Designing a multi-epitope subunit vaccine against *Fasciola gigantica* using cathepsin L by immunoinformatics approach

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Authorization

The work presented in this thesis is entirely my own and I hereby declare that I am the sole author of the thesis entitled – Development of a vaccine against *Fasciola spp* using cathepsin L by immunoinformatics approach. I also declare that it has not been previously submitted to any university for the award of a degree.

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee will be addressed

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DEDICATED TO MY HUSABAND AND BELOVED PARENTS

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Abstract

Fascioliasis, caused by the liver fluke *Fasciola spp*, is a serious zoonotic disease of animals and humans that causes great economic damage globally. Triclabendazole (TCBZ) is the FDA approved sole medicine available for the treatment of the condition, and drug resistance is on the rise. There is no commercially available safe and effective vaccine to protect against this infection. Increasing TCBZ resistance and the lack of an effective vaccination against fascioliasis necessitate vaccine research. Here, Cathepsin L protein of *Fasciola spp* was employed in the current work to create a potential vaccine candidate utilizing a structural immunoinformatics method. Cathepsin L antigen is a potential vaccine candidate which is safe, non-allergic, highly antigenic, and effective against a variety of parasitic flukes and worms. The cytotoxic T cells, helper T lymphocytes, and B-cell epitopes were chosen to design vaccine for their immunogenicity and binding affinity behavior. The developed vaccine's physicochemical characteristics, allergenicity, and antigenicity were investigated. To determine the vaccine's tertiary structure, homology modeling was used, followed by structural refinement and docking to the toll-like receptor-2 immune receptor. Molecular dynamics simulations revealed that the vaccination and receptor combination had a stable association. Finally, *in silico* cloning was used to assess the vaccine construct's expression and translation in the *E. coli* expression system. Experimental evaluation of the designed vaccine in an animal model is needed to confirm the safety and immunogenicity of the developed vaccine. It may result a novel and immunogenic vaccine that may confer protection against *Fasciola spp*. infection.

Keywords: Fascioliasis, Liver fluke, Cathepsin L, Immunoinformatics, Vaccine

Chapter I: Introduction

The influence of climate change and global change has pushed trematodiases to the forefront of infectious disease awareness (Caminade et al. 2015; Chowdhury et al. 2016). In the new World Health Organization (WHO) Roadmap 2021-2030 for neglected tropical diseases (NTDs), foodborne trematode infections (clonorchiasis, opisthorchiasis, fascioliasis, paragonimiasis) are highlighted (Casulli 2021). *Fasciola gigantica* and *Fasciola hepatica* are the platyhelminthic endoparasites, causal agent of fascioliasis, infect mainly farm animals, but human populations in poor and underdeveloped nations are also affected (Siles-Lucas et al. 2021). The most recent estimation by Fürst et al was at least 2.6 million individuals are sick with many more at risk in more than 70 nations globally (Fürst et al. 2012; Caravedo and Cabada 2020). This disease causes significant losses in the animal sector, such as milk and meat production, secondary bacterial infections, and expensive anthelmintic treatment (Soulsby 1968; Young et al. 2010). Triclabendazole (TCZ), a FDA-approved medicine that is active against both immature and adult parasites, is the primary treatment for this condition (Klinger et al. 2020; Tabari et al. 2022). Because of the rising prevalence of both animal and human fascioliasis, as well as the establishment of TCZ-resistant parasite populations, vaccination has emerged as an alternate option for disease management (Robinson et al. 2002; Kelley et al. 2016; Morales et al. 2021). The whole genome of *F. gigantica* was recently sequenced which has an assembly length of 1.04 GB and encodes 20,858 genes (Pandey et al. 2020a).

Fasciola spp. has a complex life cycle, use cysteine proteases for growth and survival, involving penetration, migration, nutrition acquisition, and immune evasion (Cwiklinski et al. 2018; Buffoni et al. 2020; Aftab et al. 2021). A number of cysteine proteases expressed in the newly excysted juvenile (NEJ) and infective stages have been discovered and investigated as vaccine candidates (Hillyer 2005; Cancela et al. 2008; Siricoon et al. 2012; Garza-Cuartero et al. 2018). Cathepsin L proteases are the primary proteases secreted by mature *Fasciola spp. in vitro* and *in vivo* (DOWD et al. 1994; López Corrales et al. 2021). In addition, cathepsin B, and cathepsin L3 are found mostly in infective stages, including newly excysted juveniles (NEJs)(Chantree et al. 2013; Wesołowska et al. 2018; Smith et al. 2020). However, major protease

cathepsin L generated by adult parasites have been demonstrated to cleave hemoglobin (Lalor et al. 2021), type I collagen (Corvo et al. 2009; Sansri et al. 2013), and immunoglobulin (SMITH et al. 1994; Sansri et al. 2013), as well as hinder eosinophil adhesion to its surface (Carmona et al. 1993; Zafra et al. 2013). FhCL3 and FhCL4 were discovered as juvenile cathepsin L proteases in *F. hepatica*, and their sequences were shown similar to *F. gigantica* cathepsin L1G and L1H (FgCatL1G, FgCatL1H) (Meemon and Sobhon 2015; Sansri et al. 2015; Ferraro et al. 2016; Pritsch et al. 2020). FhCL3 was most active and stable at neutral pH and could cleave collagen but not immunoglobulin, implying a role in parasite movement through the liver (Celias et al. 2019; Barbour et al. 2021). Thus, cathepsin L proteases are essential for parasite survival.

Fasciola spp. encounters a mixture of humoral and cellular immune reactions within the host as it makes its way via the peritoneal cavity to the liver, and then to the bile ducts. This state is combated by flukes by altering cellular functions through immune evasion and subversion (Ryan et al. 2020; Jiménez et al. 2021; Mei et al. 2022). Several investigations on cattle and buffaloes against fasciola infection have been conducted, but commercial vaccine is still unavailable (Toet et al. 2014; Akıl et al. 2022). However, researchers tried to implement immunoprophylactic control in sheep, cattle, buffaloes using fasciola specific antigen such as glutathione-S-transferase (GST), cathepsin L-like cysteine proteases, fatty acid binding protein (FABP), leucine aminopeptidases (LAP), and fluke hemoglobin (Sexton et al. 1990; Raina et al. 2011; López-Abán et al. 2012; Kalita et al. 2019; Kalita et al. 2020; Barbour et al. 2021; Villa-Mancera et al. 2021). Vaccination with GST may give 57% protection to sheep afflicted with fasciola as well as *Taenia ovis* (Johnson et al. 1989). Vaccination of cattle and sheep using Cathepsin L1 and L2 antigens resulted in 33 to 79% protection in fasciola burden and ~60% reduction in egg viability (Dalton et al. 2003). In another research, the same antigens did not diminish worm load in sheep but did lower egg production and egg viability to 70% and 80%, respectively (Wijffels et al. 1994). Cathepsin L exhibited improved (78%) protection in sheep when coupled with LAP but vaccination with LAP alone elicited the highest level of protection (89%) (Piacenza et al. 1999). Fluke loads were reduced by 47% and 63%, respectively, with recombinant *F. hepatica* cathepsin L3-1 and L3-2 (Wesołowska et al. 2018). However, *F. gigantica* recombinant cathepsin B2 and B3 inoculated mice reduced

parasites by 60% and 66%, respectively, by eliciting a mixed Th1/Th2 immune response (Chantree et al. 2013). Vaccination with *F. gigantica* Saposin-like protein, SAP-2, resulted in 76.4% to 78.5% protection in mice via a mixed Th1/Th2 response (Kueakhai et al. 2013; Kalita et al. 2019).

Recent significant advances in computational methodologies have resulted in the development of immunoinformatics tools as a viable alternative way for prediction of potential vaccine with possible translational implications (Chauhan et al. 2019; Kalita et al. 2019; Kadam et al. 2020; Kolla et al. 2021; Tosta et al. 2021; Yılmaz Çolak 2021; Ayyagari et al. 2022). The current work aims to develop a vaccine by integrating B-cell, T-cell, and Helper T Lymphocytes (HTL) epitopes from *F. gigantica* Cathepsin L protein to confer protection and potentially elicit neutralizing antibodies to prevent fasciola infection. *In silico* cloning was also used to assess the construct's expression and translation efficiency.

Chpater II: Review of literature

2.1 Fasciolosis

2.1.1 Definition

Fasciolosis known as fasciolasis, distomatosis, and liver rot caused by a parasite illness that affects sheep, goats, and cattle. It is regarded as a zoonotic illness since it can occasionally harm people (CDC 2019; Wagari 2021). They cause widespread illness and death in cattle characterized by anemia, hypoproteinemia, and weight loss (Soulsby 1968).

2.1.2 Etiology

The genus Fasciola contains many species of trematodes (often known as "flukes") that cause fascioliosis (Roberts and Suhardono 1996). Several species responsible for fasciolosis according to their taxonomic classification: Phylum: Platyhelminthes, Class: Trematoda, Sub class: Digenea, Order: Echinostomida, Super family: Fascioloidea, Genus: Fasciola, Species: *F. hepatica* and *F. gigantica* (Soulsby 1968).

2.1.3 Morphology

Fasciola is a fluke with a wide, conical-shaped front protrusion and a leaf-like form. When stored, its appearance changes from grayish brown to gray. Sharp spines are on the tegument. After the immature fluke first enters the liver, it is between one and two millimeters long and lancet-shaped when it has reached full maturity in the bile ducts. It is about 3.5 cm long and 1 cm wide, and it has a leaf-like form. Its color is gray brown (Soulsby 1968; Wagari 2021) The oval-shaped eggs of fasciola are brownish to yellowish brown in color. The eggs only develop after they are deposited and have an unclear operculum. It is important to identify fasciola eggs from those of other flukes, notably from the massive paramphistomum eggs. While paramphistomum eggs have a transparent shell, prominent operculum, embryonic clear cells, and a little knob at their posterior ends, fasciola eggs have a yellowish-brown shell, an unclear operculum, and embryonic cells (Coles et al., 2006).

F. gigantica may grow up to 7.5 cm in length, making it bigger than *F. hepatica*. The form is more like a leaf, with a very short conical front end. The eggs of *F. gigantica*

measure 190x100 micrometer (µm), bigger than those of *F. hepatica* (Roberts and Suhardono 1996; Wagari 2021).

2.2 Epidemiology

2.2.1 Geographical distribution

Fasciolosis is regarded as a significant production-limiting condition for ruminants. It is found worldwide where there is a suitable environment for Laymnaeid snails population in grazing field of cattle, sheep, and goats (Soulsby 1968) The most significant trematode of domestic ruminants and one of the main causes of liver fluke illness in temperate regions of the world is *F. hepatica*, a temperate species. As a result, it may be found in Australia, Africa, Europe, Southern and Northern America. On the other hand, *F. gigantica* is widespread and commercially significant in tropical regions of Asia and Africa . While *F. hepatica* is reported between 1200 and 2560 meters above sea level, *F. gigantica* is located below 1800 meters below sea level. Mixed infection of both species found at 1200-1800 meters above sea level (Lalrinkima et al. 2021; Pan et al. 2022).

2.2.2 Risk factors

The quantity of metacercaria that accumulate on grass is one of the main factors that affecting severity of fasciolosis. The geographical and temporal availability of snail host as well as the frequency of egg and larval maturation are particularly influenced by temperature and rainfall are the risk factors of fasciolosis (Nath et al. 2022)

2.2.3 Availability of suitable snail habitat

The existence of sufficient snail habitation is one of the most significant elements that affects the prevalence of fasciolosis in a location. The habitat for snails might be temporary or permanent. As an example, *L. truncatuala* likes moist mud to free water, and its main ecosystem includes trenches, creeks, and the borders of small ponds. The availability of these intermediate hosts is influenced by climatic circumstances. Rush clusters in fields are sometimes a sign of trouble. Although *L. truncatula* thrives best in a slightly acidic environment, extremely acidic pH values are harmful (Vázquez et al. 2018; Malatji et al. 2020).

2.2.4 Temperature

Temperature has a significant impact on the snails' rate of growth as well as the phases of the parasite that exist outside the ultimate host. The growth of fasciola inside the snail requires a temperature of at least 10°C during the day or at night, and all activity stops at 5°C. According to research, fasciola cercaria and lymnaea snails can live better between 25°C and 30°C, which at least partially reflects why autumn has a considerably higher incidence than other months (Moazeni et al. 2010; Kianifard et al. 2020; Andreyanov et al. 2021).

2.2.5 Moisture

When rainfall overcomes outflow and field wetness is reached, the perfect moisture conditions are created for fasciola growth and reproduction inside of snail. The maturation of fluke eggs, the ability of miracidium to hunt for snails, and the dissemination of cercariae shed by the snails are depend on these circumstances (Sun et al. 2020; May et al. 2022).

2.3 Host range

2.3.1 Definitive host

Cattle, sheep, several other ruminants, equidae, swine, and rabbits are some of fasciola's recognized hosts. Finally, it should be kept in mind that whereas *F. gigantica* infects ruminants, *F. hepatica* may infect a broad variety of mammals, and it is likely that occasionally these hosts may function as carriers of transmission (Vázquez et al. 2021; Kipyegen et al. 2022).

2.3.2 Intermediate host

Molluscs of the family Lymnaeidae, such as Lymnaea, can serve as intermediate hosts. The most popular species of Lymnaea snails, *L. truncatula*, are amphibious snails that are widely distributed around the world. Outside of Europe, *L. tomentosa* in Australia and New Zealand, *L. cumella* in North America, Australia, and New Zealand, *L. bulimoidis* in Southern USA and the Caribbean, *L. humlis* in North America, *L. viator* in South America, and *L. diaphena* in South America are other significant Lymnaea vectors of *F. hepatica*. However, Lymnaea snails, including *L. auricularia*, *L. natalensis*, *L. rufescens*, and *L. rubiginas*, are intermediate host of *F. gigantica*. They may all be found in marshy marshes, drainage systems, and streams (Ferreira et al., 2021; Lalrinkima et al., 2021; Prastowo et al., 2022).

2.4 Life cycle

Life is sketched in Figure 1. Flukes that are adults shed their eggs into the bile, which then travels to the gut. Eggs exit the body after going through feces. Initially after releasing, they are immature and take at least 10 days to grow to the miracidial stage (Muro and Rojas-Caraballo 2017). Fluke eggs passed through the host's feces being grow and hatch, delivering a motile, ciliated miracidium. These require 9 days at the ideal temperature range is 22–26°C, and little growth happens below 10°C (Gayo et al. 2020; Andrews et al. 2021) The freed miracidium only has a three-hour window in which to find a compatible snail and successfully penetrate its tissue. The last stage of the intermediate host, the cercaria, develops in infected snails after passing through the sporoyst and redial stages. These cercaria are discharged from the snail as motile forms that stick to surfaces like grass blades and encyst there to create the infective metacercariae (Moazeni and Ahmadi 2016; Gayo et al. 2020). The transition from the miracidium to the metacercariae requires at least 6-7 weeks. The final host consumes metacercariae, which excyst in the small intestine and then go through the gut wall, cross the peritoneum, and enter the liver capsule (Vázquez et al. 2021; Prastowo et al. 2022). For 6-8 weeks, the immature flukes passageway through the liver parenchyma before entering the bile ducts, where they spread to the major ducts and possibly the gall bladder. The preparation process lasts 10 to 12 weeks (Soulsby 1968). The minimum time required to complete one *F. hepatica* life cycle is therefore 17-18 weeks. In untreated cattle, *F. hepatica* normally lives for less than a year (Soulsby 1968). *F. gigantica* infects animals near water holes; infection is determined by numerous parameters linked to the nature of the vector, the physiology of the parasite, and flock and livestock husbandry. Humans are infrequently affected by ingesting metacercariae that have encysted in aquatic plants. Consuming raw liver foods made from fresh livers contaminated with juvenile *Fasciola spp* may also result in spreading. Major symptoms are abdominal pain, uncomfortable liver areas, and anemia (Muro and Rojas-Caraballo 2017; Singh et al. 2021; Kipyegen et al. 2022).

