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Highlights:

- PCR-RFLP has been used for genotyping *Cryptosporidium* species.
- Samples were collected from children, pre-weaned calves and broiler chickens.
- *C. parvum* was the only identified species in children and calves.
- *C. baileyi* was identified in broilers in addition to another unknown species.
- Further studies are needed to sequence and detect subtypes of this parasite.

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Title: First genotyping of *Cryptosporidium* spp. in pre-weaned calves, broiler chickens and children in Syria by PCR-RFLP analysis

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Abstract

In this study, PCR-RFLP was used for the first time in Syria for genotyping *Cryptosporidium* species of man, calves and chickens. The total of 391 fecal samples included 213 from children with diarrhea (<5 years), 67 from pre-weaned calves with diarrhea and 111 from broiler chicken farms. All samples were collected and examined with acid fast stain to detect the positive samples. Subsequently a nested-PCR test was performed on 35 positive samples (17 from calves, 11 from chicken, and 7 from children) targeting SSU rRNA gene, and was followed by RFLP analysis using three restriction enzymes *Ssp*I, *Vsp*I and *Mbo*II.

Results showed that *C. parvum* was the only identified species in children and calves, on the other hand *C. baileyi* was identified in broilers in addition to another species with unknown RFLP profile in comparison to those which have been described in chicken. Further studies using more genes are needed to sequence and detect subtypes of this parasite.

Keyword: *Cryptosporidium*, calves, chicken, children, PCR-RFLP, Syria.

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1. Introduction

Protozoan parasites of the *Cryptosporidium genus* infect epithelial cells at the microvillus border of the gastrointestinal tract and sometimes respiratory tract of vertebrates (Hunter et al., 2007).

Most human cryptosporidiosis cases are caused by one of the five *Cryptosporidium* species: *C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, and *C. canis* (Xiao, 2010; Xiao and Fayer, 2008). *Cryptosporidium* is a significant cause of diarrheal disease in both developing and industrialized countries (Sulaiman et al., 2005). *C. hominis* and *C. parvum* are by far the most common species responsible for the majority of human infections (Helmy et al., 2013).

Studies worldwide suggest that cattle are infected with at least four *Cryptosporidium* parasites: *C. parvum*, *C. bovis*, *C. andersoni*, and *C. ryanae* (previously called deer-like genotype). Some studies have shown a host age related susceptibility, *C. parvum* predominates in pre-weaned calves, *C. bovis* and *C. ryanae* in post-weaned calves, and *C. andersoni* in older calves and adult cattle (Santin et al., 2008).

The three valid *Cryptosporidium* species infect chicken birds are: *C. baileyi*, *C. meleagridis* and *C. galli*. Naturally occurring cryptosporidiosis in chicken manifests itself in three clinical presentations: respiratory disease, enteritis and renal disease, and the parasite has been found in many anatomic sites in the birds including the conjunctiva, nasopharynx, trachea, bronchi, air sacs, small intestine, large intestine,

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ceca, cloaca, bursa of fabricius, kidneys and urinary tract. Usually only one form of the disease can present as an outbreak (Sreter and Varga, 2000).

Nested-PCR is a sensitive technique for detection the oocysts in clinical and environmental samples. The most commonly used target is the 18S rRNA gene (Nichols et al., 2003). Restriction fragment length polymorphism (RFLP) technique have been described for the differentiation of *Cryptosporidium* species or genotypes, and most of studies have based on the small-subunit (SSU) rRNA gene (Coupe et al., 2005; Nichols et al., 2003; Xiao et al, 1999a). This is largely due to the fact that *Cryptosporidium* species and genotypes are mostly characterized at this locus. The SSU rRNA gene has some advantages over other genes because of the multiple copy number and presence of conserved regions interspersed with highly polymorphic regions, which facilitates the design of PCR primers.

The present study aimed, for the first time in Syria, to differentiate the species of *Cryptosporidium* by molecular technique (PCR-RFLP), as the previous studies in Syria used microscopy examination which had a weak reliability.

