition, none of the tested substances provided complete control of clinical findings and elimination of infection (Shadiduzzaman & Daugschies, 2012). For this reason, studies on the prophylaxis and treatment of *C. parvum* continue, and many active substances from different groups are still being tested. It is reported that clinoptilolite added to feeds in farm animals prevents diarrhoea. It is reported that adding clinoptilolite at a dose of 2g/kg body weight for treatment purposes in calf diarrhoea and 1g/kg body weight for protection purposes affects preventing diarrhoea. 1g/kg of clinoptilolite added to colostrum or milk reduces the occurrence of diarrhoea in calves. However, it does not affect passive immunity (Sadeghi & Shawrang, 2008). Adding 1g of clinoptilolite per kg body weight to colostrum in postpartum calves reduces respiratory and diarrheal problems and the use of antibiotics (Vrzgula et al., 1988). Lamb ration that includes 3% clinoptilolite to effectively control nematodes (Deligiannis et al., 2005). It has been reported that clinoptilolite affects the morphology of oocysts in vitro coccidiosis in sheep and reduces the excretion of oocysts and environmental contamination in vivo (Alcala-Canto et al., 2011). Ay et al. (2021) demonstrated the prophylactic effect of clinoptilolite in experimentally infected lambs with *C. parvum*. In addition, this study revealed that clinoptilolite changes environmental factors such as humidity, which causes sporulation and infection of oocysts and stimulates the immune system.

Researchers said that aluminium silicates, one of the essential components of zeolites containing clinoptilolite, have beneficial biological properties in reducing diarrhoea associated with intestinal diseases, especially in pigs, calves and rats. It is reported that aluminium silicates have the potential as a new alternative feed supplement in inflammatory conditions, the regulation of the immune system, and the prevention of immunosuppressive diseases (Sverko et al., 2004; Papaioannou et al., 2005). Although the efficacy of clinoptilolite in the prevention and treatment of diarrhoea in farm animals has been reported, there is no study on the effectiveness of clinoptilolite-containing polymer nanoparticles in both diarrhoea and *C. parvum* infection. This study is the first to evaluate the in vitro efficacy of clinoptilolite polymer nanoparticle formulation against *C. parvum*. The absence of a study in the literature assessing the efficacy of clinoptilolite-containing polymer nanoparticles against *C. parvum* limits the discussion of the results of this study. However, investigating the effects of different nanobiotechnological products on *C. parvum* has come to the fore in recent years. Cameron et al. (2016) reported that silver nanoparticles destroy *C. parvum* oocysts and reduce their survival in a dose-dependent manner. Likewise, Fallah et al. (2017) say that nano nitazoxanide is more effective than normal nitazoxanide, and that nano product can treat *Cryptosporidium.*

In this study, in which the effect of clinoptilolite-containing polymer nanoparticles on *C. parvum* oocysts was evaluated in vitro, the inhibition percentage of different concentrations (1000µg/mL, 750µg/mL, 500µg/mL, 250µg/mL) was found to be 33.54%, 65.56%, 55.99%, 42.66%, respectively. It was observed that polymer nanoparticles containing clinoptilolite had anticriptocidal activity in in vitro infection of Caco-2 cells with *C. parvum*, and clinoptilolite-containing polymer nanoparticle concentrations (IC50) that provided 50% inhibition for *Cryptosporidium parvum* were 750µg/mL and 500µg/mL.

It is thought that this effect of the polymer nanoparticle containing clinoptilolite is due to the positive effect of its activity against *C. parvum* in epithelial cells through different mechanisms concerning the prolongation of the intestinal transit time and the increase of the contact time with the intestinal mucosa. The clinoptilolite-con-

taining polymer nanoparticle used in this study, such as paromomycin, halofuginone and azithromycin, also reduces the number of *C. parvum* oocysts with direct and indirect effects. Therefore, it can be used alone or in treating combined *C. parvum* infection.

Conclusion

In conclusion, with these effects, polymer nanoparticles containing clinoptilolite may be an economical alternative to other anticryptocidal agents for prophylactic and treatment purposes in *C. parvum* infection. For this purpose, conducting in vivo studies in which clinical and parasitological improvement can be determined using nanoparticles containing clinoptilolite in farm animals with Cryptosporidiosis would be beneficial.

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Conflict of interest

The authors declare that they have no conflict of interest.

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