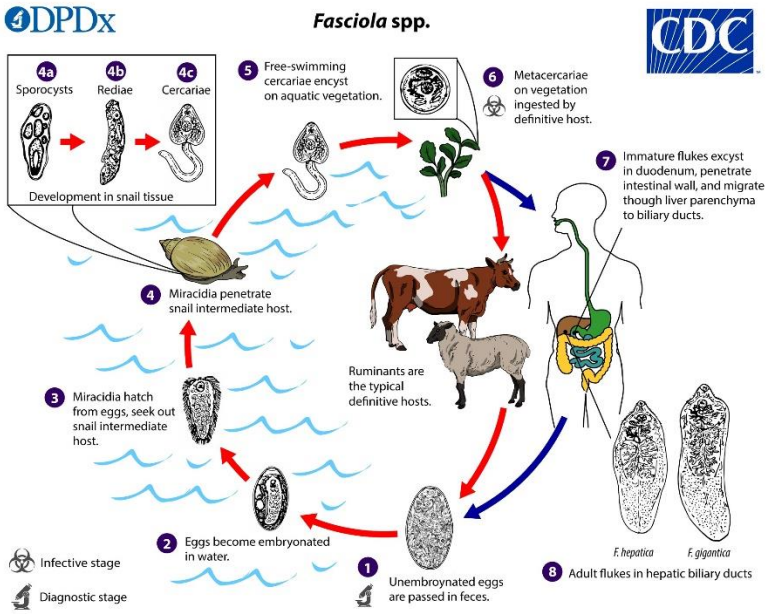

Figure 1. Life cycle of *Fasciola spp* (CDC 2022)

2.5 Pathogenesis

The pathobiology of fasciolosis differs depending on the stage of parasite growth in the liver and the host species engaged. Pathogenesis is divided into two stages. The first phase is related with liver injury and bleeding and occurs during translocation in the liver parenchyma (Pan et al. 2022). The second happens when the parasite is in the bile duct and is caused by the adult fluke's haematophagic activity as well as injury to the biliary mucosa by their tegumental spines. Pathogenesis caused by the fluke's activities might be either acute or chronic (Muro and Rojas-Caraballo 2017; Lu et al. 2018). Acute fasciolosis develops during the preadult migration of flukes in the liver parenchyma. The liver is largely damaged in chronic fasciolosis. In contrast to the acute phase of the illness, fibrosis (scar tissue), bile duct obstruction, and bile duct inflammation cause harm (Gayo et al. 2020; Lalor et al. 2021). The pathophysiology of bovine fasciolosis is analogous to that of sheep, but it includes bile duct calcification and gall bladder hypertrophy. Although acute and subacute illness can occur under extreme conditions, particularly in young calves, chronic disease is significantly more common in sheep in late winter or early spring (Andrews et al. 2021; Wagari 2021).

Fluke migration is more widespread in cattle, and encapsulated parasites are frequently observed in the lungs (Lalor et al. 2021). Migrating *F. hepatica* via hepatic

tissue bearing dormant *Clostridium nobyi* spores may result in contagious necrotic hepatitis in sheep and cattle. This movement is likely to have aided in the generation of rare causes of bacillary haemoglobinuria in cattle. Immature flukes are tissue consumers, although they may inadvertently consume some blood and the slight anemia that arises in the intestines. The 4-5 weeks of ingesting most likely represents excessive bleeding into the migratory tracts of immature flukes (Soulsby 1968; Lalor et al. 2021). Mechanical (by juvenile flukes digging into the liver and adult flukes irritating the epithelia wall of the bile ducts); toxic (by parasitic secretary and excretory output); and haemorrhage due to bleeding in the liver (acute type) and blood - sucking feeding behavior of the flukes (Hughes et al. 2020). *F. hepatica* infection might be undetectable. They may, however, cause hepatic colic with coughing and vomiting, widespread abdominal tightness, headache and sweating, irregular temperature, diarrhea, pipe clay, and anemia (Forbes 2017; Matsuda et al. 2020).

2.5.1 Secretory protein

During their maturation into adult worms, immature fluke release huge amounts of proteins and other chemicals in their digestive tracts, excretory pores, and cyst surface. These chemicals shield parasites from host defensive responses and are critical in the parasite-host relationship (Haçariz et al. 2012). Proteomics analysis is used to research parasite-host interactions, particularly when pathogens are difficult to obtain. Using proteomics analysis, 575 proteins discovered in the soluble juvenile fluke extract, 90 proteins in immature fluke E/S products, and 202 proteins in adult E/S products (Robinson et al. 2009; Cwiklinski et al. 2015). Previously it is predicted that 14,031 secretory protein in *F. hepatica* expressed as sequence tags (Robinson et al. 2009). Infective larvae, juvenile flukes, and adult fasciola release mostly proteases and a variety of antioxidants that are highly controlled in their movement through host tissues (Caffrey et al. 2018). A key proteins of the whole immature fluke secretome include proteases, protease inhibitors, antioxidants, and a variety of immunomodulators that presumably disarm host innate immune cells (Xu et al., 2020).

2.6 Clinical signs

The clinical manifestations of fasciolosis may differ depending on the type of the disease (acute, sub-acute and chronic) (Muro and Rojas-Caraballo 2017).

2.6.1 Acute fasciolosis

Acute fasciolosis is uncommon in cattle. It is less prevalent than chronic hepatitis, and it is caused by the concurrent translocation of a high number of immature flukes. Acute fasciolosis can result in sudden death. It causes widespread disease and death in cattle, including weight loss, anemia, and hypoproteinemia (Soulsby 1968; Kumar et al. 2021).

2.6.2 Subacute fasciolosis

It is caused by ingesting a modest number of metacercaria, it is characterized by anemia, jaundice, and lethargy (Soulsby 1968; Fiss et al. 2013)

2.6.3 Chronic fasciolosis

In cattle, this represents the most prevalent type of the illness. It happens when the worm gets into the hepatic bile duct. Emaciation, pale eyes and gums, and "bottle jaw" are all symptoms of edema beneath the jaw. Depending on the degree of the disease, untreated animals die in two to three months, while many live longer and may finally recover if not reinfection happened. Cattle with chronic fasciolosis may experience diarrhea, although the majority of their illnesses usually resolve within six months(Soulsby 1968; Javaregowda and Rani 2017; Alizadeh and Mohammadi 2019; Molina-hernández et al. 2021) .

2.7 Diagnosis

Fasciolosis is diagnosed based on clinical signs, grazing history and seasonal incidence, feces investigation using laboratory assays, and post mortem inspection (Soulsby 1968)

2.7.1 History and clinical signs

F. hepatica infection is typically connected with livestock and lambs grazing damp, marshy ground. *F. gigantica*, on the other hand, is found in animals consuming water from snail-infected watering holes, as well as in eating grass wet ground that is not periodically dated (Singh et al. 2021). Acute fasciolosis causes abrupt mortality and anemia due to immature flukes moving through the liver; yet, no fluke eggs are discharged in the stool. Subacute fasciolosis causes signs of dramatic decline, anemia, increased fluke egg number, and death 12-30 weeks after infection, whereas chronic

fasciolosis induces progressive wasting, severe anemia with ascites, bottles jaw, and extremely high fluke egg number, which may cause mortality more than 20 weeks after infection (Andrews et al. 2021; Wagari 2021).

2.7.2 Fecal Examination

Chronic fasciolosis is diagnosed by looking for eggs in feces using the sedimentation method. They must, however, be recognized from the eggs of other flukes, particularly the enormous eggs of *Paramphistomum paramphistomum*. Examination using the sedimentation method Because Fasciola eggs have a high specific gravity, sedimentation is preferable than flotation. *F.hepatica* oval operculated golden eggs occur in the feces 10 weeks after infection, whereas *F. gigantica* eggs arrive 15 weeks after infection (Figure 2). Fluke egg excretion varies greatly from day to day and within days, and the distribution of eggs in feces is uneven; a single fecal egg count assay may result in an inaccurate conclusion (Dias et al. 2014; Webb and Cabada 2018).

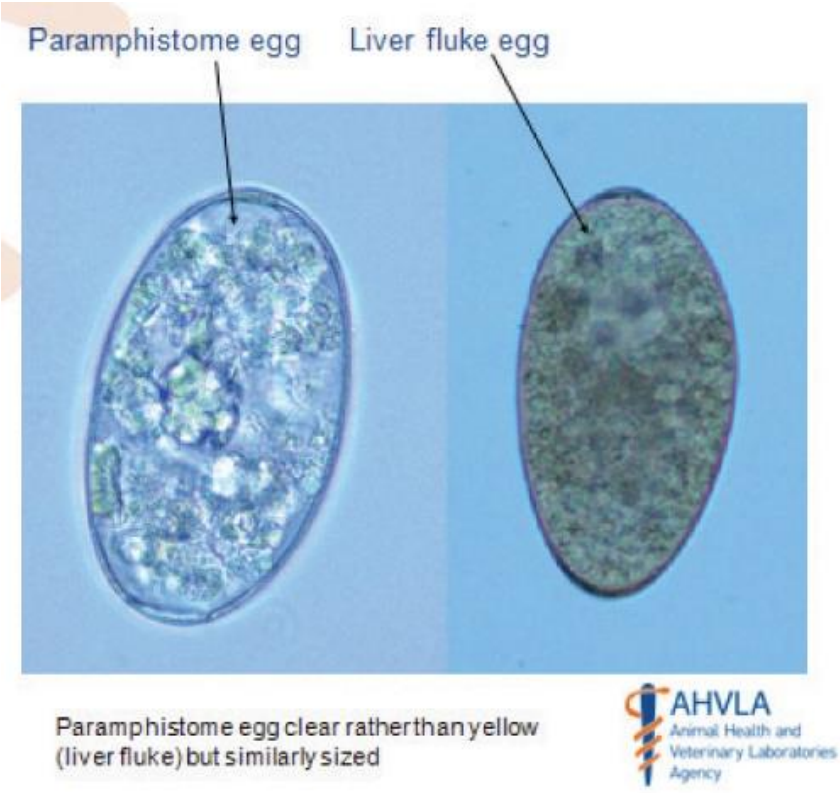

Figure 2. Eggs of Paramphistomum and liver flukes (Tilling 2013)

2.7.3 Serology

Serological diagnosis of mild and prepatent infection is achievable. Antibody detection by ELISA in serum or milk is available and particularly useful for diagnosing infection in cattle on an individual or herd basis. Antibodies begin to develop two weeks after infection and continue to grow until week six (Kishore et al., 2021; Rizwan et al., 2021; Webb & Cabada, 2018).

2.7.4 Polymerase chain reaction (PCR)

PCR is a popular technique of DNA amplification. PCR uses temperature cycling, opposing primer pairs, *Taq* polymerase, a proprietary buffer, and dNTPs to exponentially amplify a DNA sequence. DNA isolated from *F. hepatica* adult parasites and infected snails has been effectively amplified using PCR (Ai et al. 2010; Gordon et al. 2013). DNA isolated from metacercarial cysts and eggs of other helminth species has also been effectively amplified using PCR (Gordon et al. 2013; McNally et al. 2013). Real-time PCR may be used to quantify the quantity of DNA amplified (Alasaad et al. 2011). There have been just a few reports of effective amplification of DNA taken from feces of animals infected with fasciola. A conventional and a nested PCR is utilized to amplify DNA isolated from feces using primers targeted to the cytochrome C oxidase 1 gene (*cox 1*) (Martínez-Pérez et al. 2012). The normal PCR amplified DNA from three sites, whereas the nested PCR amplified DNA from two sites. It was possible to amplify DNA isolated from faeces samples from two weeks post infection using the same primer combination (Robles-Pérez et al. 2013). This assay has not yet been developed for use in real-time PCR applications.

2.7.5 Necrospy

Adult fluke discovery in the liver during necropsy represents the most accurate means of confirming fasciolosis. Prevalence studies, rather than coproscopic examination, should be conducted on slaughterhouse surveys. A severely injured and enlarged liver characterizes acute fasciolosis. There may be an increase of blood-stained serum in the peritoneum. The liver capsule has many tiny holes and sub capsular bleeding, and the parenchyma has injured tissue that is significantly more fragile than usual (Dias et al. 2014; Webb and Cabada 2018; Kumar et al. 2021).

2.8 Treatment

Not all chemicals are incredibly useful against all phases of fasciola reproduction in the body. It is critical to select a substance that is extremely efficient against juveniles that harm the liver parenchyma for the treatment of acute fasciolosis. A chemical potent towards mature fluke is essential for chronic illness (Soulsby 1968). Triclabendazole (12 mg/kg) is the most commonly used medicine due to its strong effectiveness against both adult and juvenile flukes. It is effective against adult *F. hepatica* in cattle at a dosage rate of 10mg/kg. It is ovicidal and will kill any fasciola eggs found in the bile duct or alimentary canal under therapy with ivermectin for combined fluke and roundworm control in herds (Webb and Cabada 2018; Tabari et al. 2022).

Nitroxynil is effective against the mature fluke when administered subcutaneously at 10mg/kg, but the dose must be raised by up to 50% to provide satisfactory control of acute illness (Romero et al. 2019). Until recently, therapy was not very effective due to the ineffectiveness of older medications against the early parenchymal phases. However, effective medications are now available, and one of the options is triclabendazole, which eliminates all developmental stages older than one week. Other medications that will eradicate flukes older than four weeks are Rafoxanide (7.5 mg/kg), Closantel, and Nitroxynil (Webb and Cabada 2018; Singh et al. 2021; Wagari 2021).

2.9 Control and Prevention

Using typical rainfall and temperature information of any geographical area, plan schedules for fasciolosis management may be generated. The two main management and preventive measures for fasciolosis are snail mass deportation and the use of anthelmintics (Soulsby 1968; Webb and Cabada 2018).

2.9.1 Snail Population Reduction

Before implementing any snail management strategy, a survey of the snail habitat should be conducted to identify whether the snails are isolated or widespread. The most effective long-term technique for diminishing mud snail populations, such as *L. truncatula*, is to permanently destroy snail habitat. Snail habitat can be minimized by fencing these places or treating them periodically with a molluscicide (Olkeba et al.

2022). Controlling snails with chemicals such as niclosamide and copper sulphate is conceivable, but typically not practicable owing to manpower, high costs, environmental considerations, and quick colonization of snail habitats (Lardans and Dissous 1998; Nesterkov and Grebennikov 2020).

2.9.2 Use of anthelmintic

True, seasonal strategic administration of effective trematode-specific anthelmintics, as well as early preventative and curative therapy, play a crucial role in the management of liver fluke infection (Romero et al. 2019). Prophylactic use of anthelmintics aimed at reducing pasture contamination by fluke eggs during fluke development months, April to August, and eradicating fluke populations during times of large loads or nutritional stress to the animal. Prophylactic therapy in cattle is thus aimed at lowering fluke loads throughout the winter, when the animal's nutritional state is at its lowest (Soulsby 1968; Tabari et al. 2022). Other control strategies include hygiene practices and manipulation (draining, swamping, building sewage systems, and providing safe water supply), cyclic grazing, and ignoring mixed grazing of animals of various ages (young animals are particularly susceptible to helminthes infection (Suhardono et al. 2006). Many compounds have been employed as molluscicides in the past, but currently, Niclosamide and Copper sulphate are utilized in many countries (Marques et al. 2020; Ico-Gómez et al. 2021; Kelley et al. 2021).

2.9.3 Immunity

It has been proposed that natural immunity manifests itself throughout the infection's migrating parenchymal and adult bile duct phases. This is thought to be connected to the quantity and arrangement of connective tissue in the host liver parenchyma. Cattles have higher resistance due to their liver's high connective tissue content. Perhaps the connective tissue aids in the capture of juvenile migratory flukes. Fasciola immunity has been proven, and antibodies may be identified in the blood of infected animals. Field observations revealed that elderly animals grow resistant to infection (Zhang and Song 2021). Fasciola possesses a variety of survival tactics for avoiding host immunological responses, such as altering its surface antigen during migration and producing a proteolytic enzyme capable of cleaving immunoglobins and developed host immune response (Ruiz-Campillo et al. 2017; Alba et al. 2022).

