American Journal of Bioscience and Clinical Integrity
https://biojournals.us/index.php/AJBCI
ISSN: 2997-7347

Detection of the Virulence Gene Chro.20010 for a Parasite *Cryptosporidium Spp*

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Received: 2025, 15, Jan
Accepted: 2025, 21, Feb
Published: 2025, 12, Mar

Annotation: General Background: Cryptosporidium spp. is a globally distributed protozoan parasite responsible for cryptosporidiosis, a diarrheal disease affecting both immunocompetent and immunocompromised individuals. Due to the absence of highly effective treatments, molecular studies are crucial for understanding the parasite's virulence mechanisms.

Specific Background: The heat shock protein (Hsp70), encoded by the Chro.20010 gene, plays a pivotal role in parasite survival under stressful conditions. However, its structural and genetic characteristics remain inadequately explored.

Knowledge Gap: Previous studies have identified Hsp70 as an essential chaperone protein in various pathogens, but limited research has analyzed its molecular structure and role in Cryptosporidium hominis.

Aims: This study aims to investigate the presence and structural properties of the Chro.20010 gene in Cryptosporidium hominis isolates to evaluate its potential as a diagnostic and therapeutic target.

Results: The gene was successfully amplified in 89% of positive stool samples using Nested PCR, confirming its prevalence. Sequence analysis revealed a conserved structure with no mutations, indicating its genetic stability. Structural modeling demonstrated the functional domains of Hsp70, particularly the nucleotide-

binding and substrate-binding regions, which contribute to protein stabilization under stress.

Novelty: The findings highlight the molecular stability and functional importance of Hsp70 in Cryptosporidium hominis, reinforcing its role as a virulence factor and potential therapeutic target.

Implications: This study provides foundational insights for future research targeting Hsp70 as a drug candidate, potentially aiding in the development of novel therapeutic strategies against Cryptosporidium infections.

Keywords: Cryptosporidium hominis, Hsp70, Chro.20010 gene, molecular diagnosis, virulence factor, stress response, Nested PCR, protein stability, therapeutic target.

Introduction:

Cryptosporidium is a pathogenic intestinal parasite that infects the gastrointestinal tract, first described by rat stomach mucosal swabs in 1907 from Edward Ernst Tizzer, this parasite was not linked to human infection until 1976, it is ubiquitous environmentally and geographically, and includes many species. For a wide host group, more than 40 species have been described so far, with at least 20 species diagnosed in humans, including Cryptosporidium hominis and Cryptosporidium parvum accounting for the vast majority of infections in humans Cryptosporidiosis is a zoonotic disease between humans and animals caused by different species of the genus. Cryptosporidium spp, It was not associated with human infection until 1976[1]. The intestinal parasite Cryptosporidium is a major cause of diarrhoeal disease in humans and animals worldwide, and effective treatments or vaccines are not available, so control depends on understanding the dynamics of disease transmission. The development of molecular detection mechanisms has led to the identification of a large number of mysterious species and genotypes and has facilitated our understanding of how zoonotic diseases are transmitted, and of the 44 recognized cryptosporidium species and more than 120 genotypes, some zoonotypes and genotypes are still lacking and more thorough molecular epidemiological studies are conducted in countries where the likelihood of transmission is higher[2]. What it takes to enhance our understanding of this important animal pathogen. Similarly, whole genome sequencing (WGS) and amplecon next-generation sequencing (NGS) are important for tracking transmission more accurately and understanding the mechanisms underlying host specificity . The severity of the infection depends on the host's immune system as immunocompromised individuals, such as HIV patients, transplant patients and infants, are debilitating and fatally susceptible to cryptosporidosis, which is characterized by cholera-like diarrhea that may be long-lasting, and infection in HIV patients has been linked to severe weight loss, bile duct disease, pancreatitis and respiratory diseases. The most common clinical feature of cryptosporidiosis in people with immunocompetent and immunodeficiency is diarrhea,symptoms that most often lead to a characteristically diagnosis, profuse diarrhea and watery may contain mucus, often associated with weight loss, other common clinical features include abdominal pain, nausea, vomiting and fever Sometimes, non-specific symptoms such as myalgia, weakness, malaise, headache and loss of appetite occur [3]

Molecular techniques, such as polymerase chain reaction, improved both clinical and environmental detection in some studies using molecular methods, after confirmation by staining,

parasitic DNA was extracted from stool samples using a set of molecular tests, and most of the molecular studies conducted, which can detect low-number parasites in samples, are used, and these methods are recommended for use in epidemiological and environmental studies of this parasite[4]. Therefore, molecular studies can be used to diagnose species and assess the importance of common diseases, as this method has proven high sensitivity in the diagnosis of species , as PCR technology has been developed in recent years to detect and diagnose types of cryptobacteria, as there are a large number of genes that are targeted for the purpose of diagnosis, including the gene responsible for the formation of wall protein (Cowp) and heat shock protein[5].

