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Al-Quds University

First molecular detection and genotyping of *Neospora caninum* in naturally infected cattle and sheep in Palestine

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First molecular detection and genotyping of *Neospora caninum* in naturally infected cattle and sheep in Palestine

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Declaration

I certify that this thesis submitted for the degree of Master, is the result of my own research, except where otherwise acknowledged, and that this study or any part thereof has not been submitted for a higher degree to any other university or institution.

Signature:

Heba Suleiman Salem Alfarajeen

Date: 5 / 5 / 2024

Dedication

To my parents

To my brother and sisters

To my teachers

To my friends

To everyone who supported me during my graduate studies.

Heba ALfarajeen

Acknowledgments

First of all, I would like to thank my supervisors, Dr. Suheir Ereqat and Dr. Abedelmajeed Nasereddin, not only for accepting me as a student but for all their expertise, advice, and support along the way. Also, I would like to thank Dr. Amer Al-Jawabreh for providing samples and valuable information.

My sincere thanks extend to all the individuals who helped me conduct this study. Special thanks to my brother, who has supported me all the time.

Abstract

Neosporosis has become one of the most common diseases causing abortion in dairy cattle globally. The clinical signs of *Neospora caninum* have been reported in sheep, goats, deer, and horses. Fetal abortion induced by *N. caninum* is a common reproductive problem that causes significant economic loss in cattle and sheep husbandry. This study aimed to detect and identify *N. caninum* in intermediate hosts such as cattle and sheep from Palestine using polymerase chain reaction (PCR) followed by DNA Sanger sequencing and to study the genetic diversity of the study samples, targeting the MS10 microsatellite, using phylogenetic analysis. A total of 124 brain tissue samples were collected from 106 (85%) cattle and 18 (15%) sheep from a slaughterhouse in Jericho-Palestine. The PCR technique was used to identify the *Neospora* DNA in the brain samples based on the *Nc-5* gene. *N. caninum* was detected in the brain samples; out of 124 samples, 30 (24.19%) samples were positive for *N. caninum*. The frequency of *N. caninum* in cattle (25.47%) was higher than that of sheep (16.66%). The infection caused by *N. caninum* was confirmed by DNA Sanger sequencing of (13) random positive samples. BLAST analyses of the *Nc-5* gene revealed more than 92% to 99% matching with *N. caninum* sequences available in GenBank. Microsatellite MS10 repeats were used to study the genetic diversity of the Palestinian isolates. The microsatellite genotyping was done by next-generation sequencing (NGS). It was applied to 30 positive samples, and successful results were achieved in 15 samples. Our study sequences displayed 100% similarity with *N. caninum* isolated from dogs in Liverpool and 99.34% similarity with *N. caninum* isolated from cattle in Japan. The phylogenetic analysis of 15 *N. caninum* samples from Palestine and 83 microsatellite (MS10) repeats obtained from different countries and different hosts showed that *N. caninum* isolates are genetically diverse and distributed into two main clusters. Locally, the Palestinian isolates are distributed in two clusters and share the same repeats with samples from Asia and Europe. The present study provided the first estimate of the frequency of *N. caninum* in cattle and sheep from Palestine. Additionally, it was also the first time for the investigation of the phylogenetic analysis of *N. caninum* based on microsatellite markers.

العنوان : الكشف الجزيئي والتنميط الجيني الأول لنيوسبورا كانينوم في الابقار والاغنام المصابة طبيعيا في فلسطين.

اعداد : هبة سليمان الفراجين

بإشراف : د.سهير عريقات و د.عبد المجيد ناصر الدين.

الملخص

أصبح مرض النيوسبوروسيس (Neosporosis) أحد أكثر الأمراض شيوعًا التي تسبب الإجهاض في الأبقار على مستوى العالم. حيث تم الإبلاغ عن العلامات السريرية لنيوسبورا كانينوم في الأغنام والماعز والغزلان والخيول. ومن المشاكل الإنجابية الشائعة إجهاض الجنين الناجم عن نيوسبورا كانينوم الذي تسبب بخسارة اقتصادية كبيرة في تربية الماشية والأغنام. هدفت هذه الدراسة إلى الكشف وتشخيص نيوسبورا كانينوم في العوائل الوسيطة كالأبقار والأغنام من فلسطين باستخدام تفاعل البوليميراز المتسلسل (PCR) متبوعاً بتسلسل سانجر ودراسة التنوع الوراثي لعينات الدراسة باستخدام التحليل التطوري مستهدفاً الميكروساتليت (MS10) . أجريت هذه الدراسة على 124 عينة تم جمعها من أنسجة الدماغ من 106 (85%) من الأبقار و 18 (15%) من الأغنام من مسلخ أريحا-فلسطين. ثم تم استخدام تقنية البوليميراز المتسلسل للتعرف على الحمض النووي لنيوسبورا كانينوم في عينات الدماغ بناءً على الجين *Nc-5*. وجاءت نتائج هذه الدراسة كما يلي : من أصل 124 عينة، كانت 30 (24.19%) عينة إيجابية للنيوسبورا. واظهرت النتائج وجود مرض النيوسبوروسيس في الأبقار (25.47%) أعلى من الأغنام (16.66%). ثم تم تأكيد العدوى التي تسببها النيوسبورا من خلال تسلسل الحمض النووي لثلاثة عشر عينة إيجابية عشوائية، وكشفت النتائج لجين *Nc-5* عن أكثر من 92% إلى 99% من التطابق مع تسلسلات النيوسبورا كانينوم المتوفرة في بنك الجينات. وتم استخدام المايكروساتليت لدراسة التنوع الوراثي في العزلات الفلسطينية بواسطة تقنية تسلسل الجيل القادم لدراسة تسلسل الحمض النووي (NGS) .ومن ثم تم تطبيق التنميط الجيني للمايكروساتليت في 30 عينة إيجابية، وتم تحقيق نتيجة ناجحة في 15 عينة. أظهرت النتائج تشابهًا بنسبة 100% مع النيوسبورا كانينوم المعزولة من الكلاب في ليفربول وتشابهًا بنسبة 99.34% مع النيوسبورا كانينوم المعزولة من الماشية في اليابان. و أظهر التحليل التطوري لخمسة عشر عينة فلسطينية و 83 عينة من المايكروساتليت (MS10) التي تم الحصول عليها من بلدان مختلفة و مضيفين مختلفين أن عزلات النيوسبورا كانينوم متنوعة وراثيا

وتتوزع عالميًا إلى مجموعتين رئيسيتين. محلياً، توزعت العزلات الفلسطينية في مجموعتين مع عينات من آسيا وأوروبا. قدمت الدراسة الحالية التقدير الأول لوجود النيوسبورا كانينوم في الأبقار والأغنام من فلسطين. بالإضافة إلى ذلك، كانت أيضًا المرة الأولى التي يتم فيها دراسة التحليل التطوري للنيوسبورا كانينوم بناء على المايكروساتليت.

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List of Abbreviations

N. caninum : Neospora caninum

CNS *:* Central Nervous System

Am :amylopectin

Ar 1, 2, : apical rings 1 and 2

Co : conoid

Go: Golgi complex

Im: inner membrane

Mi : mitochondrion

Mn: microneme

No : nucleolus

Nu : nucleus

Pl : plasmalemma

Pr 1, 2 : polar rings 1 and 2

Sm : subpellicular microtubule

Tm : tubulovesicular membrane network

µm : Micrometre

IHC : Immunohistochemistry

IFAT : Indirect immunofluorescent antibody test

ELISA : Enzyme-linked immunosorbent assay

DNA : Deoxyribonucleic Acid

PCR : Polymerase Chain Reaction

rtPCR : Real-time PCR

ITS1 :Internal transcribed spacer 1

SSRs : simple sequence repeats

NGS : Next generation sequencing

MS : Microsatellite marker

µl: Microliter

Chapter One

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1. Introduction

Neospora caninum is an obligate intracellular parasite belonging to the phylum Apicomplexa and was first detected in 1984 in the central nervous system and skeletal muscle of dogs in Norway (Bjerkås *et. al.,* 1984). The parasite was isolated from tissue culture and named in 1988 (Dubey *et. al.*, 1988b).

Neosporosis is caused by *N. caninum*, a polysystemic parasitic disease with worldwide distribution (Dubey *et. al.,* 2003). It is a common pathogen of cattle and dogs, but it can also infect horses, goats, sheep, and deer (Dubey *et. al.,* 1988a). The most common sites of infection are the muscles and the central nervous system (Dubey & Lappin *et. al.,* 2006). *N. caninum* infection has significant clinical consequences, most commonly in the brain, the site where most *N. caninum* cysts are found (Dubey *et. al.,* 2007, 2004; Kobayashi *et. al.,* 2001). Affected animals usually develop non-suppurative encephalomyelitis and myositis (Dubey & Lappin *et. al.,* 2006).

In several mammalian species, particularly cattle, *N. caninum* is recognized as a causative agent of abortions, stillbirths, and the birth of weak newborns (Dubey *et. al.,* 2007). The outcome of infection in pregnant cattle might be an abortion of the fetus, the birth of a stillborn calf, a calf having neurological clinical symptoms, or a clinically healthy calf that is permanently infected (Buxton *et. al.,* 2002; Innes *et. al.,* 2002). In non-pregnant cattle, the *N. caninum* infection is latent and asymptomatic. A lot of factors are responsible for the infection outcome, such as *N. caninum* virulence, transmission routes, infection types (primary infections, recrudescence, and reinfections), immune competence, and the stage of pregnancy at which the dam is infected

(Innes *et. al.*, 2002). There are no clinical signs of neosporosis in cattle infected with *N. caninum* other than abortion. However, the mechanisms that cause abortion have not yet been fully elucidated (De Meerschman *et. al.,* 2002). In the absence of typical clinical signs, diagnosing and controlling the disease are difficult.

There are several species of canines that are definitive hosts of *N. caninum*, including domestic dogs (*Canis familiaris*), coyotes, dingoes, and gray wolves (Dubey *et. al.*, 2005). The main intermediate host for *N. caninum* is cattle, but the disease has also been detected in dogs, sheep, goats, and horses (Dubey *et. al.,* 1999; Dubey & Schares *et. al.,* 2011; Donahoe *et. al.,* 2015), antelopes (Peters *et. al.*, 2001), alpacas and llamas (Chávez-Velásquez *et. al.,* 2004; Wolf *et. al.*, 2005), and in one-humped camels (Hilali *et. al.,* 1998; Sadrebazzaz *et. al.*, 2006). Additionally, dogs can serve as both definitive and intermediate hosts at the same time (Dubey *et. al.,* 2007).

1.2 History

Neosporosis was first described as *a toxoplasma*-like protozoan by Bjerkas *et. al.* (1984). It was reported that dogs between 2 and 6 months of age showed neurological disorders, including encephalomyelitis and myositis. Furthermore, they found organisms similar to *Toxoplasma gondii* in lesions of the central nervous system and muscles, but no antibodies were found in the dogs (Bjerkas *et. al.*, 1984). There was a similar parasite that was found in 10 dogs in the USA in 1988, which was named *N. caninum* (Dubey *et. al.*, 1988a). In retrospective studies, *N. caninum* has been found in dogs that died in 1957 and 1958 in the United States (Dubey *et. al.*, 1990c). Bjerkas and Dubey compared parasite structures and antigenicity in fixed tissues from Norwegian and American dogs in 1992 and determined that the Norwegian parasite was *N. caninum*. Abortions and other symptoms associated with *Neospora* have been observed in cattle throughout

Europe, America, Asia, Australia, and Africa since 1988 (Obendorf *et. al.,* 1994; Trees *et. al.*, 1994; Boulton *et. al.*, 1995; Jardine and Wells *et. al.*, 1995; Yamane *et. al.*, 1996; Perez *et. al.*, 1998).