2.9.4 Vaccination

A vaccine for fasciola is being under developed. In mice, protection against Fasciola gigantica infection was tested using individual and combination cathepsin L1H and cathepsin B3 vaccinations. When compared to an adjuvant-infected control, the percentages of protection of rproFgCatL1H, rproFgCatB3, and combination vaccinations against F. gigantica ranged from 58.8 to 75.0% (Kueakhai et al. 2021). Successful vaccination techniques stimulate a Th1 immune response rather than the Th2 immune response induced by spontaneous infection. Another trial was performed in sheed using two candidates, each one by a cocktail of antigens (rCL1, rPrx, rHDM and rLAP) formulated in two different adjuvants (Montanide ISA 61 VG (G1) and Alhydrogel®(G2)). G1 showed 37.2% protection with highest IgG (Zafra et al., 2021). Another trial in cattle was performed using *F. hepatica* tetraspanin 2 extracellular loop 2 (rFhTSP2) as a protective vaccine antigen fused with *Escherichia coli* heat-labile enterotoxin's B subunit (LTB). It reduced fluke number and produce higher IgG response (Zerna et al. 2021). Still now, no commercial vaccine are available in the market. Increasing resistance to drugs and the lack of a successful vaccine against fascioliasis demands the development of novel vaccines.

Chapter III: Materials and Methods

3.1. Sequence retrieval

The amino acid sequence of F. gigantica Cathepsin L (Q9NGW4) was retrieved in FASTA format from the UniProt database (http://www.uniprot.org/). The crystal structure of TLR2 (PDB ID: 5D3I) was obtained from the protein data bank (https://www.rcsb.org/). The antigenic propensity of CatL was predicted using the antigenic peptide prediction tool (http://imed.med.ucm.es/Tools/antigenic.pl)(Kolaskar and Tongaonkar 1990).

3.2. Antigenicity and physicochemical property analysis of the proteins

The antigenicity of the protein sequences was predicted by the online server, VaxiJen v2.0 (http://www.ddg-pharmfac.net/vaxijen/scripts/VaxiJen_scripts/VaxiJen3.pl), keeping the prediction accuracy parameter threshold at 0.5(Doytchinova and Flower 2007). The accuracy prediction threshold determines a prediction's sensitivity, specificity, and accuracy, and the 0.5 threshold enhances the server's prediction accuracy. The server predicts the antigenicity of query proteins or peptides using the auto cross covariance (ACC) transformation method, and it produces findings with an accuracy range of 70% to 89%. Therefore, for in silico determination of the antigenicity of query proteins, this server is the most extensively used and acknowledged server. Then, using the ExPASy's online program ProtParam (https://web.expasy.org/cgi-bin/protparam/protparam), several physicochemical properties of the chosen antigenic protein sequences were examined. The physicochemical properties of a query protein are computed by this site without the need for supplementary data (Duvaud et al. 2021).

3.3. T-cell epitope prediction

Cytotoxic T-cell, helper T-cell, and B-cell epitopes must be present in a multi-epitope subunit vaccine in order to trigger these immune cells during an immune response (Zhang 2017). The epitopes of the selected protein sequences were predicted using the online epitope prediction server, Immune Epitope Database or IEDB (https://www.iedb.org/) and during the predictions, all the parameters in the server were kept at their default values (Vita et al. 2019). The MHC class I restricted CD8+

cytotoxic T-lymphocyte (CTL) epitopes of the selected sequences were obtained using the recommended NetMHCpan EL 4.0 prediction method (http://tools.iedb.org/mhci/) for some common HLA alleles, i.e. HLA A01:01, HLA A02:01, HLA A02:06, HLA A03:01, HLA A11-01, and HLA A29:02 and the length of the epitopes were kept at 9 (9-mer epitopes). The MHC class-II restricted CD4+ helper T lymphocyte (HTL) epitopes (15-mer epitopes) were also obtained for some common HLA alleles i.e. DRB103:01, DRB104:01, DRB115:01, DRB501:01, DRB401:01, and DRB301:01, using the IEDB recommended 2.22 prediction method (http://tools.iedb.org/mhcii/). For extensive analysis, the top ten MHC class I and class II epitopes that were confirmed to be shared by all of the aforementioned HLA alleles were taken.

3.4. Prediction of B-cell epitopes

A humoral immune response is provided by B-cell epitopes to fight infectious pathogens. The ABCPred server is used to obtain the B-cell epitopes (http://crdd.osdd.net/raghava/abcpred/), and this is a vital step for designing an effective vaccine. Based on an artificial neural network, this website predicts the B-cell epitope region in a protein or antigen. The 16 amino acid long B-cell epitopes with a score between 0 and 1 were chosen from the ABCPred server. Epitopes from various organisms were also predicted with a prediction accuracy of roughly 65.9%. Selectable binders or epitopes have scores close to 1. (Saha and Raghava 2006).

3.5. Antigenicity, allergenicity, toxicity prediction

The antigenicity of the selected epitopes was predicted using the VaxiJen v2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) server again, keeping the prediction accuracy threshold at 0.5. After that, the allergenicity of the selected epitopes was determined using two online tools, AllerTOP v2.0 (http://www.ddg-pharmfac.net/AllerTOP/feedback.py) and AllergenFP v1.0 (http://ddg-pharmfac.net/AllergenFP/). However, the results predicted by AllerTOP v2.0 (Dimitrov et al. 2013) were given priority since the server has better accuracy of 88.7% than AllergenFP (Dimitrov et al. 2014) server (87.9%). The toxicity prediction of the selected epitopes was carried out using ToxinPred server (http://crdd.osdd.net/raghava/toxinpred/), keeping all the parameters default (Gupta et al. 2013). For the server's toxicity prediction, the support vector machine (SVM)

method was used with all the parameters left at their default values. Since it effectively distinguishes between toxic and non-toxic epitopes, the SVM is an extensively used machine learning technique for toxicity prediction.

3.6. Multi-epitope subunit vaccine candidate design

An appropriate adjuvant, B-cell epitopes, CTL and HTL epitopes, and linkers were used to create the multi-epitope subunit vaccine candidate. Linkers provide amino acid residues the most flexibility possible in order to fold into favorable conformations. An adjuvant is an immunogenic element that enhances the vaccine's immunogenicity. To assist the vaccine protein bind with the TLR-2 receptor in the experimental system and to increase the immunogenicity of the construct, a TLR-2 agonist, Lipoprotein LprA (P9WK55), of 244 amino acids, was introduced as an adjuvant at the N terminal of the construct. The B-cell and CTL epitopes generated the highest score binders, while the HTL epitopes generated the lowest ranked binders. The linkers employed were AAY, GPGPG, and KK for the CTL, HTL, and B-cell epitopes, respectively (Solanki and Tiwari 2018).

3.7 Predictions of antigenicity and allergenicity of the designed vaccine construct

The protective antigens were predicted by the VaxiJenV2.0 server, which conducts an autonomous alignment, using a threshold value of 0.5 (http://www.ddg pharmfac.net/vaxijen/VaxiJen/VaxiJen.html). This server's accuracy varies from organism to organism and spans from 70% to 89%.

The server AllerTop (http://www.ddg-pharmfac.net/AllerTOP/) was used to forecast how allergic the vaccine construct will be. The chemical composition of amino acids is used as a basis for allergen recognition in this alignment-independent method, which transforms protein sequences into equal-length vectors using auto cross-covariance (ACC). By using the k-nearest neighbor method (kNN, k = 1) and a training set that includes 2427 recognized allergens from various species and 2427 non-allergens, the proteins are classified (Dimitrov et al. 2013).

3.8. Physicochemical characterization of vaccine protein

For characterization purposes, the ProtParam (https://web.expasy.org/protparam/) server was used to predict the physiochemical properties of the vaccine protein(Duvaud et al. 2021). Multiple amino acids, molecular weight, theoretical pI,

an expected half-life in vitro and in vivo, an instability index, an aliphatic index, and the grand average of hydropathicity are among the predicted attributes. Apart from the primary amino acid sequence, ProtPram doesn't need any other data to make a prediction.

3.9. Secondary and tertiary structure prediction of the vaccine constructs

Using the online tool PRISPRED (http://bioinf.cs.ucl.ac.uk/psipred/), the secondary structures of the vaccine construct was constructed while keeping all the settings at their default values (Buchan and Jones 2019). In addition to secondary structure prediction, PRISPRED is a straightforward secondary structure generator that can also predict the transmembrane topology, transmembrane helix, fold, and domain recognition, among other things. The I-TASSER (Yang and Zhang 2015) server was used to generate the tertiary structures of the vaccine. Based on its confidence score, the best model was picked (C-score, typical range: -5, 2). A C-score of -1.5 or higher indicates that the model has an appropriate fold.

3.10. 3D structure refinement and validation

Computational approaches may generate protein 3D structures that are not accurate to their native structures. Therefore, the low-resolution projected models were upgraded to higher resolution models that closely approach the native protein structures using 3D structure refinement. The GalaxyRefine module of the GalaxyWEB server (http://galaxy.seoklab.org/) used dynamics simulation and the CASP10-tested refinement method to improve the 3D structures of the vaccine that was created (Ko et al. 2012). Following structure refinement, the PROCHECK (https://servicesn.mbi.ucla.edu/PROCHECK/) server-generated Ramachandran plots was analyzed to verify the vaccine construct (Laskowski et al. 2012). ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php), another online application, was then used for protein validation (Wiederstein and Sippl 2007). The server uses a variety of statistical techniques to generate the z-score, which is used to rate the quality of a protein structure in a query. A query protein is of higher quality if its z-score is within the range of the z-scores of all the experimentally determined protein chains in the PDB database.

3.11. Vaccine protein disulfide engineering

The online tool Disulfide by Design 2 v12.2 (http://cptweb.cpt.wayne.edu/DbD2/) was used to engineer the vaccine protein disulfide. The server makes predictions about the sites in a protein structure where disulfide bond formation is most likely to occur. The intra-chain, inter-chain, and C_β for glycine residue disulfide bonds were chosen when engineering structures. The amino acid pairings with less than 2.2 kcal/mol bond energy were selected for mutation into cysteine residues in order to establish the disulfide bonds among themselves. The X_3 vaccine angle was retained at 87 or 97° and the C_α-C_β-S_γ angle at 114.6°. In this study, the bond energy of 2.2 kcal/mol was utilized as a threshold because it is typically found that 90% of native disulfide bonds have an energy value less than 2.2 kcal/mol (Craig and Dombkowski 2013).

3.12 Conformational B-cell epitope prediction

The vaccination's discontinuous epitopes were predicted using the Ellipro (http://tools.iedb.org/ellipro/) server and were shown to be present in the vaccine (Ponomarenko et al. 2008). The protein's 3D structure's continuous and discontinuous epitopes were shown using the fully functional molecular spectator Jmol (Rose et al. 2017). The Bcells, which create antibodies when they come in contact with an antigen, are crucial to the body's humoral immunity. In order to induce significantly better immunity, the vaccines should therefore contain efficient conformational B-cell epitopes.

3.13 Protein-protein docking and molecular dynamic simulation

To comprehend the binding interactions between the receptor and the ligand, molecular docking was performed. TLR-2 (PDB ID: 5D3I) was used as the receptor molecule for the molecular docking study, and the refined tertiary model of the vaccine construct served as the ligand molecule. The PatchDock server (Schneidman-Duhovny et al. 2005) was used to predict the optimum placements and orientations in the study, and further refinement was carried out using the FireDock website (https://bioinfo3d.cs.tau.ac.il/FireDock/php.php) (Mashiach et al. 2008). The PDB files of both the receptor and the ligand were supplied in the server with default setting. The docked complexes are ranked by the FireDock server according to their global energy scores; the lower the energy score, the better the outcome. The best

vaccine construct that TLR-2 successfully docked with was taken for visualization and the molecular dynamics (MD) simulation study. One vaccine was chosen from the docking experiment for further examination and was observed visually via the Discovery Studio Visualizer. Then a molecular dynamics simulation study was carried out using iMODS (López-Blanco et al. 2014). To comprehend the dynamic mobility, binding mode, and structural stability of the vaccine construct at the atomic level as previously stated, molecular dynamics (MD) simulation was performed.

3.14 Adaptation of codon and in silico cloning

Codon optimization studies were performed by the Java Codon adaptation tool (http://www.jcat.de/) (Grote et al. 2005a) in order to clone and express the developed subunit vaccine in a bacterial expression system. E. coli K12 strain was chosen as the expression host, and the designed vaccine sequence was used as the input. During the run, the options to avoid rho independent transcription terminators, bacterial ribosome binding sites, and restriction enzyme cleavage sites were chosen (Ermolaeva et al. 2000). An optimized gene sequence is provided by this tool, which predicts the input DNA sequence. Additionally, the subunit vaccine construct's % GC content and codon adaptive index (CAI) were assessed. The GC content should be between 30% and 70%, and the ideal CAI value for a gene's expression should be between 0.8 and 1.0 (Pandey et al. 2020b). Then, the N- and C-terminal regions of the cDNA sequence were added with restriction sites by running the optimized codon sequence through NEBcutter. HindIII and BamHI restriction sites were introduced at the N- and C-terminal locations of the optimized vaccine sequence, which was then inverted. Utilizing the SnapGene restriction cloning tool, in silico cloning was carried out, and the optimized codon sequence for the protein's bacterial expression was inserted into the pET28a (+) plasmid vector.

Chapter IV: Results and Discussions

4.1 sequence retrieval

The amino acid sequence of F. gigantica Cathepsin L (Q9NGW4) were selected by reviewing literatures from the NCBI database and then the protein sequence was retrieved from the UniProt database. Table 1 lists the UniProt accession ID of the selected protein sequences**.**

Table 1. The amino acid sequence of Cathepsin L (Q9NGW4)

Amino acid sequence
MRCFVLAVLTVGVLGSNDDLWHQWKRMYNKEYNGADDEHRRNIWEENVK HIQEHNLRHDLGLVTYTLGLNQFTDMTFEEFKAKYLTEMPRASDILSHGIPYE ANNRAVPDKIDWRESGYVTEVKDQGNCGSCWAFSTTGTMEGQYMKNERTSI SFSEQQLVDCSGPWGNYGCMGGLMENAYEYLKQFGLETESSYPYTAVEGQC RYNRQLGVAKVTDYYTVHSGSEVELKNLVGAEGPAAVAVDVESDFTMYSG GIYQSRTCSSLRVNHAVLAVGYGTQGGTDYWIVKNSWGSSWGERGYIRMVR NRGNMCGIASLASLPMVARFP

4.2. Antigenicity and physicochemical property analysis of the proteins

The protein Cathepsin L was found to be potentially antigenic (probable Antigen 0.5928) exceeded the threshold (0.40), indicating that they may induce a response in the host.

The pH level at which a protein has no net charge is known as the theoretical pI. Therefore, to neutralize the proteins, a pH of less than 7.0 would be needed, which is relatively doable. The percentage volume of the amino acids in a protein's side chains occupied by the aliphatic amino acids, such as alanine, valine, etc., is known as the protein's aliphatic index. Yet again, the GRAVY value establishes a compound's hydrophilic and hydrophobic properties. The hydrophilic property of a compound is represented by a negative GRAVY value, whereas the hydrophobic property is represented by a positive GRAVY value. The protein was discovered to be hydrophilic due to its low GRAVY value (Chang and Yang 2013a). The protein would therefore be readily soluble in water. The protein's 30 h half life in mammalian

cell culture was also discovered, which is pretty excellent. It also had a high aliphatic index (over 60.00). The aliphatic index of a protein measures its thermal stability, and a protein's higher aliphatic index indicates a more thermostable state (Ikai 1980). So, the protein was determined to be thermostable due to its relatively high aliphatic index. All of these physicochemical analysis results were deemed to be reliable and satisfactory. The outcomes of the analysis of the physicochemical properties are provided in (Table 2).