The HSP70 coding gene is used as the target of studies that reported higher sensitivity, although other genes, such as the Cryptosporidium egg wall protein, 60 kDalton, and Laxer position, use interspecific discrimination because of the very close similarity in their sequences[6].

Materials and Methods:

Nested PCR of the gene PCR was performed in the reaction to amplify a 430bp piece of the 18s rRNA gene and the reaction mixture was prepared as follows.

Components	20 µl reaction volume
Template DNA	3µl
Forward primer	1µl
Reverse primer	1µl
D.W	15µl
Total volum	20µl

The reaction components were mixed in Premix tubes and then placed in a thermoplastic cycler and the following program was prepared[7].

Step	Temperature	Time	Number of cycles
Initial denaturation	$95C^0$	4 min	One cycle
Denaturation	$95C^0$	60 s	30 cycle
Annealing	$55C^0$	60 s	
Extension	$72C^0$	60 s	
Final extension	$72C^0$	10 min	One cycle

The second reaction contains a pair of nested primers (overlapping) to amplify a 553 bp piece of the Chro.20010 gene responsible for the formation of the thermal shock protein has been used 3µl of the first product PCR and used as a template for the second PCR reaction with the same components in the first reaction except for the prefixes and new prefixes were used.

F:(AGCTTGTACAGCAGCACCAT)

R (GCAGGTGCAATTGCTGGTTT)

The reaction components are mixed in premix tubes and placed in the thermopolymer core and the same program is used, but some differences as follows.

Step	Temperature	Time	Number of cycles
Initial denaturation	$95C^0$	4 min	One cycle
Denaturation	$95C^0$	50 s	30 cycle
Annealing	$55C^0$	30 s	
Extension	$72C^0$	50 s	
Final extension	$72C^0$	10 min	One cycle

After amplification of PCR, the technique of electrophoresis (Agarose Gel electrophoresis) was adopted to confirm the presence of amplification of DNA beams.

Results:

Molecular analysis of the gene encoding the thermal shock protein in Cryptosporidium hominis Molecular investigation of the Chro.20010 gene responsible for encoding the thermal shock protein (Hsp) was performed in Cryptosporidium hominis samples used in the current study. The method involved extracting and purifying the genetic material (DNA) from all the samples studied, and then measuring the purity of the extracted DNA. Electrorelay was used to analyze the resulting DNA beams and amplify the target gene using polymerase chain reaction (PCR) technology using prefixes specifically designed for that gene. The reaction products were examined by electrorelay to confirm the presence of the gene, and gene sequencing was determined to predict the structural and functional properties of the coding protein. The results of amplification of the Chro.20010 gene showed luminous beams in electrorelay, confirming the presence of the gene in the isolated studied. It was observed that the isolates showed a distinctive genotype that contributed to the molecular diagnosis of this gene associated with virulence factors. Prefixes are designed to target specific functional regions of the HSP protein encoding gene, With the exclusion of inefficient cutting. The magnified regions showed a clear genotype on the electrogel, confirming the efficiency of the initiators and the presence of the target gene.The expected molecular weight of the amplified gene was 532 base pairs per large unit of HSP. The results of the electrorelay (photo 1-1) confirmed the validity of the primers by showing characteristic DNA beams at the expected molecular weight. The beams matched the expected sizes when compared to the DNA ladder 1000 plus, indicating successful replication and primer specialization in all tested samples. These results confirm the importance of the Chro.20010 gene in encoding the functional HSP protein, which plays a crucial role in the survival of the parasite under stress conditions. . This molecular study contributes to the understanding of virulence mechanisms and provides a basis for further research targeting heat shock proteins as potential therapeutic targets in Cryptosporidium hominis. The results of this study showed that the Chro.20010 gene, responsible for encoding the heat shock protein (Hsp), plays a crucial role in the survival of the Cryptosporidium hominis parasite and its ability to cause diseases under stress conditions. Thermal shock proteins are essential "companion proteins" that help fold proteins, repair damaged proteins, and stabilize cellular processes during adverse environmental conditions. He confirmed the amplification of gene sequences through electrorelay matching the expected molecular weights of protein units, proving the accuracy of the initiators used and the efficiency of the process. Amplification of the HSP gene in C. hominis isolates indicates its primary role in parasite's adaptation and survival within hostile body environments, such as elevated gastrointestinal acidity and immune challenges. Previous studies have demonstrated that HSP proteins enhance their gene expression in response to heat, oxidative stress and immunity, making them essential for parasite's survival and pathogenesis. The isolates also showed a distinct genotype indicating the possibility of genetic variations affecting the virulence of the parasite between strains. Structural and functional predictions of the HSP protein encoded by the Chro.20010 gene show its oligomeric (multiunit) nature, which is in line with other parasites in which HSP forms multi-unit clusters to increase its efficiency in re-folding damaged proteins . Determinations of functional regions such as nucleotide binding and substrate binding regions emphasize their vital role in stress response and maintaining protein stability[8]. These findings highlight the potential for targeting HSP proteins as therapeutic targets. Inhibiting the activity of HSP can disrupt a parasite's ability to cope with stress, making it more vulnerable to immune defenses and drug treatments. Similar strategies in diseases such as malaria and leishmaniasis have shown that HSP inhibitors are effective in reducing parasite survival. Future research could focus on the development of small inhibitors or vaccines that target the CHRO.20010-encoded HSP in the C. hominis.