1.3 Prevalence of *N. caninum* Infection

1.3.1 Neosporosis in cattle

Worldwide, reports have been published on *Neospora* prevalence in cattle, including in Jordan, where the prevalence of antibodies against *N. caninum* in individual cattle herds was 66.5% (Talafha and Al-Majali *et. al.,* 2013). In Israeli dairy farms, antibodies against *N. caninum* were detected in more than 45% of dams, and 18% of abortions were linked to *N. caninum* infection, as determined by serological and molecular tests on aborted fetuses (Fish *et. al.,* 2007; Mazuz *et. al.,* 2011). Several studies reported the seroprevalence rate of neosporosis in cattle: it was 28.89% in Egypt (Selim *et. al.,* 2023). 19.64% in Algeria (Ghalmi *et. al.,* 2012), 16.57% in Canada (Paréet *et. al.,* 1998), 5.74% in Japan (Koiwai *et. al.,* 2006), 25.34% in Spain (Quintanilla-Gozalo *et. al.,* 1999), 14.88% in Australia (Stoessel *et. al.,* 2003), 12.61% in India (Sengupta *et. al.,* 2013), and 4.1% in Korea (Kim *et. al.,* 2002).

Neosporosis is globally distributed, and the reported prevalence rates range from 10.7 to 19.6% in Africa (Ghalmi *et. al.,* 2012; Ibrahim *et. al.,* 2012), 5.7–43% in Asia (Nazir *et. al.,* 2013; Koiwai *et. al.,* 2005), 7.6–76.9% in America (Sousa *et. al.,* 2012; Cedeño *et. al.,* 2013), and 0.5–27.9% in Europe (Imre *et. al.,* 2012; Bartels *et. al.,* 2006).

There is a significant variation in the prevalence of *N. caninum* infection across continents and countries worldwide (Diakoua *et. al.,* 2013; Nasir *et. al.,* 2012). There may be differences in seroprevalence in different regions due to different factors, including climatic conditions, nutritional and health management, and the use of different techniques in serological diagnosis (Dubey *et. al.,* 2020; Rodrigues *et. al.,* 2020).

1.3.2 Neosporosis in sheep and goats

In a meta-analysis study, the pooled prevalence of *N. caninum* in aborted fetuses of sheep and goats was estimated, by molecular methods, to be 15% and 7%, respectively. Additionally, *N. caninum* seroprevalence in sheep aborted fetuses was estimated to be 17% (Nayeri *et. al.,* 2022). A worldwide estimate of *N. caninum* seroprevalence in goats was 5.99% (Rodrigues *et. al.,* 2020). The overall prevalence of *N. caninum* infection in sheep that suffered an abortion was 3% (Nayeri *et. al.*, 2022).

1.3.3 Neosporosis in humans

There have been no reports of clinical neosporosis in humans (Namavari *et. al.,* 2020). Humans with serological positivity, primarily immunocompromised individuals (Namavari *et. al.,* 2020), and the detection of *N. caninum* DNA in two human umbilical cord blood samples have been recently reported (Duarte *et. al.,* 2020). *N. caninum* revealed strong biological similarity to the widespread zoonotic parasite *Toxoplasma gondii*, as well as the fact that rhesus monkeys have been experimentally infected, raises concerns that it could be zoonotic (Barr *et. al.,* 1994). In human cell lines, *N. caninum* parasites have been successfully cultured, but low antibody titers have been reported in serum samples with unconfirmed specificity against *N. caninum* (Dubey *et. al.,* 2017; Lobato *et. al.*, 2006; Robert-Gangneux *et. al.*, 2009).

1.4 Economic impact

Neosporosis can lead to significant economic loss in livestock production (Dubey *et. al.,* 2007). Even though, there is limited data on the exact loss expressed in US dollars, according to estimates, New Zealand beef suffered losses of US $1.1 million, Canadian dairy losses of US $17.1 million, and US dairy losses of US $546.3 million (Reichel *et. al.,* 2013). The global total median losses due to abortions caused by *N. caninum* are estimated at US$1.298 billion per year, with an expected loss of US$2.380 billion. There are about two-thirds of the estimated 1,298 million losses worldwide occurring in North America (US$ 852.4 million, or 65.7%), followed by South America (US$ 239.7 million, or 18.5%), and Australasia (Reichel *et. al.,* 2013).

1.5 Overview of the agent

1.5.1 The structure and morphology of *Neospora caninum*

Morphological studies of *N. caninum* by electron microscopy have shown that this parasite belongs to the family Sarcocystidae, subclass Coccidiasina, of the phylum Apicomplexa (Ellis *et. al.,* 1994). *N. caninum* shares many similarities with *T. gondii* (Barr *et. al.,* 1997). The infectious stages of *N. caninum* include tachyzoites, tissue cysts, and oocysts. The stages found in intermediate hosts are tachyzoites and tissue cysts, and they occur intracellularly (Dubey *et. al.,* 2002). According to the division stage, *N. caninum* can be found in the brain, the myocardium, the lungs, and the placenta of the host, but primarily in the central nervous system (Jardine and Wells *et. al.,* 1995; Dubey *et. al.,* 2003).

1.5.1.1 The tachyzoite stage

A tachyzoite is a rapidly dividing parasite stage. It has an ovoid, lunate, or globular shape approximately 6 x 2 µm in size and a single vesicular nucleus as shown in figure 1.1, and it has all the ultrastructural features of other cyst-forming coccidians that are closely related to it. Tachyzoites are found in the cytoplasm of the host cell, with or without parasitophorus vacuoles (Dubey *et. al.,* 1992). In tachyzoites, there are three layers of plasmalemma and 22 subpellicular microtubules, two apical rings, a conoid, a polar ring, one to three mitochondria, up to 150 micronemes, eight to twelve rhoptries anterior to the nucleus and four to six rhoptries posterior to the nucleus, a Golgi complex, a rough and smooth endoplasmic reticulum, a nucleus, and a nucleolus (Bjerkas and Prestus *et. al.,* 1989; Speer and Dubey *et. al.,* 1989; Dubey and Portfield *et. al.,* 1990; Barr *et. al.,* 1991; Conrad *et. al.,* 1993; Barr *et. al.,* 1997; Sonda *et. al.,* 2000).

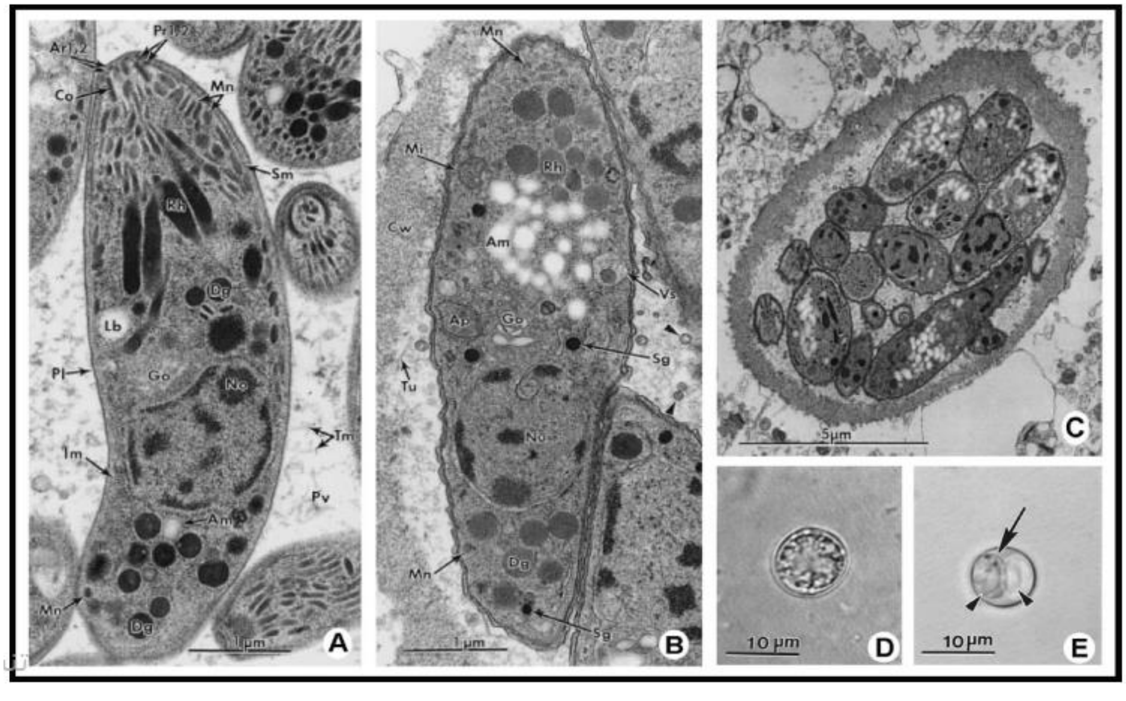

Figure 1.1. Ultrastructure of Neospora caninum: (A) tachyzoite, (B) bradyzoite, (C) tissue cyst, (D) unsporulated Oocyst, and (E) sporulated Oocyst with two sporocysts (arrowed) and

two sporozoites (arrow heads). Abbreviations: Am, amylopectin; Ar 1, 2, apical rings 1 and 2; Co, conoid; Go, Golgi complex; Im, inner membrane; Mi, mitochondrion; Mn, microneme; No, nucleolus; Nu, nucleus; Pl, plasmalemma; Pr 1, 2, polar rings 1 and 2; Sm, subpellicular microtubule; Tm, tubulovesicular membrane network. Source: (reproduced by Stephen et. al., 2013 from Speer et. al., 1999; McAllister et. al., 1998).

1.5.1.2 The tissue cyst stage

Several species are known to have tissue cysts affecting mainly neural tissues, such as the brain and spinal cord, that are usually round to oval in shape and can reach 107 µm long (Dubey *et. al.*, 1992; Wouda *et. al.,* 1997; Daft *et. al.,* 1997). A tissue cyst has a wall that can be up to 4 µm thick (see figure 1.1). There are branched tubule-like structures in tissue cysts (Bjerkas and Prestus *et. al.*, 1989). In addition, no secondary cyst wall exists, and no septa can be seen (Dubey *et. al.,*1992). In the cyst, the parasite forms a slender structure (6–8 x 1.1.8 µm) called a bradyzoite, which contains many of the same organelles as a tachyzoite but with fewer rhoptries. Moreover, tubular vesicular structures may contain micropores between bradyzoites (Bjerkas and Prestus *et. al.,* 1989; Bjerkas and Dubey *et. al.*, 1991).

1.5.1.3 The Oocyst stage

Cases of Oocyst shedding have been documented in Argentina (Basso *et. al.*, 2001) and the Czech Republic (Slapeta *et. al.*, 2002b) among naturally infected dogs. The sporulated Oocysts were thin, colorless, and generally ovoid or ellipsoidal in shape, measuring 10.6–12.4×10.5–12 µm, and had thin walls (0.6–0.8 µm), colorless, bilayered, smooth surfaces as shown in figure 1.1 Within three days of leaving the host, oocysts become sporulated and infective (Dubey *et. al.*, 2002). There are two sporocysts approximately 7.4–9.4×5.6–6.4 µm in size within each sporulated oocyst, each containing four sporozoites and a residuum (Dubey *et. al.,* 2002). The characteristics of the Oocyst of *N. caninum* resemble those of *Hammondia heydorni* Oocysts in dog feces as well as *T. gondii* and *H. hammondi* Oocysts in cat feces (Dubey *et. al.,* 2002).