Table 2. The physicochemical property of the selected protein sequence (AI = aliphatic index, GRAVY= Grand average of hydropathicity)

No. of Amino Acids	Molecular wt	Instability Index	Aliphatic Index	Gravy value	Half-life (In mammalian reticulocytes, in vitro)	Theoretical pI	Extinction co-efficient
326	36779.13	28.92	66.35	-0.476	30 hour	5.44	79800

4.3. T-cell and B-cell epitope prediction and their antigenicity, allergenicity, toxicity prediction

The T-cell and B-cell epitopes for the development of vaccines were anticipated after analyzing the physicochemical properties. While the helper T-cells aid in activating the B-cell, macrophages, and even the cytotoxic T-cells, the cytotoxic T-cells are responsible for recognizing antigens (Zhu and Paul 2008). Additionally, through producing antibodies, B-cells mediate the humoral immune response. The humoral immune response, however, is less robust than the cell-mediated immune response and could deteriorate over time (Bacchetta et al. 2005). On the other hand, the cell-mediated immune response, which secretes antiviral cytokines and recognizes explicitly and eliminates the infected cells, can offer far broader and lifelong immunity. The IEDB server-generated thousands of MHC class-I and MHC class-II

protein T-cell epitopes based on a conserved sequence that could bind to the most HLA cells with the highest affinity. The top T-cell epitopes from the server's hundreds of created epitopes were chosen for additional analyses. And after being predicted by the ABCPred linear prediction algorithm of the IEDB server, the B-cell epitopes with lengths of more than 10 amino acids were chosen. Finally, the highly antigenic, non-allergenic, and non-toxic epitopes were chosen for vaccine development. Table 3 contains a list of all probable T cell and B cell epitopes.

Table 3. Predicted T-cell (CTL and HTL) and B cell epitopes of Cathepsin L

	Peptide	Start	End	Score	Antigenicity	allergenicity	toxicity
MHC Class I epitopes	GTMEGQYMK	139	147	0.976467	**Antigen** 1.4294	Non-allergen	Non-toxic
	GLMENAYEY	175	183	0.904406	Antigen 0.7484	Allergen	Non-toxic
	TFEEFKAKY	76	84	0.8234	Non-Antigen	Allergen	Non-toxic
	GTMEGQYMK	139	147	0.811645	Antigen 1.4294	Non-allergen	Non-toxic
	FTDMTFEEF	72	80	0.803909	Antigen 1.4442	Non-allergen	Non-toxic
	MTFEEFKAK	75	83	0.798319	Antigen 0.9093	Allergen	Non-toxic
	GVAKVTDYY	211	219	0.788936	Non-Antigen	Allergen	Non-toxic
	DVESDFTMY	244	252	0.759099	Antigen 0.5433	Allergen	Non-toxic
	FTMYSGGIY	249	257	0.68462	Non-Antigen	Non-Allergen	Non-toxic
	TTGTMEGQY	137	145	0.680998	Antigen 1.4674	Non-Allergen	Non-toxic
	RVNHAVLAV	26	27	0.5513	Antigen	Non-	Non-

		6	4	64		Allergen	toxic
MHC Class II epitopes	GQCRYNRQLGVAKVT	202	216	2.20	Non-Antigen	Non-Allergen	Non-toxic
	QCRYNRQLGVAKVTD	203	217	2.30	Non-Antigen	Allergen	Non-toxic
	CRYNRQLGVAKVTDY	204	218	2.40	Non-Antigen	Non-Allergen	Non-toxic
	EGQCRYNRQLGVAKV	201	215	2.40	Non-Antigen	Non-Allergen	Non-toxic
	AAVAVDVESDFTMYS	239	253	2.80	Antigen 1.0992	Allergen	Non-toxic
	AVAVDVESDFTMYSG	240	254	2.80	Antigen 0.7329	Allergen	Non-toxic
	SYPYTAVEGQCRYNR	194	208	3.60	Antigen 0.5419	Allergen	Non-toxic
	YPYTAVEGQCRYNRQ	195	209	3.60	Antigen 0.6292	Allergen	Non-toxic
	SSYPYTAVEGQCRYN	193	207	3.80	Antigen 0.6024	Allergen	Non-toxic
	VAVDVESDFTMYSGG	241	255	3.80	Antigen 0.6212	Allergen	Non-toxic
	EHNLRHDLGLVTYTL	53	67	4.50	Antigen 0.7067	Non-Allergen	Non-Toxic
	VEGQCRYNRQLGVAK	200	214	4.90	Antigen 0.6117	Non-Allergen	Non-toxic
				Percentile rank			
B-cell epitopes	GSSWGERGYIRMVRNR	292	N/A	0.91	Antigen 1.0862	Non-Allergen	Non-toxic
	KHIQEHNLRHDLGLVT	49		0.90	Antigen	Allergen	Non-toxic
	YGTQGGTDYWIVK	27		0.90	Non-	Allergen	Non-

	NSW	6			Antigen		toxic
	HQWKRMYNKEYN GADD	22		0.90	Antigen	Allergen	Non-toxic
	GQCRYNRQLGVAK VTD	20 2		0.90	Non-Antigen	Non-Allergen	Non-Toxic
	AFSTTGTMEGQYM KNE	13 4		0.90	Antigen	Allergen	Non-toxic
	DWRESGYVTEVKD QGN	11 3		0.90	Antigen	Allergen	Non-toxic
	QQLVDCSGPWGNY GCM	15 8		0.85	Antigen	Non-Allergen	Non-toxic

4.4. Multi-epitope subunit vaccine candidate design

As previously reported, the adjuvant, HTL epitopes, B-cell epitopes, and CTL were combined to create the multi-epitope vaccine candidate (Narula et al. 2018). The 244 amino acid residue protein known as lipoprotein LprA, a TLR2 agonist, was obtained from UniProt. It was incorporated into the vaccine construct as an adjuvant to boost the antigenicity of the shot. To provide protein flexibility, specific linkers were used to connect all of the HTL, B cell, and CTL epitopes that were chosen. CTL epitopes were linked using an AAY linker, whereas B-cell epitopes were linked using a KK linker. Additionally, HTL epitopes were linked together using the GPGPG linker, and the adjuvant was attached to the epitopes using the EAAAK linker (Lee and Nguyen 2015). The final subunit vaccine consists of 386 amino acid residues that contained 1 EAAAK linker, 3 KK linkers, 5 AAY linkers, and 2 GPGPG linkers. The newly constructed vaccine was designated as: FV

Constructed Fasciola Vaccine (FV)

EAAKMKHPPCSVVAAATAILAVVLAIGGCSTEGDAGKASDTAATASNGDAA
MLLKQATDAMRKVTGMHVRLAVTGDVPNLRVTKLEGDISNTPQTVATGSAT
LLVGNKSEDAKFVYVDGHLYSDLGQPGTYTDFGNGASIYNVSVLLDPNKGL
ANLLANLKDASVAGSQQADGVATTKITGNSSADDIATLAGSRLTSEDVKTVP
TTVWIASDGSSHLVQIQIAPTKDTSVTLTMSDWGKQVTATKPV**AAY**GTMEG
QYMK**AAY**GTMEGQYMK**AAY**FTDMTFEEF**AAY**TTGTMEGQY**AAY**RVNHAV
LAV**GPGPG**EHNLRHDLGLVTYTL**GPGPG**VEGQCRYNRQLGVAK**KK**GSSW
GERGYIRMVRNR**KK**QQLVDCSGPWGNYGCM**KK**

4.5. Predictions of antigenicity, allergenicity and physiochemical property analyses of the designed vaccine construct

The antigenicity and allergenicity analyses revealed that FV was both antigenic (Antigenicity 0.7311) and non-allergenic. As a result, it could elicit strong immune responses without triggering an unintended allergic reaction in the body.

The designed subunit vaccine must be stable and immunogenic in nature; therefore, the physicochemical properties of subunit vaccine were determined. It was found that the vaccine construct was composed of 386 amino acid residues with a molecular weight of 40.54 kDa, and a theoretical pI of 7.80, suggesting the basic nature of the construct. The construct had 35 negatively charged and 36 positively charged amino acid residues. The instability index of the vaccine protein was 18.78, suggesting the stable nature of the protein. the instability indexes (less than 40) of the vaccine proteins indicated that they might be quite stable in the biological environment because a compound with instability index less than 40 is considered to be stable. The aliphatic index represents the volume occupied by the aliphatic side chains, and its obtained value of 74.61 defined the thermostable nature of the construct (Chaudhri et al. 2009). The GRAVY represents the amphipathic nature of the proteins, where negative and positive value denoted the hydrophilic and hydrophobic nature of the amino acids side chain, respectively (Chang and Yang 2013b). The subunit vaccine had a GRAVY value of -0.220, showing the hydrophilic nature of the vaccine. The hydrophilic characteristic of the protein should aid in easy purification and formulation of the vaccine. The half-life of the construct was estimated to be 1h in

mammalian reticulocytes (in vitro), 30 min in yeast (in vivo), and >10 h in E. coli (in vivo), suggesting that the construct remains stable in vivo.

4.6. Secondary and tertiary structure prediction of the vaccine constructs

The secondary structure of the vaccine protein was obtained from the PSIPRED server and confirms the presence of 24.87% helix, 46.11% coil and 29% of strands. The I-TASSER server generated the final shapes of the vaccines based on the C-score. The MODEL 1 was selected for further investigation because it was the most effective model with the greatest C-score value available (–1.11).

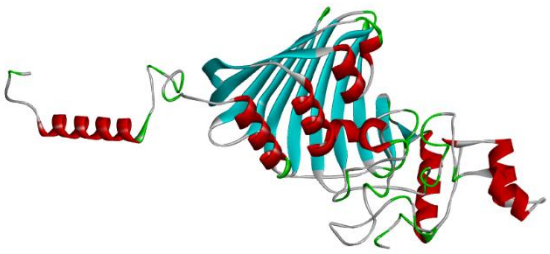

Fig 3. Predicted tertiary structure of the constructed vaccine

4.7. 3D structure refinement and validation

The protein structure generated by the I-TASSER server was refined using Galaxy-web server, which were then analyzed by Ramachandran plots generated by the PROCHECK server and the z-scores generated by the ProSA-web server. The Ramachandran plot analysis showed that vaccine had 87.2% of the amino acids in the most favored region, 11.6% of the amino acids in the additional allowed regions, 0.0% of the amino acids in the generously allowed regions, and 1.2% of the amino acids in the disallowed regions. Moreover, vaccine had the z-scores of -5.34, which represented that FV scores well within the range of experimentally proven X-ray crystal structures of proteins from the Protein Data Bank. In the tertiary structure refinement and validation study, the FV was found to possess quite good quality protein structures.

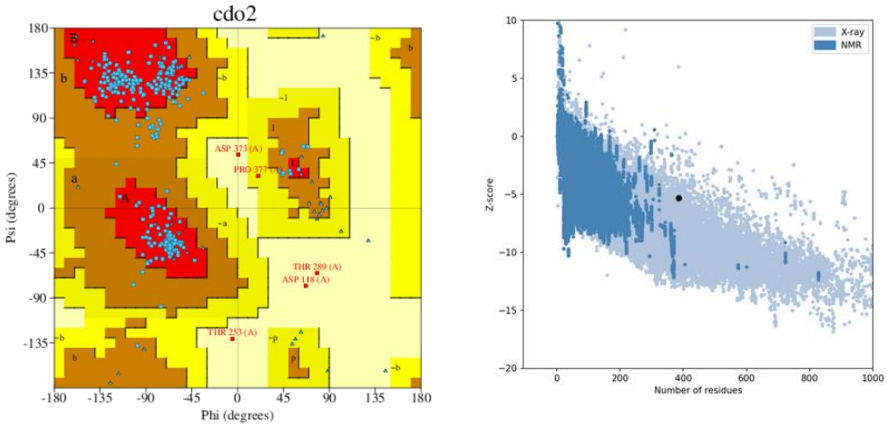

Fig. 4. Ramachandran plot(left) showing the presence of amino acid residues in favored, allowed and disallowed region and quality score or z-score graphs (right) generated by the ProSA-web server.

4.8. Vaccine protein disulfide engineering

In protein disulfide engineering, the DbD2 server identifies the pairs of amino acids that have the capability to form disulfide bonds based on the given selection criteria. In this experiment, we selected only those amino acid pairs that had bond energy value was less than 2.2 kcal/mol. The vaccine generated 7 pairs of amino acids with bond energy less than 2.2 kcal/mol: 1 Glu and 4 Lys, 56 Gln and 216 Ser, 85 Leu and 101 Ala, 163 Ala and 181 Gly, 247 Pro and 250 Ala, 318 Arg and 352 Ser, 359 Tyr and 371 Leu. The selected amino acid pairs formed the mutant version of the original vaccine (with disulfide bonds) in the DbD2 server (Figure 5). Since the FV was predicted to have 7 possible pairs of amino acid residues with the capability to form potential disulfide bonds, therefore, it can be declared that it might be most stable vaccine construct.

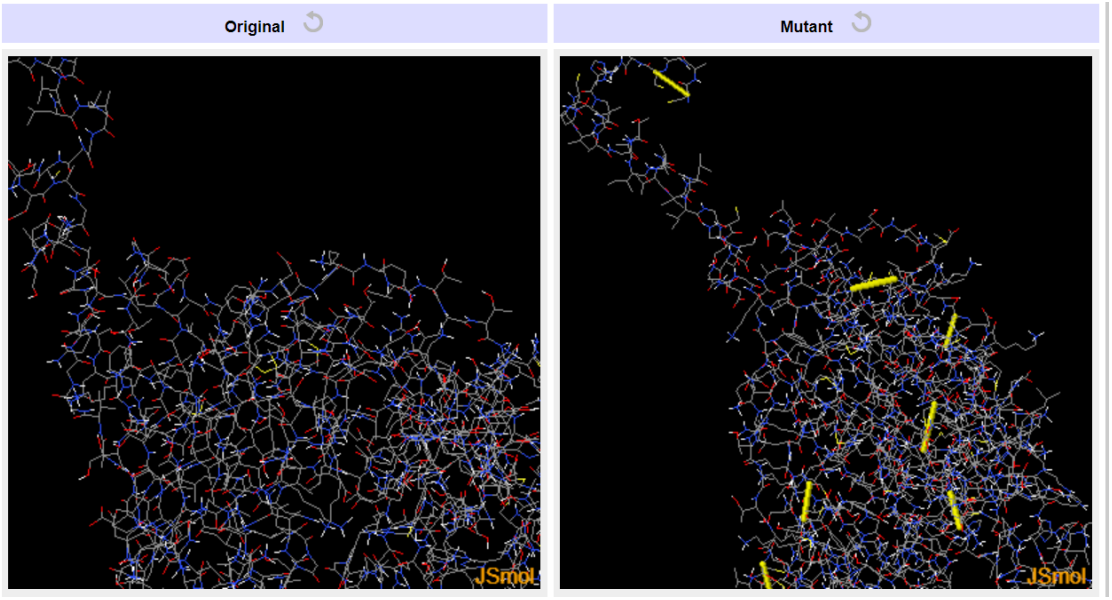

Fig.5. The disulfide engineering of the FV vaccine construct; (original form and mutant form)

Table 4. Protein Disulfide engineering Scores by DbD2 server in terms of Energy value

	Residue 1				Residue 2				Bond		
	Chain	Seq #	AA	Struct	Chain	Seq #	AA	Struct	χ_3	kcal/mol	Σ B-factor
☑	A	1	GLU		A	4	LYS		+87.49	1.26	0.00
☑	A	56	GLN		A	216	SER		-85.75	1.38	0.00
☑	A	85	LEU		A	101	ALA		+121.97	2.15	0.00
☑	A	163	ALA		A	181	GLY		-88.95	1.65	0.00
☑	A	247	PRO		A	250	ALA		+96.62	1.90	0.00
☑	A	318	ARG		A	352	SER		-80.27	2.18	0.00
☑	A	359	TYR		A	371	LEU		-89.84	1.99	0.00

4.9. Conformational B-cell epitope prediction

The Ellipro server predicted conformational B-cell epitopes from the revised 3D structure of the vaccine. Three sets of discontinuous epitopes were predicted, with values in the range from 0.624 to 0.753. A schematic depiction of the discontinuous epitopes is shown in Figure6 and Table 5.