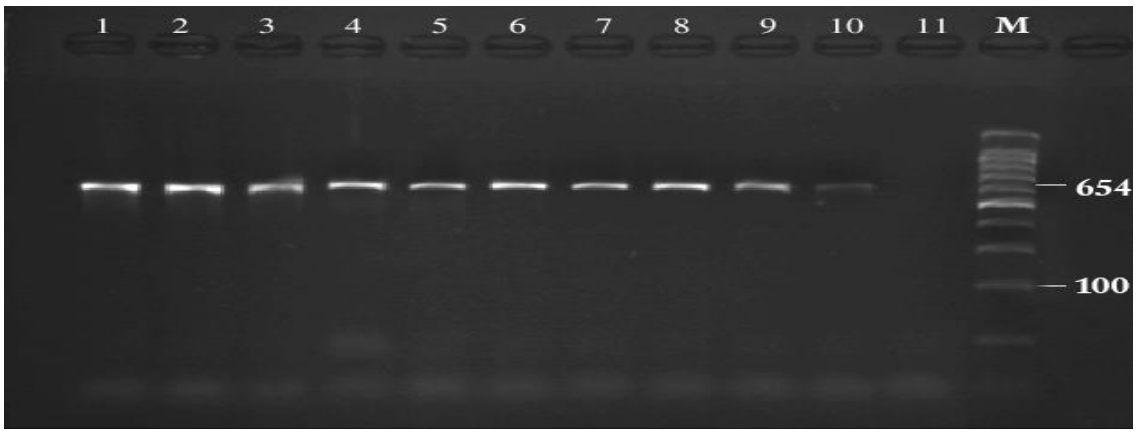

Image (1-1) The output of the electrical relay of the extraction of DNA extracted from the parasite Cryptosporidium hominis as the columns of 1-10 DNA beams of the gene Chro.20010, and the column M represents the known molecular weight Ladder DNA with a molecular weight of 100 base pairs and a concentration of acarose gel 2% for an hour and a half and a current of 5 volts per centimeter.

Chro.20010 gene diagnosis based on nitrogen base sequence and global comparison in NCBI

Gene symbol: *Chro.20010*

Gene description: heat shock protein

Locus tag: Chro.20010

Gene type: protein coding

RefSeq status: PROVISIONAL

Organism: *Cryptosporidium hominis TU502* (strain: TU502)

Lineage: *Eukaryota; Alveolata; Apicomplexa; Conoidasida; Coccidia; Eucoccidiorida; Eimeriorina; Cryptosporidiidae; Cryptosporidium*