1.5.2 Life-cycle and transmission

Neospora caninum is known for a complex facultative heteroxenous life cycle that includes a variety of intermediate hosts for asexual replication and a definitive canid host for sexual replication (Dubey and Lindsay *et. al.,* 1996; Dubey *et. al.,* 2006, 2007; Dubey and Schares *et. al.,* 2011). There are several species of canines that are definitive hosts of *N. caninum*, including domestic dogs (*Canis familiaris*), coyotes, dingoes, and gray wolves (Dubey *et. al.,* 2005). The main intermediate host for *N. caninum* is cattle, but the disease has also been detected in dogs, sheep, goats, and horses (Dubey *et. al.,* 1999; Dubey & Schares *et. al.,* 2011; Donahoe *et. al.,* 2015), antelopes (Peters *et. al.,* 2001), alpacas and llamas (Chávez-Velásquez *et. al.,* 2004; Wolf *et. al.,* 2005), and in one-humped camels (Hilali *et. al.,* 1998; Sadrebazzaz *et. al.,* 2006). In addition, dogs have the ability to serve as definitive and intermediate hosts at once.

(Dubey *et. al.,* 2007). The life cycle of *N. caninum* is characterized by three infectious stages: rapidly dividing tachyzoites, slowly growing bradyzoites within tissue cysts, and sporozoites within sporulated Oocysts (sporulated Oocysts present two sporocysts with four sporozoites each) (Dubey *et. al.,* 2006). *Neospora caninum* can be passed through the placenta (also known as vertical or congenitally transmitted) or postnatally (also known as horizontal transmission) (Trees *et. al.,* 2005). Oocysts can infect an intermediate host by entering through the transplacental route or by ingesting them through food and water; see figure 1.2 (Dubey *et. al.,* 2005, 2007). The intermediate host is the site of transformation for the Oocysts into tachyzoites, which subsequently become bradyzoites and form cysts in a variety of tissues, including the brain and muscles. After consuming neospora-tissue cysts, the definitive host creates Oocysts in its intestine that are expelled in its feces as the last stage of the life cycle (Dubey *et. al.*, 2003).

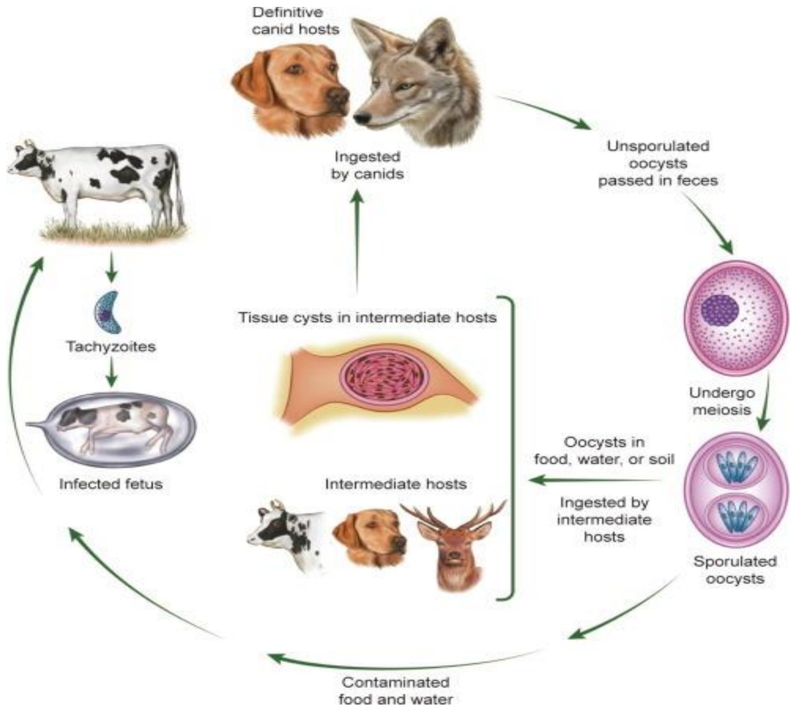

Figure 1.2. Life cycle of Neospora caninum. Source: Dubey & Schares *et. al.*, 2011.

1.6 Pathogenesis of *N. caninum*

Ingestion of sporulated oocysts may result in host infection.Sporozoites were released by excystation in the gastrointestinal tract and parasitize the intestine, where they convert into tachyzoites (Hemphill *et. al.,* 2006). The pathogenesis of neosporosis is determined by a balance between the tachyzoite's ability to penetrate and multiply inside cells and the host's ability to inhibit parasite proliferation (Buxton *et. al.,* 2002). Tachyzoites can infect many different types of nucleated host cells, such as mononuclear cells, which most likely contribute to the spread of the parasite by leukocyte trafficking (Dubey *et. al.,* 2006; Hemphill *et. al.,* 2006). Tachyzoites live and multiply inside host cells in an intracellular space called a parasitophorous vacuole, which is created when the host cell membrane is altered by the parasite to prevent it from fusing with endocytic vesicles (Buxton *et. al.,* 2002; Hemphill *et. al.,* 2006). Tachyzoites can be present in almost every host tissue during the acute stage of infection. Furthermore, lesion formation and, in certain cases, clinical disease are triggered during this phase by a series of progressive cycles of intracellular replication, lysis of infected host cells, release of tachyzoites, infection of neighboring cells, and associated immunopathologic aftereffects (Dubey *et. al.,* 2007). Tachyzoites divide for about 20 divisions in an immunocompetent host before differentiating into bradyzoites, the parasite's quiescent life stage that forms under host immune pressure and results in a tissue cyst (Goodswen *et. al.,* 2013). Bradyzoites are protected from host immune responses by tissue cysts, which also promote long-term parasite persistence and chronic infections without symptoms (Hemphill *et. al.,* 2006; Dubey *et. al.,* 2007; Dubey and Schares *et. al.,* 2011). Reactivation of the infection and transformation of bradyzoites into tachyzoites can occur when the host immune status changes (immunomodulation or immunosuppression) (Hemphill *et. al.,* 2006). This is known to happen in pregnant animals and allows tachyzoites to spread to other tissues, such as the placenta and the developing fetus (Williams *et. al.,* 2009).

1.7 Clinical features

In intermediate hosts, abortions are the initial clinical symptom. The animals that are congenitally infected and born alive frequently show no outward symptoms of their illness. In other instances, they might have had a reduced birth weight, not gained any weight, constricted or overly stretched limbs, and neurological symptoms like ataxia, a diminished patellar reflex, and disorientation (Dubey *et. al.,* 1999).

The most common indicator of neosporosis in dogs and, most likely, other hosts is severe neuromuscular dysfunction (Dubey *et. al.,* 1992). When neosporosis occurs naturally, dogs that are infected have the distinctive pattern of progressive paralysis and inflexible hind limb hyperextension. Compared to the front legs, the hind limbs suffer more damage (Dubey and Lindsay *et. al.,* 1993; Barber and Trees *et. al.,* 1996). Additional dysfunctions include trouble swallowing, jaw paralysis, muscle atrophy, flaccidity, head tremors, forelimb ataxia, and even heart failure from myocarditis (Dubey and Lindsay *et. al.*, 1993; Barber and Trees *et. al.,* 1996).

1.8 Prevention and control of Neosporosis

The main objective in *N. caninum*-free herds is to prevent the illness from spreading by using basic biosecurity precautions (Haddad *et. al.,* 2005). Control programs for *N. caninum*-infected herds focus on reducing the risk of horizontal transmission of the infection by primarily managing the definitive host population as a source of oocyst contamination and reducing the vertical transmission in a herd by reducing the number of seropositive cattle (Conraths *et. al.,* 2005; Haddad *et. al.,* 2005; Hall *et. al.,* 2005; Larson *et. al.,* 2004; Reichel and Elliset. al., 2002).

Unfortunately, there are currently neither vaccines nor treatments to prevent bovine neosporosis (Dubey *et. al.,* 2007). In the case of bovine neosporosis, a number of steps are suggested to prevent the entrance of infected animals into farms that are either free or infected and to prevent or decrease the probability of horizontal and vertical transmission in those with the presence of *N. caninum*-infected cattle. Various strategies have been proposed at the farm biosecurity level, including: (1) testing newly purchased and replacement cattle; (2) preventing transmission from definitive hosts; (3) managing rodent populations; and (4) preventing any immunosuppressive factors like virus infection, mycotoxin ingestion, or stress that could trigger the reactivation of chronic infections and vertical transmission.

1.9 Diagnosis of *N. caninum* infection

The initial years following the identification of *N. caninum* were spent diagnosing the disease based on histology and clinical indications that described lesions in the fetus's muscles, central nervous system, and other organs. *N. caninum* and *T. gondii* were misdiagnosed with this type of diagnosis because of their striking structural similarities (Dubey *et. al.,* 1992; Dubey and Lindsay *et. al.,* 1993). Molecular biology, serology, and immunohistochemistry have therefore played a significant role in providing targeted tests to accurately, quickly, and sensitively identify those animals that have Neosporosis.

1.9.1 Histopathology and immunohistochemistry (IHC)

Histopathology has a low sensitivity for detecting organisms when compared to other available diagnostic tests because parasite life stages are not always present or easily observed in tissue sections (Dubey and Schares *et. al.,* 2006). However, histopathology remains an extremely valuable diagnostic tool that is critical for improving our understanding of infection outcomes in wildlife species. It makes it possible to describe lesions, analyze tissue distribution, and identify concurrent pathology with intralesional parasites, thereby establishing a link between the presence of parasites and illness. Histopathology should be performed in conjunction with additional confirmatory diagnostic tests, as a light microscopic examination of tissue alone is not sufficient to make a definitive distinction between the various tissue-cyst-forming coccidia. By detecting parasite-specific antigens, IHC with *N. caninum*-specific antibodies can be helpful in diagnosis (Dubey and Schares *et. al.,* 2006). For the IHC diagnosis of neosporosis, polyclonal antibodies are generally recommended over monoclonal antibodies due to their increased sensitivity in detecting the presence of the *N. caninum* antigen (Dubey and Schares *et. al.,* 2006; Dubey *et. al.,* 2007).

1.9.2 Serology

Neospora stimulates the host's generation of antibodies. Therefore, identifying seropositive animals may benefit from a serological test. A positive antibody test suggests a high possibility of infection and parasite exposure. To confirm a definitive diagnosis, histological analyses of tissues from aborted fetuses may be performed. The indirect immunofluorescent antibody test (IFAT), the enzyme-linked immunosorbent assay (ELISA), and immunoblot have been the primary serological tests used in studies for *Neospora* diagnosis; however, because of their relative affordability, IFAT and ELISA are the most often used tests. The ELISAs has been utilized in the majority of studies due to their high sensitivity and specificity (Paré *et. al.,* 1995; Baszler *et. al.,* 1996; Thurmond and Hietala *et. al.,* 1996; Thurmond *et. al.,* 1997; Jenkins *et. al.,* 1997; Osawa *et. al.,* 1998; Romero *et. al.,* 2002).

1.9.3 Molecular biology tests

1.9.3.1 Polymerase chain reaction (PCR) test

Neospora DNA has been identified in cattle tissues or sera since 1993 using the polymerase chain reaction (PCR), which is particularly useful in differentiating this parasite from closely related species such as *Toxoplasma* (Brindley *et. al.,* 1993). Nucleic acid detection by PCR is a very useful tool for the detection of *N. caninum* in bovine aborted fetuses since even small amounts of parasite DNA can be amplified to detectable levels even from poor-quality tissue. The conventional PCR assay can confirm the presence of ≥3 copies of the genome with high reliability (Baszler *et. al.,* 1999). Real-time PCR (rtPCR) assays using amplicon-specific probes are highly sensitive and precise for the rapid and accurate detection of pathogens in clinical samples that frequently contain infectious agents in low quantities. Within the *N. caninum* genome, there are approximately 7121 protein-coding genes distributed across 14 chromosomes (Reid *et. al.,* 2012). Internal transcribed spacer 1 (*ITS1*) of rRNA and the *Nc5* gene are common targets of molecular assays. In the DNA genome, the *Nc5* region is a repeating sequence that has been shown to be highly specific for *N. caninum* (Kaufmann *et. al.,* 1996). In addition, this region shows a ≥95% identity across the 105 available sequences of *N. caninum*, making it an attractive target for novel molecular diagnostics. Novel, sensitive, and specific tests are desperately needed to detect the presence of *N. caninum*, as there is currently no vaccine or effective treatment for the disease.