Table 5. Conformational B-cell epitopes from FV vaccine protein using Ellipro server

No.	Residues	No. of residues	Scores
1	A:E1, A:A2, A:A3, A:K4, A:M5, A:K6, A:H7, A:P8, A:P9, A:C10, A:S11, A:V12, A:V13, A:A14, A:A15, A:A16, A:T17, A:A18, A:I19, A:L20, A:A21, A:V22, A:V23, A:L24, A:A25, A:I26, A:G27, A:G28, A:C29, A:S30, A:T31, A:E32, A:G33, A:D34, A:A35, A:G36, A:K37, A:A38, A:S39, A:D40, A:T41, A:A42, A:A43, A:T44, A:A45, A:S46, A:N47, A:G48, A:D49, A:A50, A:A51, A:M52, A:T74, A:G75, A:D76, A:V77, A:P78, A:N79, A:L80, A:R81, A:V82, A:T102, A:L103, A:L104, A:V105, A:G106, A:N107, A:K108, A:S109, A:E110, A:D111, A:L160, A:K161, A:D162, A:A163, A:S164, A:V165, A:A166, A:T180, A:G181, A:N182, A:S183, A:S184, A:A185, A:D186, A:S194, A:R195, A:L196, A:T197, A:S198, A:E199, A:D200, A:V201, A:K202, A:T203, A:V204, A:P205, A:S212, A:A224, A:P225, A:T226, A:K227, A:D228, A:T229	104	0.753
2	A:D124, A:L125, A:G126, A:Q127, A:P128, A:G129, A:T130, A:Y131, A:T132, A:A244, A:T245, A:K246, A:P247, A:V248, A:A249, A:A250, A:Y251, A:G252, A:T253, A:M254, A:E255, A:G256, A:Q257, A:M259, A:K260, A:A261, A:A262, A:Y263, A:G264, A:T265, A:M266, A:E267, A:G268, A:Q269, A:M271, A:K272, A:A273, A:A274, A:Y275, A:F276, A:G322	41	0.693
3	A:M279, A:T280, A:F281, A:E282, A:E283, A:F284, A:A285, A:A286, A:Y287, A:T288, A:T289, A:G290, A:T291, A:M292, A:E293, A:G294, A:Q295, A:Y296, A:A297, A:A298, A:Y299, A:R300, A:V301, A:H303, A:A304, A:V305, A:L306, A:A307, A:V308, A:G309, A:P310, A:G311, A:E314, A:H315, A:L323, A:G329, A:P330, A:G331, A:P332, A:G333, A:W354, A:G355, A:E356, A:R357, A:G358, A:Y359, A:I360, A:R361, A:M362, A:V363, A:R364, A:N365, A:K368, A:Q369, A:Q370, A:L371, A:D373, A:C374, A:S375, A:G376, A:P377, A:W378, A:G379, A:N380, A:Y381, A:G382, A:C383, A:M384, A:K385, A:K386	70	0.624

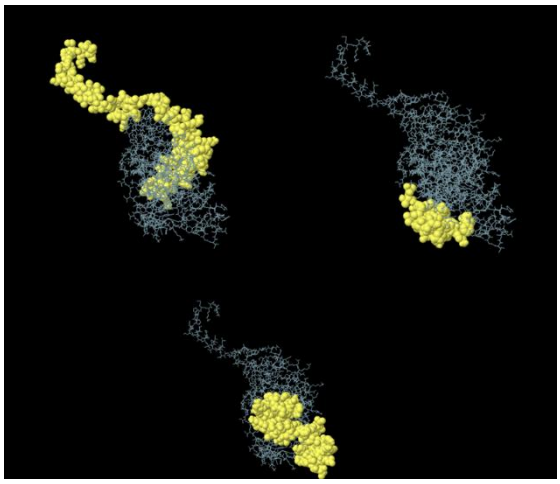

Fig.6. The graphical representation of the discontinuous epitopes

4.10. Protein protein docking

The final vaccination construct's interaction with the TLR2 agonist receptor was investigated using molecular docking (PDB ID: 5D3I). First, docking with TLR2 and then Firedock for a more complex interaction of protein receptor molecules were applied to study the binding affinities of the FV vaccine. The server then balanced the docked molecules based on global and atomic contact energy (ACE). Then, the best solution requiring the least amount of global energy was chosen. The model that required the least amount of binding energy to interact with TLR2 was chosen for MD simulation investigations.

Table 6. Protein-protein docking score of FV by Firedock

Vaccine	Receptor	Global Energy	Attractive VdW	Repulsive VdW	ACE	HB
FV	TLR2(5D3I)	-30.19	-45.80	21.66	9.37	-6.09

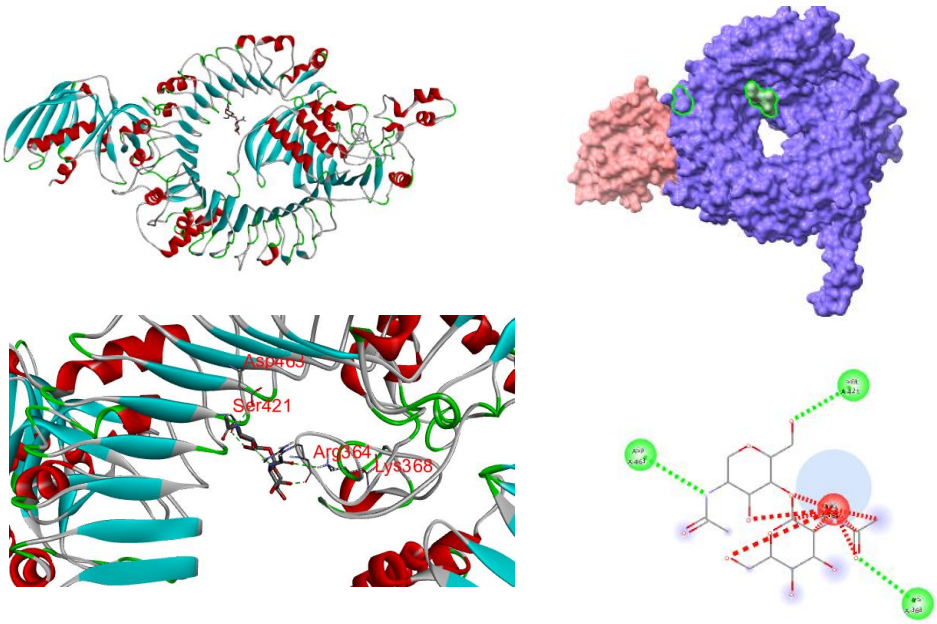

Fig. 7. Stable interaction between the vaccine construct and TLR2 after docking

4.11 Molecular dynamics simulation

iMODs were used on the FV and TLR2 receptors to carry out the simulation study of the docked complexes (FV-TLR2). The areas of the proteins that are vulnerable to deformation are represented by peaks on the deformability graphs. The eigenvalue of the docked FV and TLR2 complex was 1.968923e-05. On the variance graphs, individual variance is represented by red bars, while cumulative variance is represented by green bars. The complexes' covariance map reveals correlated motion between two residues. The color white denotes an uncorrelated motion between two residues, while the color blue denotes an anti-correlated motion. The elastic map of the complex FV-TLR2 depicts the atomic connections, with darker gray areas denoting looser connections and deeper grey areas denoting stiffer regions. Figure 8 shows the result of the molecular dynamic analysis of FV-TLR2.

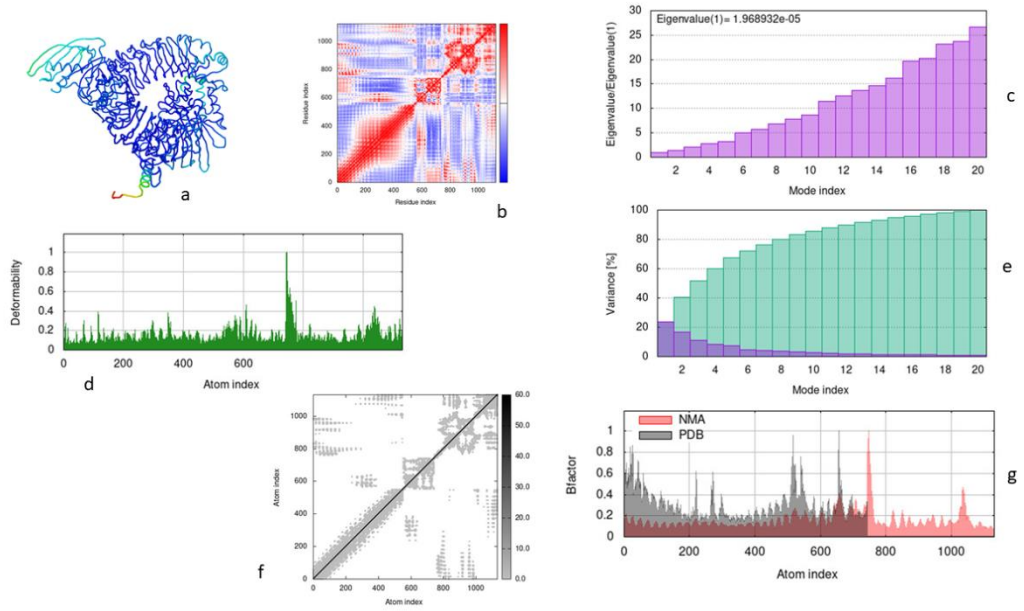

Fig. 8. The molecular dynamics simulation study of Fv-TLR2 docked complex. Here (a)NMAmobility, (b) co-variance map, (c) Eigenvalue, (d) deformability, (e) variance, (f) elastic network and (g) Bfactor

4.12 Codon adaptation and *In silico* cloning

Finally, codon adaptation and *in silico* cloning were carried out to generate a recombinant plasmid that could be utilized to produce the FV vaccine in mass in the *E. coli* strain K12. Recombinant protein production is recommended to use the *E. coli* cell culture system (Grote et al. 2005b) . For expression of the vaccine construct in bacterial cells, the pET28a(+) plasmid vector was used. Upon codon optimization, the CAI value and GC content were 0.92 (range: 0.8–1.0) and 68.48% (range: 30–70%) (Khatoon et al. 2017), respectively. Due to the absence of restriction sites in the sequence, HindIII and BamHI restriction sites were added to the reversed codon optimized vaccine sequence at N-terminal and C-terminal regions, respectively. The constructed vaccine gene was then cloned into the pET28a(+) vector by using the SnapGene tool for effective expression in bacterial (E. coli) cells. To clone the designed vaccine construct into an expression vector by in silico approach, the subunit vaccine protein was first codon optimized. The desired gene constitutes a length of 1163 base pairs, and the restriction clone has a length of 6507 base pairs.

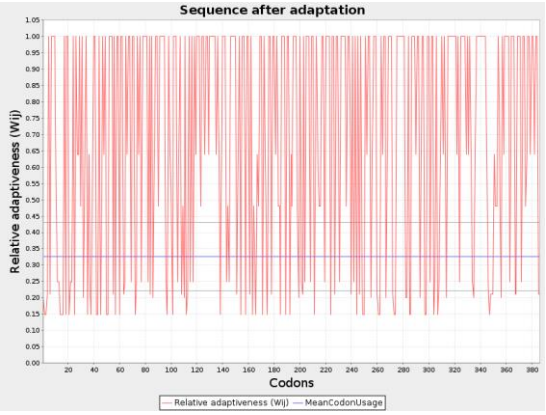

Fig. 9. Figure showing the codon adaptation graph of the FV vaccine.

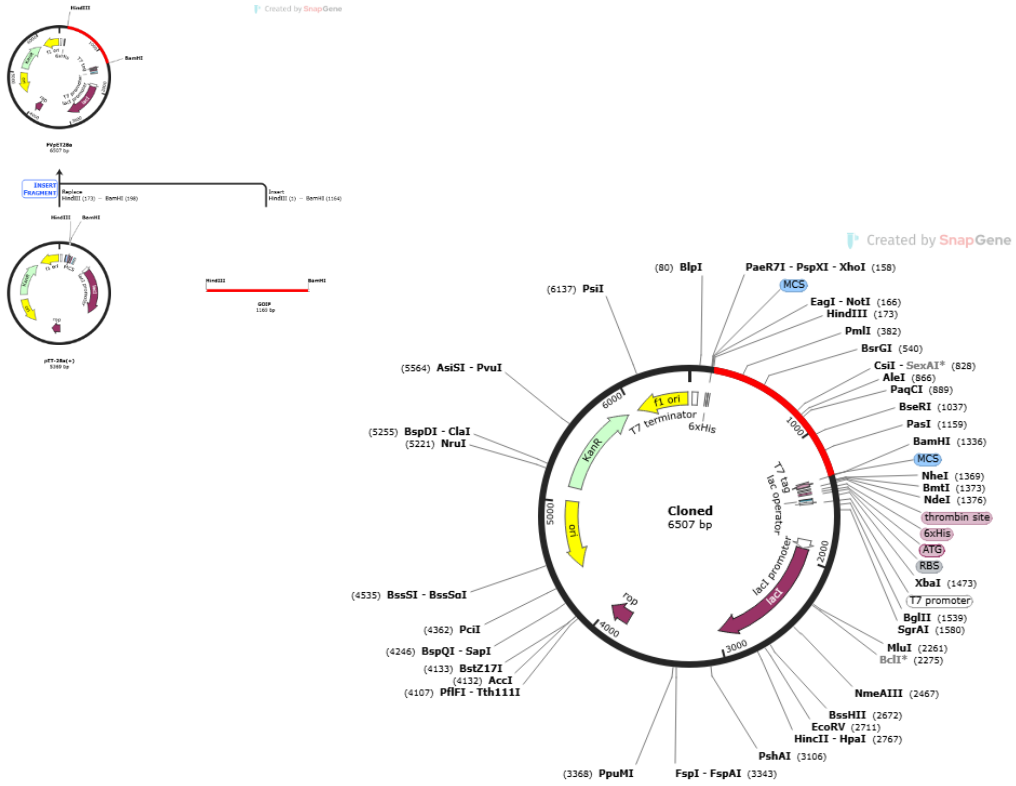

Fig. 10. *In silico* restriction cloning of the FV vaccine sequence in the pET-28a (+) plasmid between the HindIII and BamHI restriction enzyme sites. The red-colored indicated regions include DNA insertion of the vaccine.

Chapter V: Conclusions, Recommendations and future direction

5.1 Conclusions

The development of recombinant subunit vaccines against these parasites has not succeeded despite years of effort. Small peptides or proteins that are obtained from pathogens make up the subunit vaccines. Due to the low risk of side effects and their ability to provide long-lasting immunity, it can be administered to patients who have weakened immune systems. Identification of antigens with the capacity to trigger a potent immune response is necessary for the development of a subunit vaccine. To eliminate the antigens, the immune system's cellular components cooperate. CTLs and B-cells are activated once T-helper cell activation has caused the release of cytokines. The vaccine construct also includes an adjuvant, an immuno-stimulant, to promote the targeted immune response. In this study, a multi-epitope subunit vaccine against *F. gigantica* is developed. Linkers connect suitable adjuvant, B-cell, CTL, and HTL epitopes. An understanding of the binding energy, dynamics, and interaction patterns of the vaccine construct came from computational research. This work assessed the immunogenicity, allergenicity, and antigenicity of the vaccine generated from cathepsin L. In silico cloning ensured that the vaccine construct could potentially be expressed in the microbial expression system, enabling rapid scale-up of the vaccine in the event of a potential outbreak to combat *F. gigantica* infection. The experimental evaluation of this multi-epitope subunit vaccine construct's immunogenic behaviour is validated by it.

5.2 Recommendations and future direction

In vitro and in vivo investigations are recommended to determine the vaccine's effectiveness against fascialiasis.

Chapter VII: References

Aftab A, Bisen1 S, Lall R, Yadav1 S, Silamparasan M, Raina OK. 2021. Cloning and expression of fasciola gigantica cathepsin-b recombinant proteins. Indian J Anim Res. 55(3). doi:10.18805/ijar.B-3959.

Ai L, Dong SJ, Zhang WY, Elsheikha HM, Mahmmod YS, Lin RQ, Yuan ZG, Shi YL, Huang WY, Zhu XQ. 2010. Specific PCR-based assays for the identification of Fasciola species: their development, evaluation and potential usefulness in prevalence surveys. Ann Trop Med Parasitol. 104(1):65–72. doi:10.1179/136485910X12607012373713. [accessed 2023 Jan 1]. https://pubmed.ncbi.nlm.nih.gov/20149293/.