Figure (1-1) Genetic map of the gene Chro.20010

The Chro.20010 gene, responsible for encoding the heat shock protein in the Cryptosporidium hominis, shows advanced results in genetic sequencing analysis. A comparison with sequences recorded at the National Center for Biotechnology Information (NCBI) showed that the gene had significant genetic stability, as no mutations or variations in nitrogenous bases were observed. This finding reinforces the hypothesis that the thermoshock protein-coding gene has a high degree of preservation across the parasite's various isolates. The data also indicate that the gene contains only one exxon unit and is present on the parasite's second chromosome, contributing to an understanding of its essential role in the parasite's biological processes[9]. Genetic sequencing shows that the protein pieces encoded by the gene play an important role in virulence, with heavy protein units promoting parasite activity under different stress conditions, while intermediate units contribute to increased virulence effectiveness. Furthermore, the comparison with the sequences recorded in the Global Genbank (NCBI) shows .The genetic sequence of this gene is identical to that found in other studied species. This proves the importance of the gene as a potential study goal in the fight against parasites. The results of this genetic analysis support the need for additional studies to understand the functional roles of this gene, especially in the development of targeted therapeutic strategies[10].

Stereostructure and protein structure of gene output by bioinformatics programs (Figure 1-2)

Three-dimensional structure (Panel A, B, C) The SWISS-MODEL website demonstrates the prediction of the three-dimensional structure of a protein using the available reference models. The image shows that the protein consists of key domains representing the active regions (Nucleotide-Binding Domain (NBD) and substrate-binding Domain (SBD). The protein contains areas with twists (Helices) and beta-sheets, reflecting its structural integrity as a "chaperone protein" that contributes to the folding and stability of damaged proteins. The colors in the image (heat map) show the accuracy of the structural model. Blue regions represent the predicted structure with a high confidence score (>90), while red areas indicate areas that are less confident in structural prediction[11].

Results from Alpha Fold AlphaFold provides detailed and accurate protein predictions. The site shows high confidence in the active parts of the protein, especially the ATPase activity that the protein relies on to stabilize the substrates. Predicted Aligned Error (PAE) indicates the level of accuracy between different regions of the protein, showing a significant reduction in - Functional data from UniProt The UniProt database shows that the protein has multiple functions, including protecting the parasite from heat stress Stress Response HSP encoded by Chro.20010 helps the parasite survive under environmental stressors, such as changes in temperature, oxidative stress, and host immune responses, which are common during host infection. and promote its survival within the host. The data also indicate that the protein belongs to the HSP70 family with basic vital functions. Proper folding of damaged proteins (Protein Quality Control) ensures proper folding and prevents protein build-up, which is critical for parasite survival under hostile host conditions. The site also notes that the protein interacts with ATP molecules to regulate its activity, enhancing its efficiency as a molecular chaperone, and adapting to the life cycle adaptation during transitions between the host and environment phases (for example, cyst formation and exit), HSPs are essential to maintain protein integrity[12].