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2. Materials and Methods

2.1. Samples collection and examination

Fecal samples (approximately 5 ml) were collected during the period from March 2013 to June 2015 from three sources, including 67 pre-weaned calves (<2 months old), 111 broiler chicken flocks (<45 days old), and 213 from children (<5 years old), and all targeted calves and children were exhibiting diarrhea, while broiler chickens suffered from respiratory or digestive problems or both.

Samples from calves and chickens were preserved with equal volume of 2.5 % potassium dichromate, and formalin 10% had been used to preserve the human samples. All samples kept at 4 $^{\circ}$C until use.

Before the examination, 1 ml of the specimen was washed three times with distilled water by centrifugation 1100 x g for 5 min. And after washing from potassium dichromate or formalin, direct smears were prepared and stained with Kinyoun's acid fast stain (Goldman and Green, 2009), and then examined microscopically to detect presence of *Cryptosporidium* oocysts.

The human samples were collected from Damascus and Hama provinces, bovine samples were from Homs and Hama provinces, and chickens samples were collected from Hama, Homs, Aleppo, Tartos and Damascus provinces. We couldn't collect samples from all Syrian provinces because of war and conflict within.

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2.2. DNA extraction

Genomic DNA was extracted from 250 µl of washed samples that were positive by microscopy using NucleoSpin® Tissue kit (Macherey Nagel, Germany) according to the manufacturer's instructions for stool samples except the stool lysis temperature was increased to 65 °C and DNA eluted with 60 µl of BE buffer (supplied with kit) instead of 100 µl. The eluted DNA was stored at -20 °C for further use.

2.3. Nested PCR of the 18S (SSU) rRNA gene

Primary amplification of an 1325 bp fragment of the 18S small subunit ribosomal RNA gene was carried out using the primers XF1 (5'-TTCTAGAGCTAATACATGCG-3') and XR1 (5'-CCCATTTCCTTCGAAACAGGA-3') followed by a nested PCR for amplification of approximately 840 bp with the primers XF2 (5'- GGAAGGGTTGTATTTATTAGATAAAG-3') and XR2 (5'-AAGGAGTAAGGAACAACCTCCA-3') (Feng et al., 2007; Xiao et al., 1999a).

The reaction mixture was initially incubated at 94 °C for 2 min for initial denaturation and then a total 35 cycles of reaction were performed with denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s and strand extension at 72 °C for 1 min. The final extension was done at 72 °C for 7 min. PCR products were visualized by electrophoresis in 1.5 % agarose gel. One isolate of *Cryptosporidium baileyi* (No:061005- Kyorin University School of Medicine, Mitaka, Tokyo, Japan) was used as positive control, and deionized water as negative control.

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2.4. PCR-RFLP of the 18S (SSU) rRNA gene of *Cryptosporidium* spp.

Ten µl of the PCR product (approximately 840 bp) were separately subjected to the restriction endonuclease digestion with enzymes *Ssp*I, *Vsp*I and *Mbo*II (*Mbo*II for calves samples only). This is according to the manufacturer's instructions (Thermo,(EU) Lithuania) in 20 µl reaction mixture for 1.5 h in a water bath at 37 °C. The digested products were separated by electrophoresis in 2% agarose gel. And the interpretation of the profiles was carried out depending on (Feng et al., 2007; Xiao et al, 1999b) .

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3. Results

3.1. Results of acid fast stain and nested-PCR

In the positive samples under microscope the oocysts appeared as red or pink spheral bodies against blue background of methylene blue (Fig. 1). The overall number of positive samples was 35 (Table 1). All the infected samples were positive to nested-PCR. Also the amplicons of ~840 bp had been shown after electrophoresis .(Fig. 2)

3.2. Results of RFLP analysis:

C. parvum was the only species which had been identified from pre-weaned calves and children samples, and no another species were detected. Restriction digestion by *Ssp*I, *Vsp*I and *Mbo*II generated three (449, 267 and 108 bp), three (628, 115 and 104 bp) and two (771 and 76 bp) bands, respectively (Fig. 3)

In broiler chickens, nine samples out of eleven were identified as *C.baileyi* (572 and 254bp by *Ssp*I- 620, 104 and 102bp by *Vsp*I), while two samples have showed unknown profile in comparison to species previously described in chickens. (Fig. 4)

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4. Discussion

Only few papers have been found about cryptosporidiosis in Syria, but no one used molecular techniques to detect *Cryptosporidium* species.