1.9.3.2 Microsatellite analysis

Microsatellites, also known as simple sequence repeats (SSRs), are widely dispersed tandemly repeated segments of 1-6 base pair (bp) length short nucleotide motives found in the genomes of both prokaryotic and eukaryotic animals. They are present in both non-coding and protein-coding areas (To´th *et. al.,* 2000). There are high levels of polymorphism in microsatellite loci, and they mutate at rates higher than other DNA regions as a result of allelic repeat length. Microsatellites with high polymorphism are used as molecular markers. Microsatellite loci length polymorphisms can be investigated after PCR amplification with primers that anneal to the flanking regions using PAGE, MetaPhor agarose gel electrophoresis, or DNA sequencing (Ochsenreither *et. al.,* 2006; Scho¨nian *et. al.,* 2008). By analyzing length polymorphisms in regions containing microsatellite, distinct genetic patterns have been identified for various strains of *N. caninum*, and this technique is presently regarded as the gold standard for assessing *N. caninum* genetic diversity (Al-Qassab *et. al.,* 2010; Goodswen *et. al.,* 2013). The microsatellite-containing region MS10 is a very polymorphic locus that is extremely helpful for identifying divergence among different *N. caninum* strains and has shown great discriminatory power in identifying novel alleles (Basso *et. al.,* 2009; Al-Qassab *et. al.,* 2010). Currently, the majority of *N. caninum* in wildlife has focused on identifying parasite DNA in tissue, and little research has been conducted to further characterize the parasite. Molecular genotyping techniques based on mini- and microsatellite technologies will be useful for epidemiological studies involving wildlife species and emerging new isolates (Al-Qassab *et. al.,* 2010; Goodswen *et. al.,* 2013; Regidor-Cerrillo *et. al.,* 2013).

1.9.3.3 Next generation sequencing (NGS)

The next-generation sequencing (NGS) technique is an emerging high-throughput diagnostic method that amplifies a conservative DNA region found in all eukaryotic species and then sequences both short strands of host DNA and microbial DNA (Sun *et. al.,* 2011; Fournier *et. al.,* 2013). The NGS technology has developed rapidly in recent years and has become an important analytical tool used in many research studies, which include investigations of human infectious diseases and assessments of microbial community composition (Sun *et. al.*, 2011; Fournier *et. al.*, 2013; Slapeta and Linares *et. al.*, 2013). This technology offers advantages over other diagnostic tests, such as the ability to identify co-infections with other pathogens and the capacity to discriminate between genotyping and molecular epidemiologic analysis (Fournier *et. al.*, 2013; Slapeta and Linares *et. al.*, 2013).

1.10 Study Objectives

To our knowledge there are no studies have been conducted on *N. caninum* in Palestine yet. This study was the first one to study *N. caninum* infection in intermediate hosts using molecular methods.

The main aim of this study: is to detect and identify *N. caninum* in intermediate hosts as cattle and sheep from Palestine using polymerase chain reaction (PCR) followed by DNA Sanger sequencing .

Study Objectives

- To screen neosporosis infection in intermediate hosts (cattle and sheep) during the year 2023.
- To study the genetic diversity of the study samples using phylogenetic analysis.

1.11 Literature review

1.11.1 Molecular detection of *Neospora caninum*

PCR-based techniques have been developed to improve the sensitivity and specificity of parasite detection in low-level infections, focusing on the *ITS1* region and the *Nc5* sequence (Collantes-Fernández *et. al.,* 2002). The detection of *N. caninum* DNA allows for studying the genetic diversity and phylogenetic relationships of *N. caninum* (Amdouni *et. al.,* 2018; Li *et. al.,* 2014; Nardoni *et. al.,* 2019; Rocchigiani *et. al.,* 2017). Pathogenicity (virulence) and growth rates vary significantly among *N. caninum* isolates (Atkinson *et. al.,* 1999; Quinn *et. al.,* 2002). As a result, the biological and genetic variety of *N. caninum* isolates may have an impact on the pathogenesis of neosporosis in both cattle and dogs. However, there is limited information available about the genetic diversity of *N. caninum* isolates from different geographical regions. DNA sequencing was used to detect genetic diversity among *N. caninum* isolates, as well as the examination of inverted repetitive DNA and microsatellite repetitive DNA (Atkinson *et. al.,* 1999; Schock *et. al.,* 2001; Gondim *et. al.,* 2004; Regidor-Cerrillo *et. al.,* 2006).

Salehi *et. al.,* (2021) detected the DNA of *Neospora caninum* from brain tissue samples of aborted fetuses of cattle and sheep by a nested-PCR assay based on the *Nc-5* gene . 20.5% of cattle and 15.6% of sheep were positive for *Neospora caninum*. In cattle, neosporosis prevalence was significantly higher than in sheep.

Neospora caninum has been detected in naturally infected cattle and sheep in a study conducted in North Africa. A total of 348 samples were tested for the presence of the *N. caninum ITS1* gene using PCR. The overall molecular infection prevalence of *N. caninum* was significantly higher in cattle than in sheep (22 and 10.6%, respectively) (Amdouni *et, al.,* 2018).

Paula *et. al.,* (2004) reported a 31.3% (10/32) positive rate for *N. caninum* in the brain of aborted fetuses using PCR primers targeting the *Nc5* gene.

1.11.2 Genetic diversity among *Neospora caninum* isolates

Regidor-Cerrillo *et. al.,* (2006) first found and analyzed 12 microsatellites in nine *N. caninum* isolates from various hosts and geographical locations. Multi-locus microsatellite analysis revealed distinct profiles for each isolate, and 11 of the 12 markers were then applied to clinical samples, resulting in the identification of novel alleles (Pedraza-Diaz *et. al.,* 2009).

A large-scale genotyping investigation assessed nine microsatellite markers for a total of 108 reference and clinical *N. caninum* isolates. Based on data collected from four countries across two continents over a ten-year period (Regidor-Cerrillo *et. al.,* 2013). A total of 96 microsatellite multilocus genotypes from seven loci demonstrated a high amount of genetic variation among the samples. Data analysis revealed that these microsatellite markers were partially associated with geographical origin, with sub-structures found in each country population of samples. Abortion was also linked to genetically different clustering of microsatellite genotypes, and the study's findings revealed that microsatellite markers are propagated clonally in Spanish *N. caninum* isolates in cattle.

Basso *et. al.,* (2010) used ten microsatellite markers to characterize DNA collected from the brains of 18 *N. caninum*-infected foetuses during pandemic abortion storms. All foetuses from each abortion outbreak shared the same microsatellite pattern, which was unique to each herd.

These findings, together with avidity analysis, gave evidence that infection came from a single source, implying that horizontal transmission was a factor in epidemic abortions.

Cabrera *et. al.,* (2019) discovered that four isolated Uruguayan strains of *N. caninum* represented three separate genetic lineages using microsatellite typing. Phylogenetic analysis showed that three of these four isolates were closely related to strains from regional Argentina and Brazil. The fact that the last Uruguayan strain grouped with an unconnected cluster suggested that the local strains had various origins.

Regidor-Cerrillo *et. al.,* (2020) reported a significant level of diversity among Italian *N. caninum* isolates using microsatellite genotyping analysis. The high genetic variety discovered in Northern Italy was consistent with what has previously been documented in other *N. caninum* country populations (Regidor-Cerrillo *et. al.,* 2013). The study's findings show that *N. caninum* has a high genetic diversity and has been involved in bovine reproductive failure, as well as the existence of various *N. caninum* subpopulations that may spread throughout Northern Italy via vertical and horizontal transmission. Genetic differences between Italian *N. caninum* subpopulations and other American and European populations show that *N. caninum* is unique to Northern Italy and various subpopulations circulating in Europe.

Chapter Two

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2. Materials and Methods

2.1 Study area and sample collection

A cross-sectional study was conducted between January and March 2023. A total of 124 brain tissue samples were collected by a veterinarian in sterile containers from 106 cattle and 18 sheep slaughtered for food in the regional slaughterhouse of Jericho, then stored at -20°C until the analysis. Jericho District (A'riha) is a Palestinian city in the West Bank. It is located in the Jordan Valley, with the Jordan River to the east and Jerusalem to the west. The total population of Jericho was 22,609 (Palestinian Central Bureau of Statistics, 2015a). Jericho is located 273m below sea level and receives an average yearly rainfall of 133mm. The average yearly humidity is approximately 49.3%, and the average annual temperature is 24 °C (Applied Research Institute Jerusalem, 2011c). The overall area of the Jericho District is around 593 km2, and it has the lowest population density of all Palestinian districts, with 71 people per km2 (Palestinian Central Bureau of Statistics, 2009, 2015a).

Research ethics subcommittee of the faculty of medicine at Al-Quds University approved the study protocol under reference number Ref#: R2-23-22

2.2 Study procedure

2.2.1 DNA extraction

Genomic DNA was extracted from brain tissue samples using the Nucleospin Tissue DNA Extraction Kit (Macherey-Nagel), following the manufacturer's instructions. In brief, tissue samples were prepared by cutting 25 mg of animal tissue into small pieces using surgical blades, then 180 µl of Buffer T1 and 25 µl of proteinase K were added for lyse tissues samples and digest the proteins respectively, followed by vertexing. The samples were then incubated overnight at 56°C. After vertexing the samples, 200 µl of Buffer B3 was added and incubated at 70°C for 10 minutes. Then, 210 µl of ethanol was added and mixed by pipetting to adjust DNA binding conditions. After that, one NucleoSpin® tissue column was placed in a collection tube, and samples were loaded. The samples were then centrifuged at 11,000 x g for 2 minutes. The column was placed into a new 2 ml collecting tube, and the collection tube with the flow-through was thrown away. The silica membranes were washed with 500 µl of buffer BW and centrifuged for two minutes at 11000 rpm. After discarding the flow-through, the column was then put back into the collecting tube, filled with 600 µl of buffer B5, and centrifuged for two minutes at 11000 rpm. The column was returned to the collecting tube, the flow-through was discarded once more, and the centrifuge was run for three minutes at 11000 rpm. After that, the column was placed into a sterile 1.5-ml microcentrifuge tube, and 60 µl of preheated elution buffer (BE) was added to elute genomic DNA. The tube was then incubated for one minute at room temperature and centrifuged for three minutes at 11000 rpm. Finally, the nanodrop spectrophotometer 1000 (ND-1000) was used to quantify and assess the purity of nucleic acid.

2.2.2 Screening of *N. caninum* DNA

For the diagnosis of *N. caninum*, a 337 bp fragment sequence of the *Nc-5* gene was selected for DNA amplification (Müller *et. al.,* 1996). It has been demonstrated that the *Nc5* gene is extremely specific for *N. caninum* (Kaufmann *et. al.,* 1996). Two previously published specific primers were used to target the *Nc-5* gene: forward primer NP6+ (5′-CTCGCCAGTCAACCTACGTCTTCT-3′) and reverse primer NP21+ (5′CCCAGTGCGTCCAATCCTGTAAC-3′).

Briefly, we used 5µl of the extracted DNA in a final volume of 25µl, which contained 12.5µl of PCRBIO HS Taq Mix Red (PCR Biosystems, Ltd.), 6.5µl of nuclease-free water, and 1µl of each primer. The amplification conditions were as follows: First, the denaturation step was carried out for four minutes at 94 °C, followed by 35 cycles of 94 °C for 30 seconds, 60 °C for 40 seconds, and 72 °C for 40 seconds, followed by a final extension step at 72 °C for 10 minutes. For each PCR run, a negative control was included. The product was loaded on a 2% agarose gel.