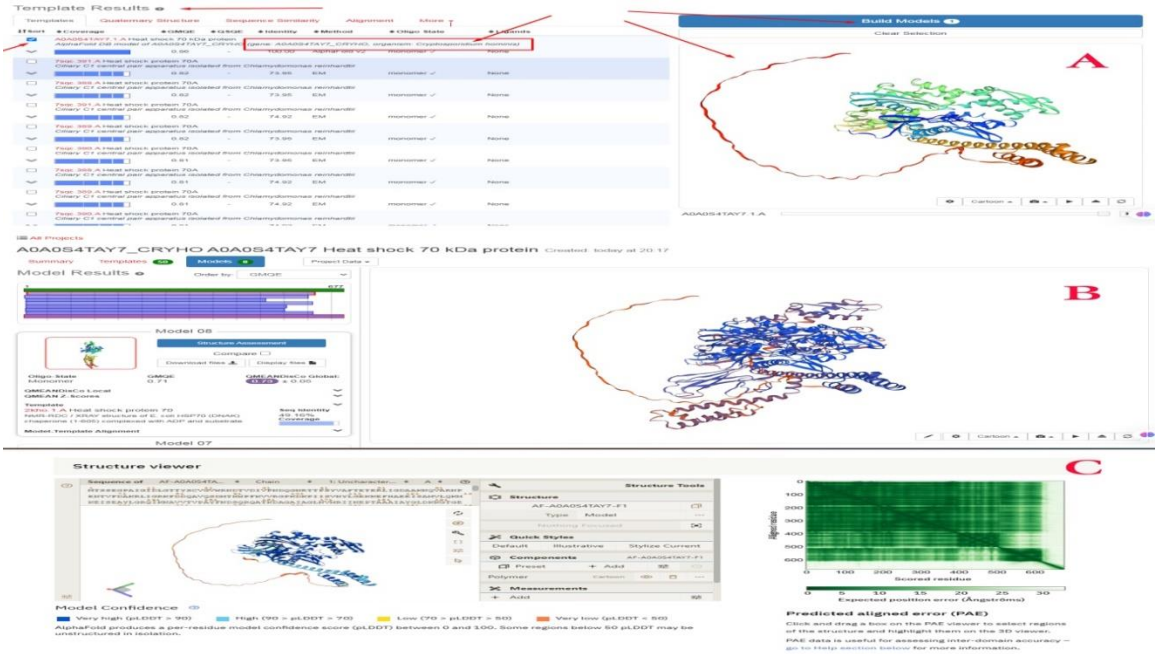

Figure (1-2) Data Analysis of the Expected 3D Structure of Heat Shock Protein 70

Discussion:

The Hsp70 protein encoded by the Chro.20010 gene is shown to play a pivotal role in parasite survival, acting as a key factor that helps adapt to conditions of heat and immune stress. The projected three-dimensional structure provides strong support for the design of studies targeting this protein to develop new drugs that block the parasite's ability to survive in the host[13].

Through models available via SWISS-MODEL and AlphaFold, the projected three-dimensional structure of the Hsp70 protein showed high accuracy in the active regions (Nucleotide-Binding Domain (NBD) and Substrate-Binding Domain (SBD). These areas are the basis for the protein to act as a "chaperone protein" that helps in the correct folding of damaged proteins. Previous studies have confirmed that the NBD domain is responsible for ATPase activity that enables the protein to bind and release substrates, enhancing its role in supporting the stability of proteins under stress[14]. The binding areas also act in particular to catch unfolded proteins, protecting the parasite in difficult environments. The data shows that Hsp70 is necessary for parasites such as Cryptosporidium hominis, as it protects the parasite from heat stress and environmental stresses within the host's body. This finding is consistent with studies such as those by Boorstein et al. [15], where it has been observed that heat shock proteins are excreted in conditions of heat stress and stabilize proteins necessary for the survival of the organism. Pathogenic parasites often experience hostile immune responses within the host's body, such as increased temperature and immune changes. Hsp70 acts as a parasite's defense mechanism, This enhances its survival in extreme conditions. From the findings from UniProt, Hsp70 acts as a key factor in the rapid adaptation of the parasite to host cells, facilitating adhesion, entry, and survival within cells. This role has been highlighted in previous studies, where thermal shock proteins have shown a pivotal role in supporting the life cycle of parasites, including their entry and reproduction mechanisms within the host[16]. HSP proteins have the ability to repair damaged proteins that may be affected by immune or therapeutic stress, making them a key factor in enhancing the parasite's resistance to treatment. The predictive structure of AlphaFold has shown high confidence domains, supporting the possibility of targeting Hsp70 with therapeutic compounds that block its activity. Previous studies have shown that inhibition of HSP70 It may impair the ability of parasites to survive and multiply, making this protein a promising target in the development of antiparasitic drugs[17].The current findings confirm their compatibility with previous studies that demonstrated the importance of Hsp70 in other parasites such as Plasmodium falciparum, where it has been successfully targeted in laboratory studies to inhibit parasite activity[9]. The difference in secondary structure between different types of parasites may contribute to the development of customized therapies to target this protein precisely.

Conclusions:

The Hsp70 protein encoded by the gene Chro.20010 plays a crucial role in the survival and reproduction of the Cryptosporidium hominis parasite in the host. The precise three-dimensional structure and compatibility of the results with previous studies make it a promising target for the development of new drugs. These drugs can disrupt the vital functions of the parasite, reducing its severity and ability to resist treatments.

Based on the findings of this study, the Chro.20010 gene encoding the Hsp70 protein plays a pivotal role in the survival and pathogenicity of *Cryptosporidium hominis* under stress conditions. The study demonstrated a high prevalence of this gene in clinical isolates, confirming its utility as a molecular marker for *Cryptosporidium* detection. The structural analysis of the Hsp70 protein revealed conserved functional domains crucial for protein stabilization and stress response, reinforcing its significance in parasite survival. These findings have important implications for the development of novel therapeutic interventions, as targeting Hsp70 could disrupt the parasite’s ability to withstand environmental and immune stresses. Furthermore, the genetic stability of the Chro.20010 gene across isolates highlights its potential as a reliable diagnostic target. Future research should explore the development of small-molecule inhibitors targeting Hsp70, as well as vaccine-based strategies aimed at mitigating *Cryptosporidium* infections, thereby contributing to improved public health outcomes

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