Following PCR-RFLP analysis only *C.parvum* species was detected in total of 24 isolates from calves and children. But larger number of samples are needed for representative survey and detecting the prevalence of all existed species.

C. hominis and *C.parvum* are the most common species responsible for the majority of human infections (about 90% of all cases worldwide), and the proportion between the two species is approximately equal among humans in Europe, *C. hominis* is more prevalent in developing countries, but *C. parvum* was found to be even more prevalent in the Middle East (Xiao, 2010) and that agree with our result. Differences in the distribution of the species *C. hominis* and *C. parvum* are considered as an indication of differences in source of infection. The predominance of *C. hominis* in a population is considered to be the result of anthroponotic transmission, whereas both anthroponotic and zoonotic transmission are possible routes for *C. parvum* infections, and there is a relation between the subtype of *C. parvum* and ability to infect man, or both man and animals, so, amplification and sequencing of GP60 gene is needed for subtype detection (Plutzer and Karanis, 2009; Helmy et al., 2013).

In samples from broiler farms we identified after RFLP analysis *C. baileyi* profile and another species with unknown profile. molecular characterization is needed by sequencing of more of one target gene for identification.

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Tables:

Table 1. Number of samples collected and samples positive using acid fast stain.

Source of samples	No. of samples collected	No. of positive samples	%
Children <5 years	213	7	3.2
Pre-weaned calves	67	17	25.3
Broiler chickens	111	11	9.9

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Figure captions:

Fig. 1. Fecal smear was stained with Kinyoun's acid fast method showing *Cryptosporidium* oocysts.

Fig. 2. Secondary PCR amplicons of 6 samples collected from calves (photos from chickens and children not showed), M: DNA ladder marker(100bp), PC: positive control, NC: negative control

Fig. 3. RFLP profiles of 6 *Cryptosporidium parvum* samples, showing digested nested PCR products with *Ssp*I, *Vsp*I and *Mbo*II restriction enzymes.

Fig. 4. *Cryptosporidium baileyi* profile after RFLP analysis in comparison to unknown profile which was reported in two samples from chicken.