2.2.3 Gel electrophoresis

To load the PCR product, 2% was prepared as followed, a mixture of approximately 2 grams of agarose was boiled for 30 seconds with 100 ml of Tris-acetate-EDTA (TAE) buffer, and 3 drops (approximately 50 µl per drop) of ethidium bromide were added to stain the DNA. After that, the mixture was poured into an agarose gel casting system (Bio-Rad, SUBCELL®GT). In each well, 5 μl of each PCR product were loaded. Samples were then run at 120 volts for 40 minutes. PCR products were visualized by using the gel documentation software (GelDoc).

2.2.4 Sanger sequencing

PCR positive samples were randomly selected for sanger sequencing to confirm specificity. PCR products from the positive samples were sent to Macrogen company for purification and sequencing. Sequencing was done using one primer. The obtained nucleotide sequences were blasted against available nucleotide sequences deposited in the GenBank database using BLAST (Altschul *et. al.*,1990).

2.2.5 *Neospora caninum* genotyping and DNA library preparation

Two PCR steps of library preparation were done for *Neospora* genotyping. The first one included the amplification of microsatellite-containing regions from PCR-positive samples, and the second PCR was applied for barcoding the samples using the Nextera index kit (Illumina).

2.2.5.1 First PCR for amplification of microsatellite

Neospora caninum genotyping was done on PCR-positive samples targeting the microsatellite marker (MS10). This marker contains three different repeat numbers (n), as follows: (ACT)n-(AGA)n-(TGA)n. The reference microsatellite MS10 sequence of *N. caninum* isolate from GenBank sequences from Japan (accession number: MT782059.1), as shown in figure 2.1 and table 2.1, was used to design the PCR primers. MS10 microsatellite primers were designed using Primer 3 software on Biowulf (nih.gov). The designed primers were modified with over hanged Illumina adapter sequences at the 5′ ends of the forward (5′-CGTCGGCAGCGTCAGATGTGTATAAGAGACA-3′) and reverse primers

(5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-3′) for attachment with the machine flow cell. The first PCR was performed as described by (https://emea.support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html). In brief, the total reaction was 25 µl containing 12.5 µl of PCR master mix (PCRBIO HS Taq Mix Red), 0.4 µM of each primer, 8.5 µl dH2O, and 3 µl of template DNA. The reaction conditions were as follows: 1 cycle of 94 °C for 2 minutes; 35 cycles of 95 °C for 15 seconds; 55 °C for 15 seconds; 72 °C for 30 seconds; and a final 72 °C for 2 minutes. Both positive and negative controls were included in every batch of amplifications. 5µl of the obtained PCR products were separated by 2% agarose gel electrophoresis and visualized under UV light.

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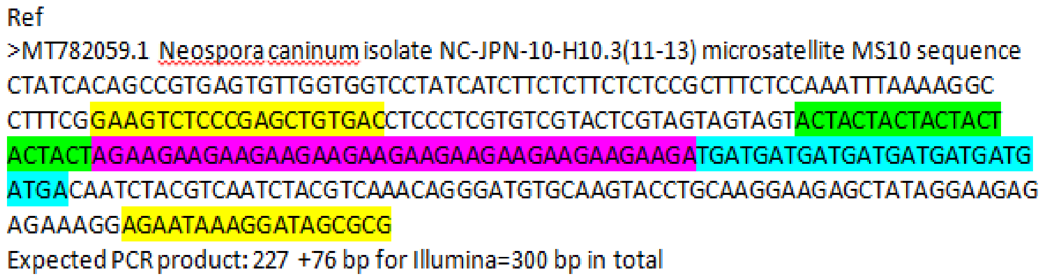

Ref
>MT782059.1 Neospora caninum isolate NC-JPN-10-H10.3(11-13) microsatellite MS10 sequence
CTATCACAGCCGTGAGTGTTGGTGGTCCTATCATCTTCTCTTCTCTCCGCTTTCTCCAAATTTAAAAGGC
CTTTCGGAAGTCTCCCGAGCTGTGACCTCCCTCGTGTCGTACTCGTAGTAGTAGTACTACTACTACT
ACTACTAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGATGATGATGATGATGATGATGATG
ATGACAATCTACGTCAATCTACGTCAAACAGGGATGTGCAAGTACCTGCAAGGAAGAGCTATAGGAAGAG
AGAAAGGAGAATAAAGGATAGCGCG
Expected PCR product: 227 +76 bp for Illumina=300 bp in total

Figure 2.1. The designed primers and PCR target regions for microsatellite marker (MS10). Primers sequences are in yellow, the microsatellite repeats are in green (ACT), purple (AGA) and blue (TGA).

Table 2.1. The primer sequences used in PCR for the amplification of microsatellite

Primer name	Primer sequence (5′-3′)	Target
F2_NEO_Mic10	TCG TCG GCA GCG TCA GAT GTG TAT AAGAGACAG GAAGTCTCCCGAGCTGTGAC	microsatellite marker(MS10)
R_NEO_Mic10	GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G CG CGCTATCCTTTATTCT	

F, forward; R, reverse

2.2.5.2 Cleaning of PCR product

25 µl of PCR products were cleaned with 1X AMPureXP beads (Beckman Coulter, Brea, CA, USA) and eluted by 25 µl elution buffer following company protocol. The samples were purified by adding 50 µl of AMPure XP beads and 30 µl of dH20 to 20 µl of each sample and incubated for 10 minutes at room temperature. Samples were set on a magnetic plate and allowed to become clear. After that, the supernatant was discarded. The attached pellets to the magnetic plate were then rinsed twice with 80% ethanol (180 µl) and allowed to dry for 10 minutes. The final step was to incubate each sample for five minutes at room temperature after adding 25 µl of elution buffer (EB). Following that, 25 µl of the supernatant was transferred to new collection tubes and stored frozen at -20 °C until used again.

2.2.5.3 The second PCR for barcoding samples

The purified PCR (7.5 ul) was subjected to a second round of amplification for barcoding using the Nextera XT Index Kit (Illumina). In brief, the purified products were applied to a second round of amplification for barcoding using specific unique combination indexes (2 indexes for each sample) sequences complementary to the oligos in the flow cell to allow for the index PCR products (libraries) to bind to the Illumina flow cell for sequencing. In brief, 7.5 µl of purified products were added to 12.5 µl of PCR master mix (PCRBIO HS Taq Mix Red), and 2.5 µl of each index was added to the mixture. The PCR conditions for barcoding were: 3 minutes at 72°C, 30 seconds at 95°C, followed by 10 cycles of 95°C for 10 seconds, 30 seconds at 55°C, 30 seconds at 72°C, and 5 minutes at 72°C. Finally, from each PCR product, 5 µl was collected from 30 tubes into a single pool tube, and X1 AMPure XP beads were used to apply PCR purification. 100 µl of the pool were added to 100 µl of AMPure XP beads and finally eluted with 25 µl of elution buffer. After that, samples were sent for deep sequencing by the NextSeq 500 machine (Illumina, San Diego, CA, USA), targeting 100,000 reads per sample.

2.2.6 Bioinformatics analysis

DNA sequences were received from the sequencing company (Macrogen) in FASTQ format files. The online Galaxy software (Galaxy Version 0.7.17.1) (https://usegalaxy.eu/) was used to analyze FASTQ files. Initially, we built a workflow to utilize the same instructions for all sequences in the study. Figure 2.2 shows the workflow and the different steps that were used to analyze the obtained sequences. The first command was input data set collection; the FASTQ files were uploaded, and the reads were extracted in FASTQ format. According to the Illumina Nextseq machine sequencing error rate, the highest quality reads (reads above 100 bp) and a quality score of >30 indicate an error rate of 1 in 1000, with a corresponding call accuracy of

99%. The next step was to convert the FASTQ files into tabular, and then we selected a specific sequence related to *Neospora* that was used to exclude all the artifacts. The filtered data were based on the *Neospora* microsatellite MS10-specific primers, and the number of sequences read for each sample was determined. Finally, the files were converted to the FASTA format. After that, the top sequences (with the highest read count) were gathered and uploaded to Microsoft Word software (https://www.microsoft.com). Then the find command was used to count the number of microsatellite MS10 repeats.

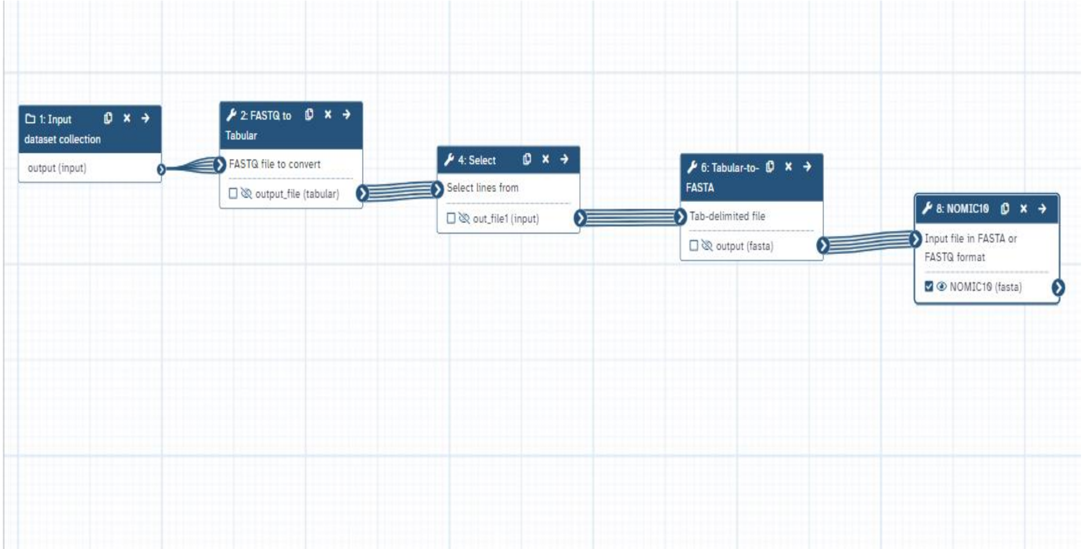

Figure 2.2. The outlined workflow for sequence analysis as shown in Galaxy software.

2.2.7 Phylogenetic analysis

The genetic diversity and phylogenetic analysis of the study samples were based on the **MS10** microsatellite repeats. The study included the microsatellite genotyping data of the Palestinian samples and the microsatellite genotyping database from different countries that was obtained from previous published articles. The phylogenetic tree was constructed by DARwin 6 program (https://darwin.cirad.fr/) according to the weighted neighbor joining method based on a simple matching dissimilarity matrix with bootstrapping analysis of 1000 replicates.

Chapter Three

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3. Results

3.1 Study population

The brain tissue samples, including 106 (85%) cattle and 18 (15%) sheep, were surveyed by PCR for the detection of *N. caninum*; out of 124 brain samples, 30 samples (24.19%) were positive in this regard. As shown in table 3.1, the frequency of *N. caninum* in cattle (25.47%) was higher than that of sheep (16.66%).

Table 3.1. *Neospora caninum* infection frequency in cattle and sheep as detected by PCR.

Type of animals	Samples (n)	Origin	Positive N	(%)	negative n	(%)
Cattle	106	Palestine(Jericho)	27	25.47%	79	74.52%
Sheep	18	Palestine(Jericho)	3	16.66%	15	83.33%
Total	124		30		94	

3.2 Nanodrop measurements of DNA extractions

The purity of the DNA obtained was determined through two ratios of 260/280 nm and 260/230 nm absorbance measures using the NanoDrop spectrophotometer 1000 (ND-1000). Table 3.2 represents the concentrations of random samples in the study.

Table 3.2. DNA concentrations by Nanodrop spectrophotometer.

Sample ID	Concentration (ng/ul)	260/280	260/230
1	77.13	1.89	2.24
2	278.18	1.98	2.38
5	42.08	1.93	1.61
7	243.22	1.96	2.15
60	35.55	1.85	1.72
72	61.89	1.93	2.06
89	188.49	1.92	2.49
102	63.10	1.91	1.69

3.3 Amplification of *Nc-5* gene and gel electrophoresis

The PCR findings showed one band with a size of 337 bp targeting the *Nc-5* gene of *Neospora caninum*. Figure 3.1 shows the agarose gel electrophoresis of representative samples to visualize the PCR-positive samples.