Akıl M, Aykur M, Karakavuk M, Can H, Döşkaya M. 2022. Construction of a multiepitope vaccine candidate against Fasciola hepatica: an in silico design using various immunogenic excretory/secretory antigens. Expert Rev Vaccines. 21(7). doi:10.1080/14760584.2022.1996233.

Alasaad S, Soriguer RC, Abu-Madi M, el Behairy A, Jowers MJ, Baños PD, Píriz A, Fickel J, Zhu XQ. 2011. A TaqMan real-time PCR-based assay for the identification of Fasciola spp. Vet Parasitol. 179(1–3):266–271. doi:10.1016/J.VETPAR.2011.01.059. [accessed 2023 Jan 1]. https://europepmc.org/article/med/21334813.

Alba A, Vázquez AA, Sánchez J, Gourbal B. 2022. Immunological Resistance of Pseudosuccinea columella Snails From Cuba to Fasciola hepatica (Trematoda) Infection: What We Know and Where We Go on Comparative Molecular and Mechanistic Immunobiology, Ecology and Evolution. Front Immunol. 13. doi:10.3389/fimmu.2022.794186.

Alizadeh S, Mohammadi T. 2019. Ultrasonographic Liver Findings in a Sheep Flock Involved in Chronic Fasciolosis. Iranian Journal of Veterinary Medicine (International Journal of Veterinary Research). 13(100759).

Andrews SJ, Cwiklinski K, Dalton JP. 2021. The Discovery of Fasciola hepatica and its Life Cycle. In: Fasciolosis II.

Ayyagari VS, Venkateswarulu TC, Abraham Peele K, Srirama K. 2022. Design of a multi-epitope-based vaccine targeting M-protein of SARS-CoV2: an immunoinformatics approach. J Biomol Struct Dyn. 40(7). doi:10.1080/07391102.2020.1850357.

Bacchetta R, Gregori S, Roncarolo MG. 2005. CD4+ regulatory T cells: Mechanisms of induction and effector function. Autoimmun Rev. 4(8):491–496. doi:10.1016/J.AUTREV.2005.04.005.

Barbour T, Cwiklinski K, Lalor R, Dalton JP, Verissimo CDM. 2021. The zoonotic helminth parasite fasciola hepatica: Virulence-associated Cathepsin B and Cathepsin L cysteine peptidases secreted by infective newly excysted juveniles (NEJ). Animals. 11(12). doi:10.3390/ani11123495.

Buchan DWA, Jones DT. 2019. The PSIPRED Protein Analysis Workbench: 20 years on. Nucleic Acids Res. 47(W1):W402–W407. doi:10.1093/NAR/GKZ297. [accessed 2023 Jan 5]. https://academic.oup.com/nar/article/47/W1/W402/5480136.

Buffoni L, Garza-Cuartero L, Pérez-Caballero R, Zafra R, Javier Martínez-Moreno F, Molina-Hernández V, Pérez J, Martínez-Moreno Á, Mulcahy G. 2020. Identification of protective peptides of Fasciola hepatica-derived cathepsin L1 (FhCL1) in vaccinated sheep by a linear B-cell epitope mapping approach. Parasit Vectors. 13(1). doi:10.1186/s13071-020-04260-6.

Caffrey CR, Goupil L, Rebello KM, Dalton JP, Smith D. 2018. Cysteine proteases as digestive enzymes in parasitic helminths. PLoS Negl Trop Dis. 12(8). doi:10.1371/journal.pntd.0005840.

Caminade C, van Dijk J, Baylis M, Williams D. 2015. Modelling recent and future climatic suitability for fasciolosis in Europe. Geospat Health. 9(2). doi:10.4081/gh.2015.352.

Cancela M, Acosta D, Rinaldi G, Silva E, Durán R, Roche L, Zaha A, Carmona C, Tort JF. 2008. A distinctive repertoire of cathepsins is expressed by juvenile invasive Fasciola hepatica. Biochimie. 90(10). doi:10.1016/j.biochi.2008.04.020.

Caravedo MA, Cabada MM. 2020. Human Fascioliasis: Current Epidemiological Status and Strategies for Diagnosis, Treatment, and Control. Res Rep Trop Med.

11:158. doi:10.2147/RRTM.S237461. [accessed 2023 Jan 5]. /pmc/articles/PMC7705270/.

Carmona C, Dowd AJ, Smith AM, Dalton JP. 1993. Cathepsin L proteinase secreted by Fasciola hepatica in vitro prevents antibody-mediated eosinophil attachment to newly excysted juveniles. Mol Biochem Parasitol. 62(1). doi:10.1016/0166-6851(93)90172-T.

Casulli A. 2021. New global targets for NTDs in the WHO roadmap 2021–2030. PLoS Negl Trop Dis. 15(5):e0009373. doi:10.1371/JOURNAL.PNTD.0009373. [accessed 2023 Jan 4]. https://journals.plos.org/plosntds/article?id=10.1371/journal.pntd.0009373.

CDC. 2019. Fasciola - Biology.

CDC. 2022. Fasciola - Biology. [accessed 2022 Dec 29]. https://www.cdc.gov/parasites/fasciola/biology.html.

Celias DP, Corvo I, Silvane L, Tort JF, Chiapello LS, Fresno M, Arranz A, Motrán CC, Cervi L. 2019. Cathepsin L3 from fasciola hepatica induces NLRP3 inflammasome alternative activation in murine dendritic cells. Front Immunol. 10(MAR). doi:10.3389/fimmu.2019.00552.

Chang KY, Yang JR. 2013a. Analysis and Prediction of Highly Effective Antiviral Peptides Based on Random Forests. PLoS One. 8(8):e70166. doi:10.1371/JOURNAL.PONE.0070166. [accessed 2023 Jan 5]. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0070166.

Chang KY, Yang JR. 2013b. Analysis and Prediction of Highly Effective Antiviral Peptides Based on Random Forests. PLoS One. 8(8):e70166. doi:10.1371/JOURNAL.PONE.0070166. [accessed 2023 Jan 5]. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0070166.

Chantree P, Phatsara M, Meemon K, Chaichanasak P, Changklungmoa N, Kueakhai P, Lorsuwannarat N, Sangpairoj K, Songkoomkrong S, Wanichanon C, et al. 2013. Vaccine potential of recombinant cathepsin B against Fasciola gigantica. Exp Parasitol. 135(1). doi:10.1016/j.exppara.2013.06.010.

Chaudhri G, Quah BJ, Wang Y, Tan AHY, Zhou J, Karupiah G, Parish CR. 2009. T cell receptor sharing by cytotoxic T lymphocytes facilitates efficient virus control. Proc Natl Acad Sci U S A. 106(35):14984–14989. doi:10.1073/PNAS.0906554106/SUPPL_FILE/0906554106SI.PDF. [accessed 2023 Jan 5]. https://www.pnas.org/doi/abs/10.1073/pnas.0906554106.

Chauhan V, Rungta T, Goyal K, Singh MP. 2019. Designing a multi-epitope based vaccine to combat Kaposi Sarcoma utilizing immunoinformatics approach. Sci Rep. 9(1). doi:10.1038/s41598-019-39299-8.

Chowdhury QMMK, Hasan M, Ahmed J, Shykat CA, Islam MS, Hossain M. 2016. IMPACT OF CLIMATE CHANGE ON LIVESTOCK IN BANGLADESH: A REVIEW OF WHAT WE KNOW AND WHAT WE NEED TO KNOW. American Journal of Agricultural Science, Engineering and Technology. 3(2):18–25. doi:https://doi.org/10.5281/zenodo.1197032.

Coles GC, Jackson F, Pomroy WE, Prichard RK, von Samson-Himmelstjerna G, Silvestre A, Taylor MA, Vercruysse J. 2006. The detection of anthelmintic resistance in nematodes of veterinary importance. Vet Parasitol. 136(3–4). doi:10.1016/j.vetpar.2005.11.019.

Corvo I, Cancela M, Cappetta M, Pi-Denis N, Tort JF, Roche L. 2009. The major cathepsin L secreted by the invasive juvenile Fasciola hepatica prefers proline in the S2 subsite and can cleave collagen. Mol Biochem Parasitol. 167(1). doi:10.1016/j.molbiopara.2009.04.005.

Craig DB, Dombkowski AA. 2013. Disulfide by Design 2.0: A web-based tool for disulfide engineering in proteins. BMC Bioinformatics. 14(1):1–7. doi:10.1186/1471-2105-14-346/FIGURES/4. [accessed 2023 Jan 5]. https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-14-346.

Cwiklinski K, Jewhurst H, McVeigh P, Barbour T, Maule AG, Tort J, O'Neill SM, Robinson MW, Donnelly S, Dalton JP. 2018. Infection by the helminth parasite fasciola hepatica requires rapid regulation of metabolic, virulence, and invasive factors to adjust to its mammalian host. Molecular and Cellular Proteomics. 17(4). doi:10.1074/mcp.RA117.000445.

Cwiklinski K, de La Torre-Escudero E, Trelis M, Bernal D, Dufresne PJ, Brennan GP, O'Neill S, Tort J, Paterson S, Marcilla A, et al. 2015. The extracellular vesicles of the helminth pathogen, Fasciola hepatica: Biogenesis pathways and cargo molecules involved in parasite pathogenesis. Molecular and Cellular Proteomics. 14(12). doi:10.1074/mcp.M115.053934.

Dalton JP, Neill SO, Stack C, Collins P, Walshe A, Sekiya M, Doyle S, Mulcahy G, Hoyle D, Khaznadji E, et al. 2003. Fasciola hepatica cathepsin L-like proteases: Biology, function, and potential in the development of first generation liver fluke vaccines. Int J Parasitol. 33(11):1173–1181. doi:10.1016/S0020-7519(03)00171-1. [accessed 2023 Jan 5]. https://pubmed.ncbi.nlm.nih.gov/13678633/.

Dias AS, de Araújo JV, Braga FR, Calian OA, Vilela VLR, Miranda VZ. 2014. Comparison among three techniques in the parasitological diagnosis of Fasciola hepatica in cattle. Archives of Veterinary Science. 19(1). doi:10.5380/avs.v19i1.31372.

Dimitrov I, Flower DR, Doytchinova I. 2013. AllerTOP - a server for in silico prediction of allergens. BMC Bioinformatics. 14(SUPPL6):1–9. doi:10.1186/1471-2105-14-S6-S4/FIGURES/4. [accessed 2023 Jan 4]. https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-14-S6-S4.

Dimitrov I, Naneva L, Doytchinova I, Bangov I. 2014. AllergenFP: allergenicity prediction by descriptor fingerprints. Bioinformatics. 30(6):846–851. doi:10.1093/BIOINFORMATICS/BTT619. [accessed 2023 Jan 4]. https://academic.oup.com/bioinformatics/article/30/6/846/286438.

DOWD AJ, SMITH AM, McGONIGLE S, DALTON JP. 1994. Purification and characterisation of a second cathepsin L proteinase secreted by the parasitic trematode Fasciola hepatica. Eur J Biochem. 223(1). doi:10.1111/j.1432-1033.1994.tb18969.x.

Doytchinova IA, Flower DR. 2007. VaxiJen: A server for prediction of protective antigens, tumour antigens and subunit vaccines. BMC Bioinformatics. 8(1):1–7. doi:10.1186/1471-2105-8-4/TABLES/2. [accessed 2023 Jan 4]. https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-8-4.

Duvaud S, Gabella C, Lisacek F, Stockinger H, Ioannidis V, Durinx C. 2021. Expasy, the Swiss Bioinformatics Resource Portal, as designed by its users. Nucleic Acids

Res. 49(W1):W216–W227. doi:10.1093/NAR/GKAB225. [accessed 2023 Jan 4]. https://academic.oup.com/nar/article/49/W1/W216/6225225.

Ermolaeva MD, Khalak HG, White O, Smith HO, Salzberg SL. 2000. Prediction of transcription terminators in bacterial genomes. J Mol Biol. 301(1):27–33. doi:10.1006/JMBI.2000.3836.

Ferraro F, Merlino A, dell´Oca N, Gil J, Tort JF, Gonzalez M, Cerecetto H, Cabrera M, Corvo I. 2016. Identification of Chalcones as Fasciola hepatica Cathepsin L Inhibitors Using a Comprehensive Experimental and Computational Approach. PLoS Negl Trop Dis. 10(7). doi:10.1371/journal.pntd.0004834.

Ferreira APPN, Costa ALO, Becattini RM, Ferreira MAND, da Paixão HPR, Coscarelli D, Vidigal THDA, Lima WDS, Pereira CA de J. 2021. Integrative taxonomy: combining molecular and morphological characteristics to identify Lymnaea (Galba) cubensis, intermediate host of Fasciola hepatica. Revista Brasileira de Parasitologia Veterinaria. 30(2). doi:10.1590/S1984-29612021052.

Fiss L, de Lourdes Adrien M, Marcolongo-Pereira C, Assis-Brasil ND, Sallis ESV, Riet-Correa F, Ruas JL, Schild AL. 2013. Subacute and acute fasciolosis in sheep in southern Brazil. Parasitol Res. 112(2). doi:10.1007/s00436-012-3096-2.

Forbes A. 2017. Liver fluke infections in cattle and sheep. Livestock. 22(5). doi:10.12968/live.2017.22.5.250.

Fürst T, Keiser J, Utzinger J. 2012. Global burden of human food-borne trematodiasis: a systematic review and meta-analysis. Lancet Infect Dis. 12(3):210–221. doi:10.1016/S1473-3099(11)70294-8. [accessed 2023 Jan 5]. https://pubmed.ncbi.nlm.nih.gov/22108757/.

Garza-Cuartero L, Geurden T, Mahan SM, Hardham JM, Dalton JP, Mulcahy G. 2018. Antibody recognition of cathepsin L1-derived peptides in Fasciola hepatica-infected and/or vaccinated cattle and identification of protective linear B-cell epitopes. Vaccine. 36(7). doi:10.1016/j.vaccine.2018.01.020.

Gayo V, Cancela M, Acosta D. 2020. Maintenance of life cycle stages of Fasciola hepatica in the laboratory. In: Methods in Molecular Biology. Vol. 2137.

Gordon DK, Roberts LCP, Lean N, Zadoks RN, Sargison ND, Skuce PJ. 2013. Identification of the rumen fluke, Calicophoron daubneyi, in GB livestock: possible implications for liver fluke diagnosis. Vet Parasitol. 195(1–2):65–71. doi:10.1016/J.VETPAR.2013.01.014. [accessed 2023 Jan 1]. https://pubmed.ncbi.nlm.nih.gov/23411375/.

Grote A, Hiller K, Scheer M, Münch R, Nörtemann B, Hempel DC, Jahn D. 2005a. JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. Nucleic Acids Res. 33(suppl_2):W526–W531. doi:10.1093/NAR/GKI376. [accessed 2023 Jan 5]. https://academic.oup.com/nar/article/33/suppl_2/W526/2505472.

Grote A, Hiller K, Scheer M, Münch R, Nörtemann B, Hempel DC, Jahn D. 2005b. JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. Nucleic Acids Res. 33(suppl_2):W526–W531. doi:10.1093/NAR/GKI376. [accessed 2023 Jan 5]. https://academic.oup.com/nar/article/33/suppl_2/W526/2505472.

Gupta S, Kapoor P, Chaudhary K, Gautam A, Kumar R, Raghava GPS. 2013. In Silico Approach for Predicting Toxicity of Peptides and Proteins. PLoS One. 8(9):e73957. doi:10.1371/JOURNAL.PONE.0073957. [accessed 2023 Jan 4]. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0073957.

Haçariz O, Sayers G, Baykal AT. 2012. A proteomic approach to investigate the distribution and abundance of surface and internal fasciola hepatica proteins during the chronic stage of natural liver fluke infection in cattle. J Proteome Res. 11(7). doi:10.1021/pr300015p.

Hillyer GV. 2005. Fasciola antigens as vaccines against fascioliasis and schistosomiasis . J Helminthol. 79(3). doi:10.1079/joh2005304.