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Figure 1

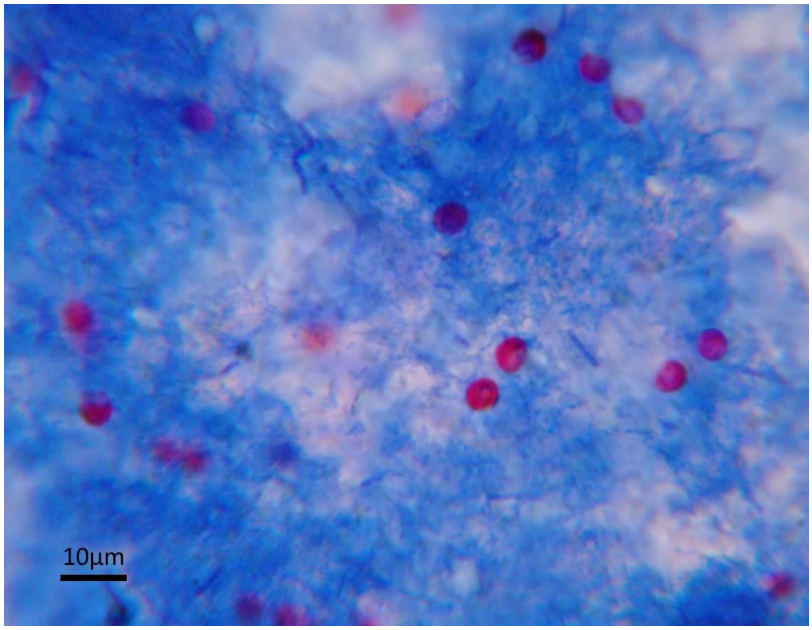

Figure 2

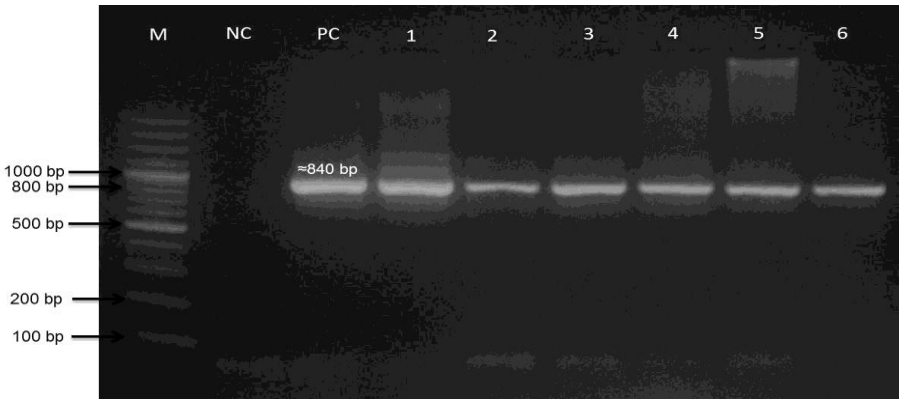

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Figure 3

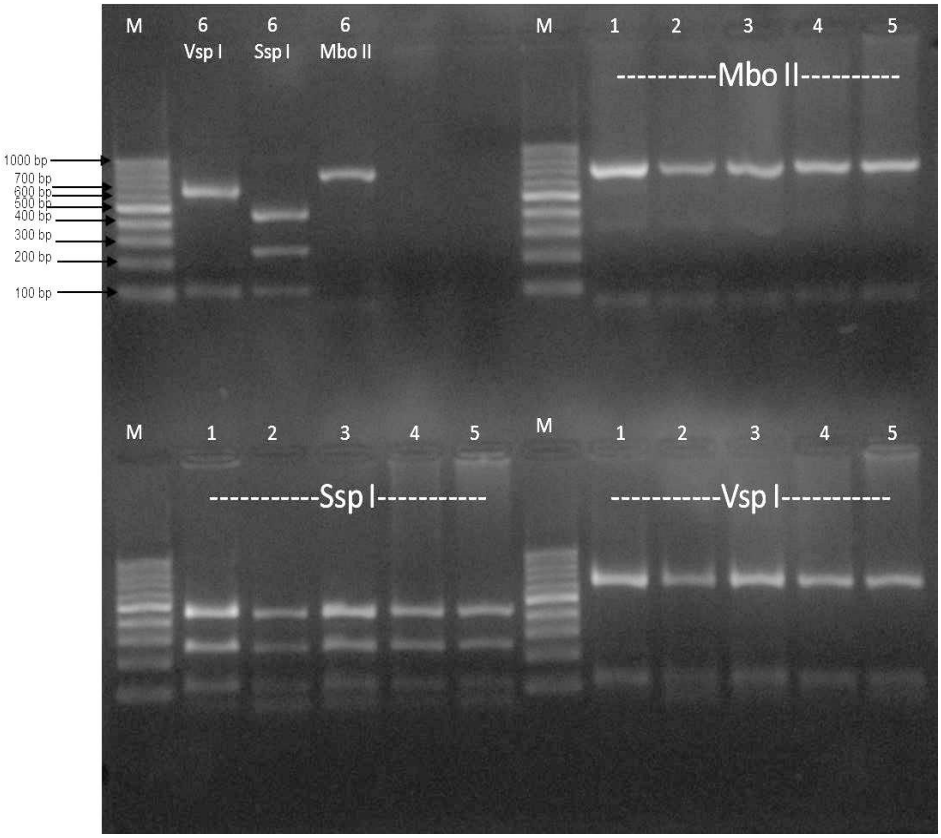

Figure 4