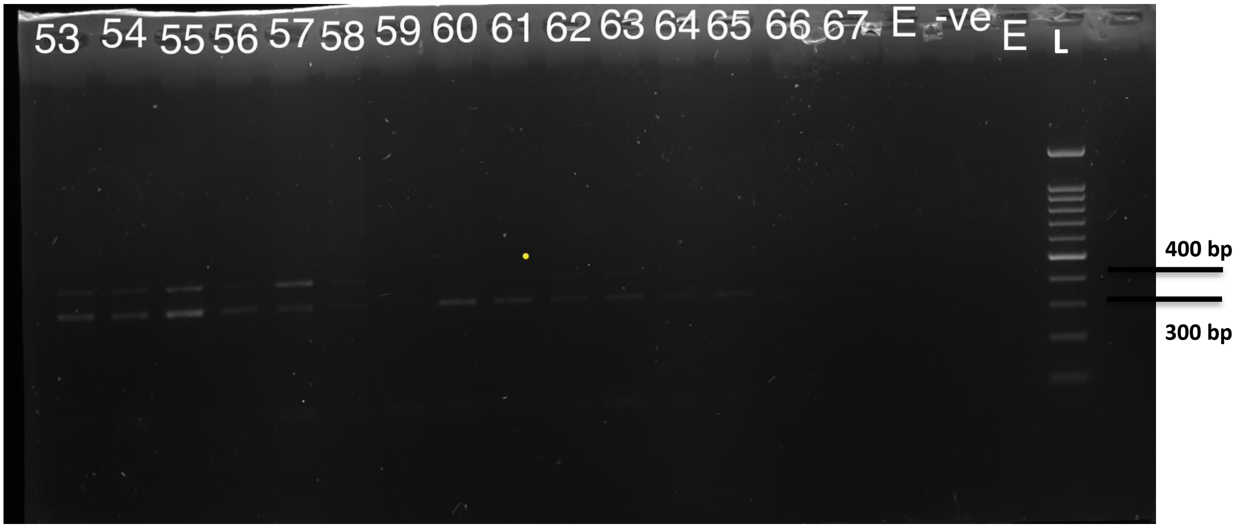
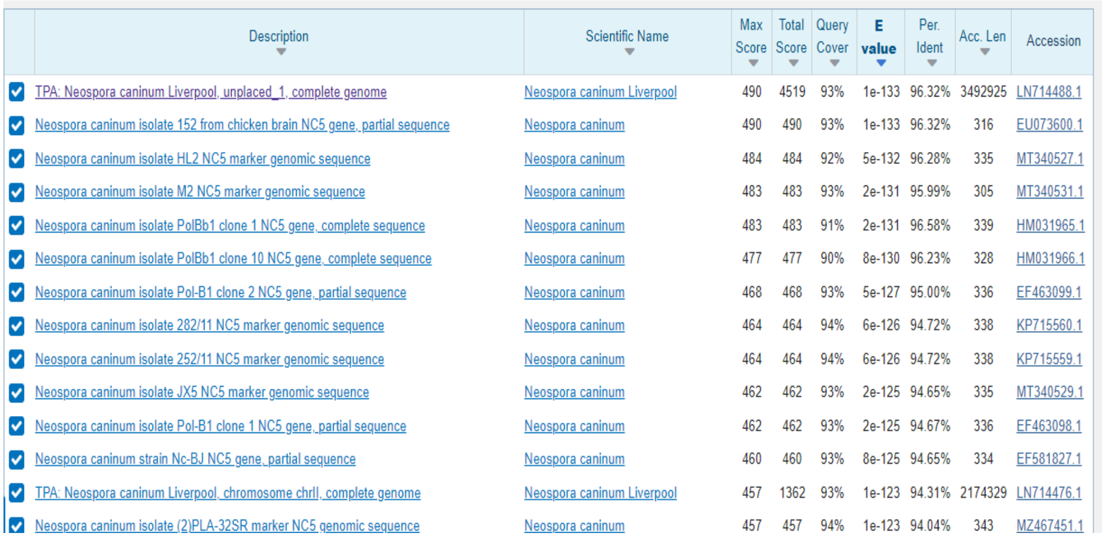


Figure 3.1. Agarose gel electrophoresis of *Nc-5* gene of representative samples isolates from cattle and sheep. Lane L refers to the DNA ladder (100 bp); 53, 54,55,56,57,58,60,61,62,63,64,65,: positive PCR products; 59,66,67 : negative PCR products; -VE: negative control ; E:empty.

3.4 Sanger sequencing

For Sanger sequencing, 13 positive samples were randomly selected, the sequencing results are shown in Appendix 1. BLAST analyses of the *Nc-5* gene showed more than 92% to 99% matching with *N. caninum* sequences available in GenBank. For example, sample number 60 isolated from cattle showed 96% similarity with *N. caninum* Liverpool (GenBank: LN714488.1), as shown in figure 3.2.



	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	TPA: Neospora caninum Liverpool, unplaced_1, complete genome	Neospora caninum Liverpool	490	4519	93%	1e-133	96.32%	3492925	LN714488.1
☑	Neospora caninum isolate 152 from chicken brain NC5 gene, partial sequence	Neospora caninum	490	490	93%	1e-133	96.32%	316	EU073600.1
☑	Neospora caninum isolate HL2 NC5 marker genomic sequence	Neospora caninum	484	484	92%	5e-132	96.28%	335	MT340527.1
☑	Neospora caninum isolate M2 NC5 marker genomic sequence	Neospora caninum	483	483	93%	2e-131	95.99%	305	MT340531.1
☑	Neospora caninum isolate PolBb1 clone 1 NC5 gene, complete sequence	Neospora caninum	483	483	91%	2e-131	96.58%	339	HM031965.1
☑	Neospora caninum isolate PolBb1 clone 10 NC5 gene, complete sequence	Neospora caninum	477	477	90%	8e-130	96.23%	328	HM031966.1
☑	Neospora caninum isolate Pol-B1 clone 2 NC5 gene, partial sequence	Neospora caninum	468	468	93%	5e-127	95.00%	336	EF463099.1
☑	Neospora caninum isolate 282/11 NC5 marker genomic sequence	Neospora caninum	464	464	94%	6e-126	94.72%	338	KP715560.1
☑	Neospora caninum isolate 252/11 NC5 marker genomic sequence	Neospora caninum	464	464	94%	6e-126	94.72%	338	KP715559.1
☑	Neospora caninum isolate JX5 NC5 marker genomic sequence	Neospora caninum	462	462	93%	2e-125	94.65%	335	MT340529.1
☑	Neospora caninum isolate Pol-B1 clone 1 NC5 gene, partial sequence	Neospora caninum	462	462	93%	2e-125	94.67%	336	EF463098.1
☑	Neospora caninum strain Nc-BJ NC5 gene, partial sequence	Neospora caninum	460	460	93%	8e-125	94.65%	334	EF581827.1
☑	TPA: Neospora caninum Liverpool, chromosome chrII, complete genome	Neospora caninum Liverpool	457	1362	93%	1e-123	94.31%	2174329	LN714476.1
☑	Neospora caninum isolate (2)PLA-32SR marker NC5 genomic sequence	Neospora caninum	457	457	94%	1e-123	94.04%	343	MZ467451.1

Figure 3.2. *Nc-5* gene of *Neospora caninum* with the highest sequence similarity from GenBank (sample ID : N60 C).

3.5 Genetic characterizations of *N. caninum* isolates

3.5.1 *N. caninum* microsatellite genotyping

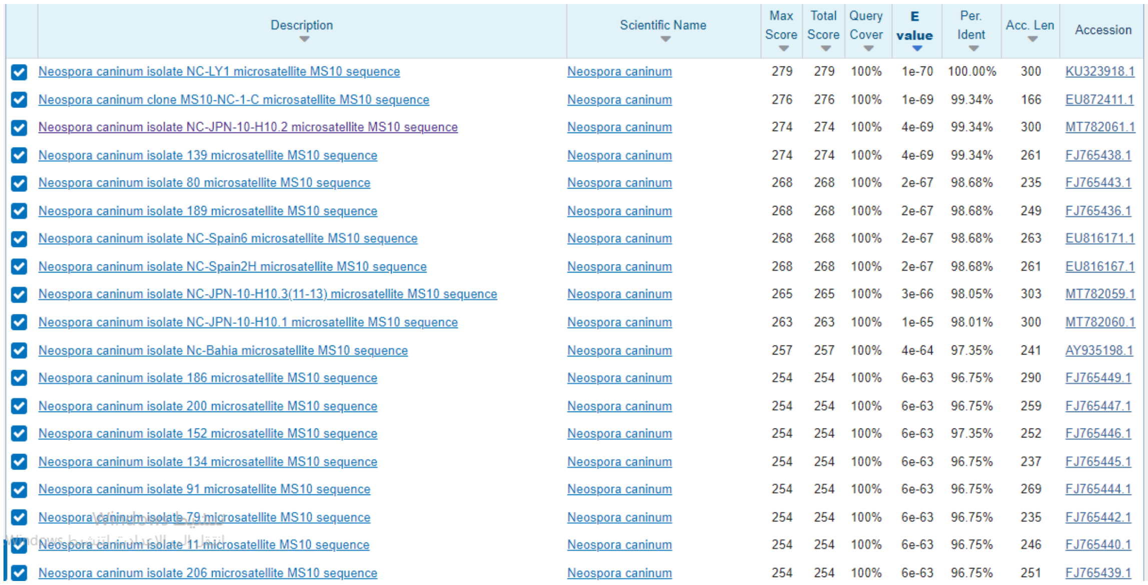
Microsatellite genotyping was applied to 30 positive samples. The genotyping successful rate was 50% (15/30); the microsatellite sequencing results are provided in Appendix 2. The *N. caninum* genotype was determined based on the microsatellite MS 10 marker. The MS10 profile of the Palestinian samples that were obtained in this study was [(ACT)7–(AGA)12–(TGA)9], [(ACT)7–(AGA)10–(TGA)9] as shown in table 3.3.

Table 3.3. Microsatellite genotype identified in 15 samples from cattle and sheep in this study.

Sample ID	Geographic Origin (Country)	Year of sample collection	Host sampled	Microsatellite Genotype
				MS10 (ACT)-(AGA)-(TGA)
N6C	Palestine/Jericho	2023	Cattle	7 12 9
N36C	Palestine/Jericho	2023	Cattle	7 12 9
N39C	Palestine/Jericho	2023	Cattle	7 12 9
N49C	Palestine/Jericho	2023	Cattle	7 12 9
N58C	Palestine/Jericho	2023	Cattle	7 12 9
N60C	Palestine/Jericho	2023	Cattle	7 12 9
N61S	Palestine/Jericho	2023	Sheep	7 12 9
N62C	Palestine/Jericho	2023	Cattle	7 12 9
N69C	Palestine/Jericho	2023	Cattle	7 12 9
N72C	Palestine/Jericho	2023	Cattle	7 10 9
N75C	Palestine/Jericho	2023	Cattle	7 12 9
N86C	Palestine/Jericho	2023	Cattle	7 12 9
N92C	Palestine/Jericho	2023	Cattle	7 12 9
N113C	Palestine/Jericho	2023	Cattle	7 10 9
N119C	Palestine/Jericho	2023	Cattle	7 12 9

3.5.2 Sequence alignments

BLAST (Basic Local Alignment Search Tool) was used to compare sequences from this study and sequences from the gene bank. Analysis of our Microsatellite (MS10) sequences showed high similarity (96–100%) with *N. caninum* sequences available in GenBank. For example, sample number 119 isolated from cattle in this study shows 100% similarity with *N. caninum* isolated from liverpool (Accession number: KU323918.1) and 99.34% similarity with *N. caninum* isolated from Japan (Accession number: MT782061.1), as shown in figure 3.3. All other sequence homologies are shown in table 3.3.