Hughes G, Green CA, Street D, Maurice Y, Henderson J, Woodhouse A, Nicholl D, Scriven JE. 2020. Case report: Subarachnoid hemorrhage and eosinophilic meningitis due to disseminated fascioliasis. American Journal of Tropical Medicine and Hygiene. 102(3). doi:10.4269/ajtmh.19-0360.

Ico-Gómez R, González-Garduño R, Ortiz-Pérez D, Mosqueda-Gualito JJ, Flores-Santiago EDJ, Sosa-Pérez G, Salazar-Tapia AA. 2021. Assessment of anthelmintic

effectiveness to control Fasciola hepatica and paramphistome mixed infection in cattle in the humid tropics of Mexico. Parasitology. doi:10.1017/S0031182021001153.

Ikai A. 1980. Thermostability and Aliphatic Index of Globular Proteins. The Journal of Biochemistry. 88(6):1895–1898. doi:10.1093/OXFORDJOURNALS.JBCHEM.A133168. [accessed 2023 Jan 5]. https://academic.oup.com/jb/article/88/6/1895/773432.

Javaregowda AK, Rani KB. 2017. Chronic bovine fasciolosis associated cholangiolithiasis: a case report. Journal of Parasitic Diseases. 41(1). doi:10.1007/s12639-016-0755-8.

Jiménez M, Hidalgo C, Stoore C, Corrêa F, Pereira I, Hernández M, Sáenz L, Benavides J, Ferreras MC, Royo M, et al. 2021. Fasciola hepatica co-infection enhances Th1 immune response in the adventitial layer of non-fertile Echinococcus granulosus cysts. Vet Parasitol. 290. doi:10.1016/j.vetpar.2021.109343.

Johnson KS, Harrison GBL, Lightowlers MW, O'Hoy KL, Cougle WG, Dempster RP, Lawrence SB, Vinton JG, Heath DD, Rickard MD. 1989. Vaccination against ovine cysticercosis using a defined recombinant antigen. Nature. 338:585–587. doi:10.1038/338585a0. [accessed 2023 Jan 5]. https://www.nature.com/articles/338585a0.

Kadam A, Sasidharan S, Saudagar P. 2020. Computational design of a potential multi-epitope subunit vaccine using immunoinformatics to fight Ebola virus. Infection, Genetics and Evolution. 85. doi:10.1016/j.meegid.2020.104464.

Kalita J, Padhi AK, Tripathi T. 2020. Designing a vaccine for fascioliasis using immunogenic 24 kDa mu-class glutathione s-transferase. Infection, Genetics and Evolution. 83. doi:10.1016/j.meegid.2020.104352.

Kalita P, Lyngdoh DL, Padhi AK, Shukla H, Tripathi T. 2019. Development of multi-epitope driven subunit vaccine against Fasciola gigantica using immunoinformatics approach. Int J Biol Macromol. 138. doi:10.1016/j.ijbiomac.2019.07.024.

Kelley JM, Elliott TP, Beddoe T, Anderson G, Skuce P, Spithill TW. 2016. Current Threat of Triclabendazole Resistance in Fasciola hepatica. Trends Parasitol. 32(6). doi:10.1016/j.pt.2016.03.002.

Kelley JM, Rawlin G, Beddoe T, Stevenson M, Spithill TW. 2021. Fasciola hepatica Control Practices on a Sample of Dairy Farms in Victoria, Australia. Front Vet Sci. 8. doi:10.3389/fvets.2021.669117.

Khatoon N, Pandey RK, Prajapati VK. 2017. Exploring Leishmania secretory proteins to design B and T cell multi-epitope subunit vaccine using immunoinformatics approach. Scientific Reports 2017 7:1. 7(1):1–12. doi:10.1038/s41598-017-08842-w. [accessed 2023 Jan 5]. https://www.nature.com/articles/s41598-017-08842-w.

Kianifard L, Yakhchali M, Imani M. 2020. Activity of serine proteases from fasciola Hepatica EGGS in relation to pH and temperature. Bulg J Vet Med. 23(3). doi:10.15547/bjvm.2218.

Kipyegen CK, Muleke CI, Otachi EO. 2022. Human and animal fasciolosis: Coprological survey in Narok, Baringo and Kisumu counties, Kenya. Onderstepoort Journal of Veterinary Research. 89(1). doi:10.4102/ojvr.v89i1.1954.

Kishore GR, Malakondaiah P, Sreedevi C, Subramanyam K v., Sudhakar K. 2021. Evaluation of flow through assay as a point-of-care test for detection of antibodies against fasciola gigantica in sheep. Journal of Veterinary Parasitology. 35(1). doi:10.5958/0974-0813.2021.00004.8.

Klinger AE, Kronen RJ, Barak T, Mophuthegi P, Makhema J, Zash R, Shapiro R, York N. 2020. 770. Otherwise Unavailable Non-Malarial Parasitic Disease Treatment Drugs in the United States: an Update from CDC Parasitic Diseases Drug Service. Open Forum Infect Dis. 7(Supplement_1):S429–S430. doi:10.1093/OFID/OFAA439.960. [accessed 2023 Jan 5]. https://academic.oup.com/ofid/article/7/Supplement_1/S429/6056806.

Ko J, Park H, Heo L, Seok C. 2012. GalaxyWEB server for protein structure prediction and refinement. Nucleic Acids Res. 40(W1):W294–W297. doi:10.1093/NAR/GKS493. [accessed 2023 Jan 5]. https://academic.oup.com/nar/article/40/W1/W294/1078340.

Kolaskar AS, Tongaonkar PC. 1990. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett. 276(1–2):172–174. doi:10.1016/0014-5793(90)80535-Q.

Kolla HB, Tirumalasetty C, Sreerama K, Ayyagari VS. 2021. An immunoinformatics approach for the design of a multi-epitope vaccine targeting super antigen TSST-1 of Staphylococcus aureus. Journal of Genetic Engineering and Biotechnology. 19(1). doi:10.1186/s43141-021-00160-z.

Kueakhai P, Changklungmoa N, Cheukamud W, Osotprasit S, Chantree P, Preyavichyapugdee N, Sobhon P, Meemon K. 2021. The combined recombinant cathepsin L1H and cathepsin B3 vaccine against Fasciola gigantica infection. Parasitol Int. 83. doi:10.1016/j.parint.2021.102353.

Kueakhai P, Changklungmoa N, Riengrojpitak S, Chaichanasak P, Meemon K, Chaithirayanon K, Chantree P, Sansri V, Itagaki T, Sobhon P. 2013. Vaccine potential of recombinant saposin-like protein 2 against Fasciolosis gigantica in mice. Vaccine. 31(47). doi:10.1016/j.vaccine.2013.09.027.

Kumar R, Sharma D, Masand R, Kumar A, Asrani RK. 2021. Pathological study of acute fasciolosis in a buffalo. Buffalo Bulletin. 40(1).

Lalor R, Cwiklinski K, Calvani NED, Dorey A, Hamon S, Corrales JL, Dalton JP, de Marco Verissimo C. 2021. Pathogenicity and virulence of the liver flukes Fasciola hepatica and Fasciola Gigantica that cause the zoonosis Fasciolosis. Virulence. 12(1). doi:10.1080/21505594.2021.1996520.

Lalrinkima H, Lalchhandama C, Jacob SS, Raina OK, Lallianchhunga MC. 2021. Fasciolosis in India: An overview. Exp Parasitol. 222. doi:10.1016/j.exppara.2021.108066.

Lardans V, Dissous C. 1998. Snail control strategies for reduction of schistosomiasis transmission. Parasitology Today. 14(10). doi:10.1016/S0169-4758(98)01320-9.

Laskowski RA, MacArthur MW, Thornton JM. 2012 Apr 1. Abstract. urn:isbn:684–687. doi:10.1107/97809553602060000882.

Lee S, Nguyen MT. 2015. Recent Advances of Vaccine Adjuvants for Infectious Diseases. Immune Netw. 15(2):51–57. doi:10.4110/IN.2015.15.2.51. [accessed 2023 Jan 5]. https://doi.org/10.4110/in.2015.15.2.51.

López-Abán J, Esteban A, Vicente B, Rojas-Caraballo J, Olmo E del, Martínez-Fernández AR, Hillyer G v., Muro A. 2012. Adaptive immune stimulation is required

to obtain high protection with fatty acid binding protein vaccine candidate against fasciola hepatica in balb/C mice. Journal of Parasitology. 98(3). doi:10.1645/GE-2891.1.

López-Blanco JR, Aliaga JI, Quintana-Ortí ES, Chacón P. 2014. iMODS: internal coordinates normal mode analysis server. Nucleic Acids Res. 42(W1):W271–W276. doi:10.1093/NAR/GKU339. [accessed 2023 Jan 5]. https://academic.oup.com/nar/article/42/W1/W271/2435308.

López Corrales J, Cwiklinski K, de Marco Verissimo C, Dorey A, Lalor R, Jewhurst H, McEvoy A, Diskin M, Duffy C, Cosby SL, et al. 2021. Diagnosis of sheep fasciolosis caused by Fasciola hepatica using cathepsin L enzyme-linked immunosorbent assays (ELISA). Vet Parasitol. 298. doi:10.1016/j.vetpar.2021.109517.

Lu XT, Gu QY, Limpanont Y, Song LG, Wu ZD, Okanurak K, Lv ZY. 2018. Snail-borne parasitic diseases: An update on global epidemiological distribution, transmission interruption and control methods. Infect Dis Poverty. 7(1). doi:10.1186/s40249-018-0414-7.

Malatji MP, Pfukenyi DM, Mukaratirwa S. 2020. Fasciola species and their vertebrate and snail intermediate hosts in East and Southern Africa: A review. J Helminthol. 94. doi:10.1017/S0022149X19000531.

Marques LT, Guedes RA, Rodrigues WD, Archanjo AB, Severi JA, Martins IVF. 2020. Chemical composition of various plant extracts and their in vitro efficacy in control of Fasciola hepatica eggs. Ciencia Rural. 50(5). doi:10.1590/0103-8478cr20190363.

Martínez-Pérez JM, Robles-Pérez D, Rojo-Vázquez FA, Martínez-Valladares M. 2012. Comparison of three different techniques to diagnose Fasciola hepatica infection in experimentally and naturally infected sheep. Vet Parasitol. 190(1–2):80–86. doi:10.1016/J.VETPAR.2012.06.002. [accessed 2023 Jan 1]. https://pubmed.ncbi.nlm.nih.gov/22749330/.

Mashiach E, Schneidman-Duhovny D, Andrusier N, Nussinov R, Wolfson HJ. 2008. FireDock: a web server for fast interaction refinement in molecular docking. Nucleic

Acids Res. 36(suppl_2):W229–W232. doi:10.1093/NAR/GKN186. [accessed 2023 Jan 5]. https://academic.oup.com/nar/article/36/suppl_2/W229/2505768.

Matsuda K, Kogame S, Niki H, Saito M, Ishiguro Y, Sano Y. 2020. Gross and histological lesions in the livers of sika deer with particular emphasis on fascioliasis. Journal of Veterinary Medical Science. 82(2). doi:10.1292/jvms.19-0544.

May K, Raue K, Blazejak K, Jordan D, Strube C. 2022. Pasture rewetting in the context of nature conservation shows no long-term impact on endoparasite infections in sheep and cattle. Parasit Vectors. 15(1). doi:10.1186/s13071-022-05155-4.

McNally J, Callan D, Andronicos N, Bott N, Hunt PW. 2013. DNA-based methodology for the quantification of gastrointestinal nematode eggs in sheep faeces. Vet Parasitol. 198(3–4):325–335. doi:10.1016/J.VETPAR.2013.09.014. [accessed 2023 Jan 1]. https://pubmed.ncbi.nlm.nih.gov/24149045/.

Meemon K, Sobhon P. 2015. Juvenile-specific cathepsin proteases in Fasciola spp.: their characteristics and vaccine efficacies. Parasitol Res. 114(8). doi:10.1007/s00436-015-4589-6.

Mei X, Zhang Y, Quan C, Liang Y, Huang W, Shi W. 2022. Characterization of the Pathology, Biochemistry, and Immune Response in Kunming (KM) Mice Following Fasciola gigantica Infection. Front Cell Infect Microbiol. 11. doi:10.3389/fcimb.2021.793571.

Moazeni M, Ahmadi A. 2016. Controversial aspects of the life cycle of Fasciola hepatica. Exp Parasitol. 169. doi:10.1016/j.exppara.2016.07.010.

Moazeni M, Ansari-Lari M, Masoodfar M, Hosseinzadeh S, Mootabi Alavi AM. 2010. Lethal effect of high temperatures on the eggs of Fasciola hepatica. Iran J Vet Res. 11(2).

Molina-hernández V, Ruiz-campillo MT, Martínez-moreno FJ, Buffoni L, Martínez-moreno Á, Zafra R, Bautista MJ, Escamilla A, Pérez-caballero R, Pérez J. 2021. A partially protective vaccine for fasciola hepatica induced degeneration of adult flukes associated to a severe granulomatous reaction in sheep. Animals. 11(10). doi:10.3390/ani11102869.

Morales ML, Tanabe MB, White. AC, Lopez M, Bascope R, Cabada MM. 2021. Triclabendazole Treatment Failure for Fasciola hepatica Infection among Preschool and School-Age Children, Cusco, Peru. Emerg Infect Dis. 27(7). doi:10.3201/eid2707.203900.

Muro A, Rojas-Caraballo J. 2017. Fasciola and Fasciolosis. In: Laboratory Models for Foodborne Infections.

Narula A, Pandey RK, Khatoon N, Mishra A, Prajapati VK. 2018. Excavating chikungunya genome to design B and T cell multi-epitope subunit vaccine using comprehensive immunoinformatics approach to control chikungunya infection. Infection, Genetics and Evolution. 61:4–15. doi:10.1016/J.MEEGID.2018.03.007.

Nath TC, Eom KS, Choe S, Islam S, Sabuj SS, Saha E, Tuhin RH, Ndosi BA, Kang Y, Kim S, et al. 2022. Insights to helminth infections in food and companion animals in Bangladesh: Occurrence and risk profiling. Parasite Epidemiol Control. 17. doi:10.1016/j.parepi.2022.e00245.

Nesterkov A v., Grebennikov ME. 2020. Grassland Land Snail Communities after Reduction of Emissions from a Copper Smelter. Russ J Ecol. 51(6). doi:10.1134/S1067413620060065.

Olkeba BK, Boets P, Mereta ST, Mandefro B, Debesa G, Ahmednur M, Ambelu A, Korma W, Goethals PLM. 2022. Malacological and parasitological surveys on ethiopian rift valley lakes: Implications for control and elimination of snail-borne diseases. Int J Environ Res Public Health. 19(1). doi:10.3390/ijerph19010142.

Pandey T, Ghosh A, Todur VN, Rajendran V, Kalita P, Kalita J, Shukla R, Chetri PB, Shukla H, Sonkar A, et al. 2020a. Draft Genome of the Liver Fluke Fasciola gigantica. ACS Omega. 5(19). doi:10.1021/acsomega.0c00980.

Pandey T, Ghosh A, Todur VN, Rajendran V, Kalita P, Kalita J, Shukla R, Chetri PB, Shukla H, Sonkar A, et al. 2020b. Draft Genome of the Liver Fluke Fasciola gigantica. ACS Omega. 5(19):11084–11091. doi:10.1021/ACSOMEGA.0C00980/SUPPL_FILE/AO0C00980_SI_005.XLSX. [accessed 2023 Jan 5]. https://pubs.acs.org/doi/full/10.1021/acsomega.0c00980.

Pan M, Bai SY, Ji TK, Fan YM, Liu DD, Yang Y, Tao JP, Huang SY. 2022. Epidemiology of Fasciola spp. in the intermediate host in China: A potential risk for fasciolosis transmission. Acta Trop. 230. doi:10.1016/j.actatropica.2022.106394.

Piacenza L, Acosta D, Basmadjian I, Dalton JP, Carmona C. 1999. Vaccination with cathepsin L proteinases and with leucine aminopeptidase induces high levels of protection against fascioliasis in sheep. Infect Immun. 67(4). doi:10.1128/iai.67.4.1954-1961.1999.