	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Neospora caninum isolate NC-LY1 microsatellite MS10 sequence	Neospora caninum	279	279	100%	1e-70	100.00%	300	KU323918.1
☑	Neospora caninum clone MS10-NC-1-C microsatellite MS10 sequence	Neospora caninum	276	276	100%	1e-69	99.34%	166	EU872411.1
☑	Neospora caninum isolate NC-JPN-10-H10.2 microsatellite MS10 sequence	Neospora caninum	274	274	100%	4e-69	99.34%	300	MT782061.1
☑	Neospora caninum isolate 139 microsatellite MS10 sequence	Neospora caninum	274	274	100%	4e-69	99.34%	261	FJ765438.1
☑	Neospora caninum isolate 80 microsatellite MS10 sequence	Neospora caninum	268	268	100%	2e-67	98.68%	235	FJ765443.1
☑	Neospora caninum isolate 189 microsatellite MS10 sequence	Neospora caninum	268	268	100%	2e-67	98.68%	249	FJ765436.1
☑	Neospora caninum isolate NC-Spain6 microsatellite MS10 sequence	Neospora caninum	268	268	100%	2e-67	98.68%	263	EU816171.1
☑	Neospora caninum isolate NC-Spain2H microsatellite MS10 sequence	Neospora caninum	268	268	100%	2e-67	98.68%	261	EU816167.1
☑	Neospora caninum isolate NC-JPN-10-H10.3(11-13) microsatellite MS10 sequence	Neospora caninum	265	265	100%	3e-66	98.05%	303	MT782059.1
☑	Neospora caninum isolate NC-JPN-10-H10.1 microsatellite MS10 sequence	Neospora caninum	263	263	100%	1e-65	98.01%	300	MT782060.1
☑	Neospora caninum isolate Nc-Bahia microsatellite MS10 sequence	Neospora caninum	257	257	100%	4e-64	97.35%	241	AY935198.1
☑	Neospora caninum isolate 186 microsatellite MS10 sequence	Neospora caninum	254	254	100%	6e-63	96.75%	290	FJ765449.1
☑	Neospora caninum isolate 200 microsatellite MS10 sequence	Neospora caninum	254	254	100%	6e-63	96.75%	259	FJ765447.1
☑	Neospora caninum isolate 152 microsatellite MS10 sequence	Neospora caninum	254	254	100%	6e-63	97.35%	252	FJ765446.1
☑	Neospora caninum isolate 134 microsatellite MS10 sequence	Neospora caninum	254	254	100%	6e-63	96.75%	237	FJ765445.1
☑	Neospora caninum isolate 91 microsatellite MS10 sequence	Neospora caninum	254	254	100%	6e-63	96.75%	269	FJ765444.1
☑	Neospora caninum isolate 79 microsatellite MS10 sequence	Neospora caninum	254	254	100%	6e-63	96.75%	235	FJ765442.1
☑	Neospora caninum isolate 11 microsatellite MS10 sequence	Neospora caninum	254	254	100%	6e-63	96.75%	246	FJ765440.1
☑	Neospora caninum isolate 206 microsatellite MS10 sequence	Neospora caninum	254	254	100%	6e-63	96.75%	251	FJ765439.1

Figure 3.3. Sequence alignment of *Neospora caninum* MS 10 sequence identified in this study (sample ID : N119 C) with the highest sequence similarity from GenBank.

Table 3.4.The percentage of homology in the study samples with the highest sequence similarity from GenBank.

Sample ID	Type of animal	Homology	Accession number
N6C	Cattle	100%	KU323918.1
N36C	Cattle	100%	KU323918.1
N39C	Cattle	100%	KU323918.1
N49C	Cattle	100%	KU323918.1
N58C	Cattle	100%	KU323918.1
N60C	Cattle	100%	KU323918.1
N61S	Sheep	100%	KU323918.1
N62C	Cattle	98.05%	KU323918.1
N69C	Cattle	100%	KU323918.1
N72C	Cattle	99.34%	KU323918.1
N75C	Cattle	100%	KU323918.1
N86C	Cattle	100%	KU323918.1
N92C	Cattle	100%	KU323918.1
N113C	Cattle	96.25%	FJ824930.1
N119C	Cattle	100%	KU323918.1

3.5.3 Phylogenetic tree

The phylogenetic tree of *N. caninum* was constructed using microsatellite (MS 10) repeats obtained from study samples (n = 15) and the microsatellite repeats database (n = 83), which included the Swedish, British, German, Japanese, Korean, Iranians, Portuguese, Chinese, American, Australians, Italian, and Spanish MS10, (see Appendix 3). Globally, the results of the phylogenetic tree showed that *N. caninum* isolated from different hosts and geographical areas are distributed into two clusters, as shown in figure 3.4. At local level, the Palestinian isolates are distributed in two clusters, as shown in figure 3.5. In cluster I, which has the majority of the Palestinian isolates, repeats were close to those from Asia (Japan and China). The second cluster included repeats from Europe (Italy and Germany).

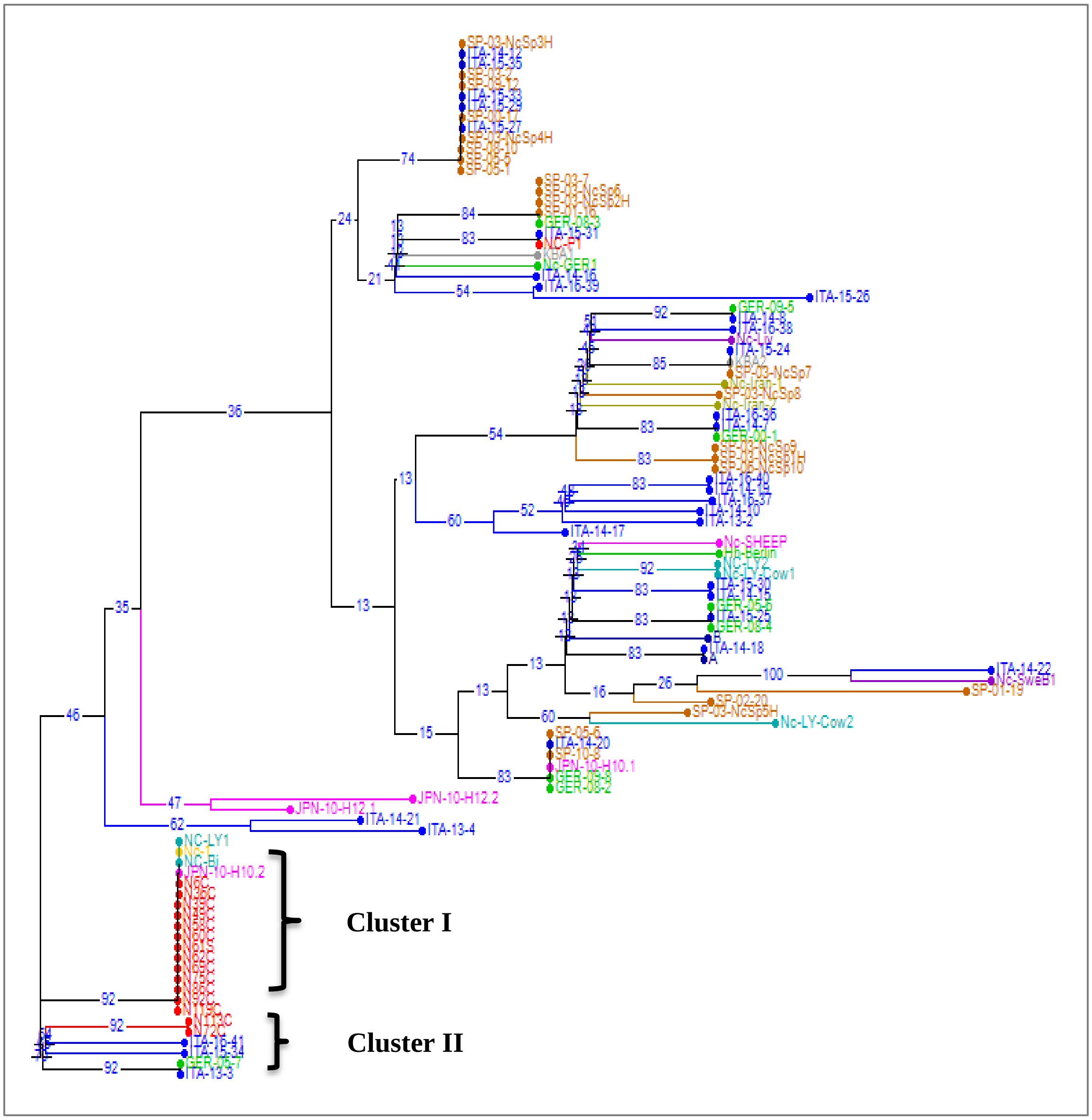


Figure 3.4. Neighbor-joining phylogeny of microsatellite (MS 10) repeats of *N. caninum* isolates. Each country's isolates are represented by a different color.

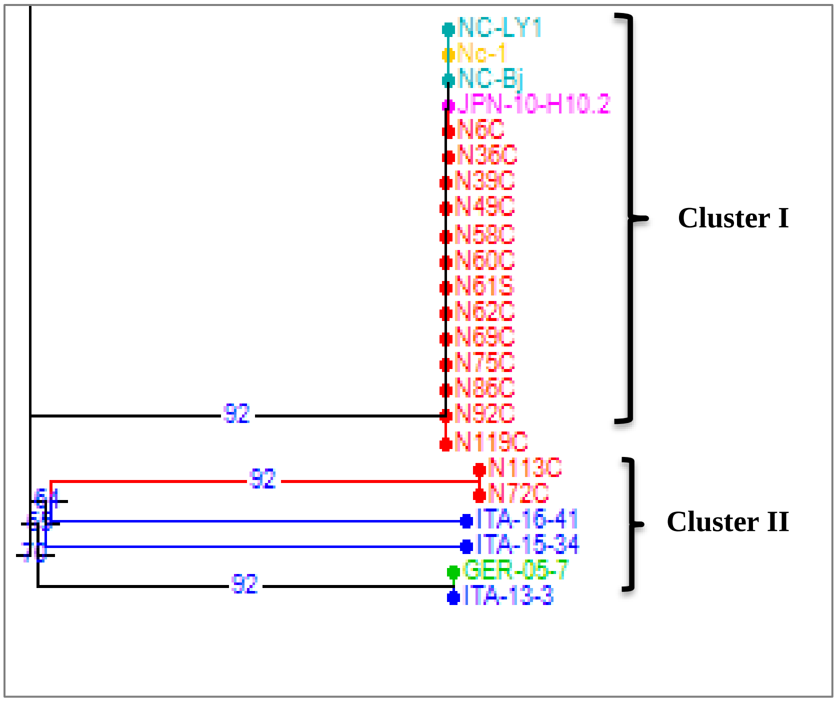


Figure 3.5. The two clusters of the Palestinian isolates. The red color indicate isolates from the present study.