Ponomarenko J, Bui HH, Li W, Fusseder N, Bourne PE, Sette A, Peters B. 2008. ElliPro: A new structure-based tool for the prediction of antibody epitopes. BMC Bioinformatics. 9(1):1–8. doi:10.1186/1471-2105-9-514/FIGURES/3. [accessed 2023 Jan 5]. https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-9-514.

Prastowo J, Priyowidodo D, Sahara A, Nurcahyo W, Nugraheni YR, Awaludin A. 2022. Molecular identification of cercaria Fasciola gigantica in lymnaeid snails in Kulon Progo, Yogyakarta. Vet Parasitol Reg Stud Reports. 30. doi:10.1016/j.vprsr.2022.100707.

Pritsch IC, Tikhonova IG, Jewhurst HL, Drysdale O, Cwiklinski K, Molento MB, Dalton JP, Verissimo CDM. 2020. Regulation of the Fasciola hepatica newly excysted juvenile cathepsin L3 (FhCL3) by its propeptide: a proposed ‘clamp-like’ mechanism of binding and inhibition. BMC Mol Cell Biol. 21(1). doi:10.1186/s12860-020-00335-5.

Raina OK, Nagar G, Varghese A, Prajitha G, Alex A, Maharana BR, Joshi P. 2011. Lack of protective efficacy in buffaloes vaccinated with Fasciola gigantica leucine aminopeptidase and peroxiredoxin recombinant proteins. Acta Trop. 118(3). doi:10.1016/j.actatropica.2011.02.008.

Rizwan HM, Sajid MS, Abbas H, Khan MN, Akram Q, Shamim A. 2021. Epidemiology, burden and seasonal variation of fasciolosis determined through faecal examination and excretory/secretory antigens based ELISA. Journal of the Hellenic Veterinary Medical Society. 72(4). doi:10.12681/jhvms.29394.

Roberts JA, Suhardono. 1996. Approaches to the control of fasciolosis in ruminants. Int J Parasitol. 26(8–9):971–981. doi:10.1016/S0020-7519(96)80074-9.

Robinson MW, Menon R, Donnelly SM, Dalton JP, Ranganathan S. 2009. An Integrated Transcriptomics and Proteomics Analysis of the Secretome of the Helminth Pathogen Fasciola hepatica. Molecular & Cellular Proteomics. 8(8). doi:10.1074/mcp.m900045-mcp200.

Robinson MW, Trudgett A, Hoey EM, Fairweather I. 2002. Triclabendazole-resistant Fasciola hepatica: β-tubulin and response to in vitro treatment with triclabendazole. Parasitology. 124(3). doi:10.1017/S003118200100124X.

Robles-Pérez D, Martínez-Pérez JM, Rojo-Vázquez FA, Martínez-Valladares M. 2013. The diagnosis of fasciolosis in feces of sheep by means of a PCR and its application in the detection of anthelmintic resistance in sheep flocks naturally infected. Vet Parasitol. 197(1–2):277–282. doi:10.1016/J.VETPAR.2013.05.006. [accessed 2023 Jan 1]. https://pubmed.ncbi.nlm.nih.gov/23743420/.

Romero J, Villaguala C, Quiroz F, Landaeta-Aqueveque C, Alfaro G, Pérez Fernández R. 2019. Flukicide efficacy against Fasciola hepatica of Triclabendazole and Nitroxynil in cattle of the central valley of Chile. Revista Brasileira de Parasitologia Veterinaria. 28(1). doi:10.1590/s1984-296120180089.

Rose PW, Prlić A, Altunkaya A, Bi C, Bradley AR, Christie CH, Costanzo L di, Duarte JM, Dutta S, Feng Z, et al. 2017. The RCSB protein data bank: integrative view of protein, gene and 3D structural information. Nucleic Acids Res. 45(D1):D271–D281. doi:10.1093/NAR/GKW1000. [accessed 2023 Jan 5]. https://academic.oup.com/nar/article/45/D1/D271/2333880.

Ruiz-Campillo MT, Molina-Hernandez V, Escamilla A, Stevenson M, Perez J, Martinez-Moreno A, Donnelly S, Dalton JP, Cwiklinski K. 2017. Immune signatures of pathogenesis in the peritoneal compartment during early infection of sheep with Fasciola hepatica. Sci Rep. 7(1). doi:10.1038/s41598-017-03094-0.

Ryan S, Shiels J, Taggart CC, Dalton JP, Weldon S. 2020. Fasciola hepatica-Derived Molecules as Regulators of the Host Immune Response. Front Immunol. 11. doi:10.3389/fimmu.2020.02182.

Saha S, Raghava GPS. 2006. Prediction of continuous B-cell epitopes in an antigen using recurrent neural network. Proteins: Structure, Function, and Bioinformatics.

65(1):40–48. doi:10.1002/PROT.21078. [accessed 2023 Jan 4]. https://onlinelibrary.wiley.com/doi/full/10.1002/prot.21078.

Sansri V, Changklungmoa N, Chaichanasak P, Sobhon P, Meemon K. 2013. Molecular cloning, characterization and functional analysis of a novel juvenile-specific cathepsin L of Fasciola gigantica. Acta Trop. 128(1). doi:10.1016/j.actatropica.2013.06.013.

Sansri V, Meemon K, Changklungmoa N, Kueakhai P, Chantree P, Chaichanasak P, Lorsuwannarat N, Itagaki T, Sobhon P. 2015. Protection against Fasciola gigantica infection in mice by vaccination with recombinant juvenile-specific cathepsin L. Vaccine. 33(13). doi:10.1016/j.vaccine.2015.02.010.

Schneidman-Duhovny D, Inbar Y, Nussinov R, Wolfson HJ. 2005. PatchDock and SymmDock: servers for rigid and symmetric docking. Nucleic Acids Res. 33(suppl_2):W363–W367. doi:10.1093/NAR/GKI481. [accessed 2023 Jan 5]. https://academic.oup.com/nar/article/33/suppl_2/W363/2505698.

Sexton JL, Milner AR, Panaccio M, Waddington J, Wijffels G, Chandler D, Thompson C, Wilson L, Spithill TW, Mitchell GF. 1990. Glutathione S-transferase. Novel vaccine against Fasciola hepatica infection in sheep. J Immunol. 145(11).

Siles-Lucas M, Becerro-Recio D, Serrat J, González-Miguel J. 2021. Fascioliasis and fasciolopsiasis: Current knowledge and future trends. Res Vet Sci. 134. doi:10.1016/j.rvsc.2020.10.011.

Singh DK, Singh VK, Singh RN, Kumar P. 2021. Fasciolosis: Causes, Challenges and Controls.

Siricoon S, Grams SV, Grams R. 2012. Efficient inhibition of cathepsin B by a secreted type 1 cystatin of Fasciola gigantica. Mol Biochem Parasitol. 186(2). doi:10.1016/j.molbiopara.2012.10.003.

SMITH AM, CARMONA C, DOWD AJ, McGONIGLE S, ACOSTA D, DALTON JP. 1994. Neutralization of the activity of a Fasciola hepatica cathepsin L proteinase by anti-cathepsin L antibodies. Parasite Immunol. 16(6). doi:10.1111/j.1365-3024.1994.tb00356.x.

Smith D, Cwiklinski K, Jewhurst H, Tikhonova IG, Dalton JP. 2020. An atypical and functionally diverse family of Kunitz-type cysteine/serine proteinase inhibitors secreted by the helminth parasite Fasciola hepatica. Sci Rep. 10(1). doi:10.1038/s41598-020-77687-7.

Solanki V, Tiwari V. 2018. Subtractive proteomics to identify novel drug targets and reverse vaccinology for the development of chimeric vaccine against Acinetobacter baumannii. Scientific Reports 2018 8:1. 8(1):1–19. doi:10.1038/s41598-018-26689-7. [accessed 2023 Jan 4]. https://www.nature.com/articles/s41598-018-26689-7.

Soulsby EJL. 1968. Helminths, arthropods and protozoa of domesticated animals. Baillière Tindall & Cassell Ltd.

Suhardono, Roberts JA, Copeman DB. 2006. Biological control of Fasciola gigantica with Echinostoma revolutum. Vet Parasitol. 140(1–2). doi:10.1016/j.vetpar.2006.02.028.

Sun P, Wronski T, Apio A, Edwards L. 2020. A holistic model to assess risk factors of fasciolosis in Ankole cattle. Vet Parasitol Reg Stud Reports. 22. doi:10.1016/j.vprsr.2020.100488.

Tabari MA, Vahdati SAF, Samakkhah SA, Araghi A, Youssefi MR. 2022. Therapeutic efficacy of triclabendazole in comparison to combination of triclabendazole and levamisole in sheep naturally infected with Fasciola sp. Journal of Parasitic Diseases. 46(1). doi:10.1007/s12639-021-01422-w.

Tilling O. 2013. Rumen fluke in cattle in the UK: a review. Livestock. 18(6):223–227. doi:10.12968/LIVE.2013.18.6.223. [accessed 2023 Jan 1]. https://onlinelibrary.wiley.com/doi/full/10.12968/live.2013.18.6.223.

Toet H, Piedrafita DM, Spithill TW. 2014. Liver fluke vaccines in ruminants: Strategies, progress and future opportunities. Int J Parasitol. 44(12). doi:10.1016/j.ijpara.2014.07.011.

Tosta SF de O, Passos MS, Kato R, Salgado Á, Xavier J, Jaiswal AK, Soares SC, Azevedo V, Giovanetti M, Tiwari S, et al. 2021. Multi-epitope based vaccine against yellow fever virus applying immunoinformatics approaches. J Biomol Struct Dyn. 39(1). doi:10.1080/07391102.2019.1707120.

Vázquez AA, Alda P, Lounnas M, Sabourin E, Alba A, Pointier JP, Hurtrez-Boussès S. 2018. Lymnaeid snails hosts of Fasciola hepatica and Fasciola gigantica (Trematoda: Digenea): A worldwide review. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources. 13. doi:10.1079/PAVSNNR201813062.

Vázquez AA, Sabourin E, Alda P, Leroy C, Leray C, Carron E, Mulero S, Caty C, Hasfia S, Boisseau M, et al. 2021. Genetic diversity and relationships of the liver fluke Fasciola hepatica (Trematoda) with native and introduced definitive and intermediate hosts. Transbound Emerg Dis. 68(4). doi:10.1111/tbed.13882.

Villa-Mancera A, Olivares-Pérez J, Olmedo-Juárez A, Reynoso-Palomar A. 2021. Phage display-based vaccine with cathepsin L and excretory-secretory products mimotopes of Fasciola hepatica induces protective cellular and humoral immune responses in sheep. Vet Parasitol. 289. doi:10.1016/j.vetpar.2020.109340.

Vita R, Mahajan S, Overton JA, Dhanda SK, Martini S, Cantrell JR, Wheeler DK, Sette A, Peters B. 2019. The Immune Epitope Database (IEDB): 2018 update. Nucleic Acids Res. 47(D1):D339–D343. doi:10.1093/NAR/GKY1006. [accessed 2023 Jan 4]. https://academic.oup.com/nar/article/47/D1/D339/5144151.

Wagari A. 2021. A Review on Cattle Fasciolosis. J Vet Med Surg. 5(4). [accessed 2023 Jan 1]. https://www.imedpub.com/veterinary-medicine-and-surgery/.

Webb CM, Cabada MM. 2018. Recent developments in the epidemiology, diagnosis, and treatment of Fasciola infection. Curr Opin Infect Dis. 31(5). doi:10.1097/QCO.0000000000000482.

Wesołowska A, Basałaj K, Norbury LJ, Sielicka A, Wędrychowicz H, Zawistowska-Deniziak A. 2018. Vaccination against Fasciola hepatica using cathepsin L3 and B3 proteases delivered alone or in combination. Vet Parasitol. 250. doi:10.1016/j.vetpar.2017.12.007.

Wiederstein M, Sippl MJ. 2007. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Res. 35(suppl_2):W407–W410. doi:10.1093/NAR/GKM290. [accessed 2023 Jan 5]. https://academic.oup.com/nar/article/35/suppl_2/W407/2920938.

Wijffels GL, Salvatore L, Dosen M, Waddington J, Wilson L, Thompson C, Campbell N, Sexton J, Wicker J, Bowen F, et al. 1994. Vaccination of sheep with purified cysteine proteinases of fasciola hepatica decreases worm fecundity. Exp Parasitol. 78(2):132–148. doi:10.1006/expr.1994.1014. [accessed 2023 Jan 5]. https://pubmed.ncbi.nlm.nih.gov/8119370/.

Xu J, Wu L, Sun Y, Wei Y, Zheng L, Zhang J, Pang Z, Yang Y, Lu Y. 2020. Proteomics and bioinformatics analysis of Fasciola hepatica somatic proteome in different growth phases. Parasitol Res. 119(9):2850. doi:10.1007/S00436-020-06833-X. [accessed 2022 Dec 30]. /pmc/articles/PMC7403185/.

Yang J, Zhang Y. 2015. I-TASSER server: new development for protein structure and function predictions. Nucleic Acids Res. 43(W1):W174–W181. doi:10.1093/NAR/GKV342. [accessed 2023 Jan 5]. https://academic.oup.com/nar/article/43/W1/W174/2467872.

Yılmaz Çolak Ç. 2021. Computational Design of a Multi-epitope Vaccine Against Clostridium chauvoei: An Immunoinformatics Approach. Int J Pept Res Ther. 27(4). doi:10.1007/s10989-021-10279-9.

Young ND, Hall RS, Jex AR, Cantacessi C, Gasser RB. 2010. Elucidating the transcriptome of Fasciola hepatica - A key to fundamental and biotechnological discoveries for a neglected parasite. Biotechnol Adv. 28(2). doi:10.1016/j.biotechadv.2009.12.003.

Zafra R, Buffoni L, Pérez-Caballero R, Molina-Hernández V, Ruiz-Campillo MT, Pérez J, Martínez-Moreno Á, Martínez Moreno FJ. 2021. Efficacy of a multivalent vaccine against Fasciola hepatica infection in sheep. Vet Res. 52(1). doi:10.1186/s13567-021-00895-0.

Zafra R, Pérez-Écija RA, Buffoni L, Moreno P, Bautista MJ, Martínez-Moreno A, Mulcahy G, Dalton JP, Pérez J. 2013. Early and Late Peritoneal and Hepatic Changes in Goats Immunized with Recombinant Cathepsin L1 and Infected with Fasciola hepatica. J Comp Pathol. 148(4). doi:10.1016/j.jcpa.2012.08.007.

Zerna G, Rathinasamy VA, Toet H, Anderson G, Dempster R, Spithill TW, Beddoe T. 2021. Evaluation of immunogenicity and efficacy of fasciola hepatica tetraspanin 2 (Tsp2) fused to e. coli heat-labile enterotoxin b subunit ltb adjuvant following

intranasal vaccination of cattle. Vaccines (Basel). 9(11). doi:10.3390/vaccines9111213.

Zhang BY, Song ZY. 2021. Research progress on host cell immunity in Fasciola hepatica infection. Chinese Journal of Parasitology and Parasitic Diseases. 39(1). doi:10.12140/j.issn.1000-7423.2021.01.016.

Zhang L. 2017. Multi-epitope vaccines: a promising strategy against tumors and viral infections. Cellular & Molecular Immunology 2018 15:2. 15(2):182–184. doi:10.1038/cmi.2017.92. [accessed 2023 Jan 4]. https://www.nature.com/articles/cmi201792.

Zhu J, Paul WE. 2008. CD4 T cells: fates, functions, and faults. Blood. 112(5):1557–1569. doi:10.1182/BLOOD-2008-05-078154. [accessed 2023 Jan 5]. https://ashpublications.org/blood/article/112/5/1557/25394/CD4-T-cells-fates-functions-and-faults.

Andreyanov ON, Postevoy AN, Sidor EA. 2021. The effect of ambient temperature on biological properties and energy metabolism of Fasciola hepatica metacercariae. Vet Parasitol. 299. doi:10.1016/j.vetpar.2021.109576.