Chapter Four

4.1 Discussion

Neosporosis has become one of the most common diseases causing abortion in dairy cattle globally. The clinical signs of *N. caninum* have been reported in sheep, goats, deer, and horses (Dubey *et. al.,* 2003). Fetal abortion induced by *N. caninum* is a common reproductive problem that causes significant economic loss in cattle and sheep husbandry (Reichel *et. al.,* 2013). There are two main methods used for neosporosis diagnosis in animals: the serology assay, which is the most commonly used method (Dubey *et. al.,* 2007), but it can produce false positive and false-negative results in early or chronic cases (Yao *et. al.,* 2009), and the molecular biology methods by using PCR-based assays. The PCR has been used since 1993 to identify *Neospora* DNA in cattle tissues or sera. This method is particularly useful for distinguishing *Neospora* from closely related species such as Toxoplasma (Brindley et. al., 1993). For detecting *N. caninum,* several target genes have been used, including *18S rDNA*, *28S rDNA*, *ITS1,* and *Nc-5* (Yao *et. al.,* 2009). The *Nc-5* gene was widely validated and utilized for PCR detection of *N. caninum* since it is specific to the parasite and its sequence appears many times in its genome (Okeoma *et. al.,* 2004; Müller *et. al.,* 1996; Yao *et. al.,* 2009). Therefore, we targeted the *Nc-5* gene for the detection of *Neospora* in animal samples. Detecting *N. caninum* DNA even in the absence of antibodies provides valuable information about infection (Mclnnes *et. al.,* 2006; Ramos *et. al.,* 2017). Furthermore, it allows the study of *N. caninum* phylogenetic relationships and genetic diversity across a variety of hosts and geographical origins (Amdouni *et. al.,* 2018; Nardoni *et. al.,* 2019; Rocchigiani *et. al.,* 2017).

To our knowledge, no studies have been conducted on *N. caninum* in Palestine yet. This study was the first to study *N. caninum* infection in slaughtered cattle and sheep from Jericho district using molecular methods.

In the present study, *N. caninum* was detected in the brain tissue samples of cattle and sheep. Out of 124 samples, 30 (24.19%) samples were positive for *N. caninum*. The frequency of *N. caninum* in cattle was 25.47%, and in sheep it was 16.66%. These results highlight the abortion risk due to *N. caninum* in livestock. Other countries have detected *N. caninum* in brain samples: 20.5% and 15.6% in cattle and sheep, respectively, from Iran; 22% and 10.6% in cattle and sheep, respectively, from North Africa (Amdouni *et. al.,* 2018; Salehi *et. al.,* 2021); 18.9% of aborted bovine fetuses in England (Hughes *et. al.,* 2006); and 5% in cattle from Brazil (Santos *et. al.,* 2009). The primary causes of varied results can be attributed to the number of definitive hosts in the examined areas (Gharekhani *et. al.,* 2014) and the use of various diagnostic methods, as well as factors related to climate and the environment. The farmers should therefore maintain hygienic conditions and keep dogs away from livestock food. They should be educated about the role of dogs in transmitting the *N. caninum* infection.

In this study, we found that the frequency of neosporosis in cattle was higher than in sheep. The greater susceptibility of cattle to neosporosis infection could explain the significant disparity in prevalence rates between these animals, as shown by Pan *et. al*, 2004. These findings are similar to the findings of other two studies in North Africa and Iran (Amdouni *et. al*., 2018; Salehi *et. al.,* 2021).

Infection caused by *N. caninum* was confirmed by Sanger sequencing of random positive samples. BLAST analyses of the *Nc-5* gene revealed more than 92% to 99% matching with *N. caninum* sequences available in GenBank. The results revealed that the amplified sequences were *N. caninum*-specific. The *Nc-5* gene is sensitive for detecting *Neospora*, but it may not be suitable for characterizing heterogeneity or for phylogenetic analyses due to the high degree of genetic similarity found among circulating *Neospora* isolates (Muradian *et. al.,* 2012). The use of microsatellite markers would be helpful for phylogenetic analyses since this technique is presently regarded as the gold standard for assessing *N. caninum* genetic diversity. In recent years, microsatellite markers have generated a lot of interest as genetic markers. Regidor-Cerrillo *et. al.,* (2006) genotyped various *N. caninum* isolates using microsatellite markers and found differences in 12 microsatellite markers amongst nine isolates. Each of these nine isolates exhibited a distinct profile, according to multilocus analysis, although there was no correlation between genotype and host or origin. A large-scale genotyping investigation assessed nine microsatellite markers for a total of 108 references and clinical *N. caninum* isolates. A total of 96 microsatellite multilocus genotypes demonstrated a high amount of genetic variation among the samples. Data analysis revealed that these microsatellite markers were partially associated with geographical origin, with sub-structures found in each country's population of samples (Regidor-Cerrillo *et. al.,* 2013). There has been a lack of research on these topics on *N. caninum* in the Middle East. In Palestine, no studies have been conducted on the prevalence or information obtained on the genetic diversity of *Neospora*. In the present study, we used the next-generation sequencing approach to reveal the M10 repeats and study the genetic diversity in the Palestinian isolates. MS10 is a very polymorphic locus that is extremely helpful for identifying divergence among different *N. caninum* strains (Basso *et. al.,* 2009; Al-Qassab *et. al.,* 2010). The main advantage of NGS targeting microsatellites is that it is less expensive and less time-consuming than traditional Sanger sequencing. In addition, by utilizing NGS to detect microsatellite repeats, we were able to not just save time but also increase the efficiency of the methodology, allowing it to evaluate the targeted regions with a very high depth of coverage. As a result, the sequencing method would be more accurate and reliable.

Microsatellite genotyping was applied to 30 positive samples, and a successful result was achieved in 15 samples. This could be due to low DNA concentrations in some samples, or less sensitivity of the primers. The sequence results displayed 100% similarity with *N. caninum* isolated from dogs in Liverpool and 99.34% similarity with *N. caninum* isolated from cattle in Japan that also clustered with them in the phylogenetic tree.

The phylogenetic tree was based on MS10 repeats, and the results indicated that globally*, N. caninum* isolates from different hosts and geographical areas are genetically diverse and distributed into two main clusters. Locally, the Palestinian isolates are distributed in two clusters. Therefore, we have two genotypes of *Neospora* in Palestine. In the first cluster, which has the majority of the Palestinian isolates, the isolates share the same genotype with one isolate from Japan (JPN-10-H10.2) (El-Alfy *et. al.,* 2021), two isolates from China (NC-Bj) (NC-LY1) (Qian *et. al.,* 2016) (Hao *et. al.,* 2014), and one isolate from the USA (Nc-1) (Regidor-Cerrillo *et. al.,* 2013).

These isolates share with this marker the same origin, which could indicate the same source of infection. In the second cluster, three isolates from Italy (ITA-16-41, ITA-15-34, and ITA-13-3) (Regidor-Cerrillo *et. al.,* 2020) and one isolated from Germany (GER-05-7) (Regidor-Cerrillo *et. al.,* 2013) shared the same genotype as the Palestinian isolates. This could indicate a European source of infection. Moreover, the majority of samples in Palestine showed a similar MS10 profile, which indicates stability in the selected marker among the Palestinian isolates.

In terms of the study limitation, we should note that the number of samples was relatively small, especially in sheep samples and the unavailability of aborted fetal samples in traditional farms.

In addition, we don’t have clinical data on the animals or source farms. Another limitation of this was the sampling area only one city and one slaughterhouse were included in this study.

4.2 Conclusions

In conclusion, the present study provided the first estimate of the frequency of *N. caninum* in cattle and sheep from Palestine. Additionally, it was also the first time for the investigation of the phylogenetic analysis of *N. caninum* based on microsatellite markers. These findings provide information on *N. caninum* isolates in Palestine and facilitate comparison with *N. caninum* isolates from other regions. The results provided evidence for the presence of *N. caninum* in Palestine. Therefore, livestock that are imported must be inspected by the Ministry of Agriculture to ensure they do not introduce neosporosis into Palestine. In addition, it is important to create veterinarian preventative programs that inform both veterinarians and farmers about the risks associated with this parasite.

Further studies with a larger sample size are required to evaluate the prevalence of *N. caninum* among various hosts and farms in Palestine. In addition, we can confirm the findings by combining molecular methods with serology and histopathology assays. It is also necessary to conduct studies on stray dogs in order to prevent economic losses caused by the spread of infection to livestock.

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Appendices

Appendix 1 : Sanger sequencing results

>Neo_Liverpool

TGCATAATCTCCCCCGTCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTGGATAAGTA

TCATTGACACACTGTCCACACCCTGACGCAGGCTGATTTCAACGTGACGAATGACTAACCACAAACCACG

TATCCCACCTCTCACCGCTACCAACTCCCTCGGTTCACCCGTTCACACACTATAGCCACAAACAAAAAAG

GAGCCTTGCTGCCGCAGGCTGCGCCCAACAACGACACGTCAGCA

>Neo60

TGCATAATCTCCACCGTCATCAGTGCCTCCGGTGT

TGCCTCAACACAGAACACTGAACTCTGGATAAGTATCATTGACACACTGTCCACAGCCTG

ACGCAGGCTCATTTCAACGTGACGAATGACTAACCACAAACCACGTATCCCACCTCTCAC

CGCTACCAACTCCCTCGGTTCACCCGTTCACACACTATAGCCACAAACAAAAAAGGAGCC

TTGCTGCCGCAGGCTGCGCCTAACAATGACACGTCCGCAT

>Neo61

TGCATAATCTCCACCGTCATCAGTGCCTCCGGTGTTG

CCTCAACACAGAACACTGAACTCTGGATAAGTATCATTGACACACTGTCCACAGCCTGAC

GCAGGCTCATGTCAACGTGACGAATGACTAACCACAAACCACGTATCCCACCTCTCACCG

CTACCAACTCCCTCGGTTCACCCGTTCACACACTATAGCCACAAACAAAAAAGGAGCCTT

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Appendix 2 : Microsatellite (MS10) sequencing results

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Appendix 3: The microsatellite genotyping databases, including the Swedish, British, German, Japanese, Korean, Iranians, Portuguese, Chinese, American, Australians, Italian, and Spanish microsatellites (MS 10), were used for the genetic relationship analyses in this study.

Sample ID	Isolation year	Geographic Origin (Country)	Host sampled	Microsatellite Genotype	References
				MS10	
Nc-SweB1	1997	Sweden	Cattle	8 23 8	Regidor-Cerrillo et al.,2006
Nc-Liv	1995	England	Dog	6 26 10	Regidor-Cerrillo et al.,2006
Nc-GER1	2000	Germany	Dog	6 25 9	Regidor-Cerrillo et al.,2006
Hh-Berlin	2001	Germany	Dog	6 17 8	Regidor-Cerrillo et al.,2006
Nc-SHEEP	2001	Japan	Sheep	6 24 8	Regidor-Cerrillo et al.,2006
KBA1	2000	South Korea	Cattle	6 22 9	Regidor-Cerrillo et al.,2006
KBA2	2000	South Korea	Cattle	6 23 10	Regidor-Cerrillo et al.,2006
Nc-1	1996	USA	Dog	7 12 9	Regidor-Cerrillo et al., 2013
NC-P1	2009	Portugal	Dog	6 15 9	Basso et al.,2009
Nc-LY-Cow1	2017	China	Cattle	6 12 8	Qian et al.,2017
Nc-LY-Cow2	2017	China	Cattle	6 16 6	Qian et al.,2017
NC-LY1	2016	China	Dog	7 12 9	Qian et al.,2016
NC-LY2	2016	China	Dog	6 12 8	Qian et al.,2016
NC-Bj	2014	China	Cattle	7 12 9	Hao et al.,2014
Nc-Iran-1	2015	Iran	Cattle	6 17 10	Salehi et al.,2015
Nc-Iran-2	2015	Iran	Cattle	6 15 10	Salehi et al.,2015

JPN-10-H10.1	2010	Japan	Cattle	6 14 8	El-Alfy et al.,2021
JPN-10-H10.2	2010	Japan	Cattle	7 12 9	El-Alfy et al.,2021
JPN-10-H12.1	2010	Japan	Cattle	7 14 9	El-Alfy et al.,2021
JPN-10-H12.2	2010	Japan	Cattle	7 14 7	El-Alfy et al.,2021
A	2016	Australia	dog	6 21 8	da Costa Reis et al., 2016
B	2016	Australia	Dog	6 22 8	da Costa Reis et al., 2016
N6C	2023	Palestine	Cattle	7 12 9	This study
N36C	2023	Palestine	Cattle	7 12 9	This study
N39C	2023	Palestine	Cattle	7 12 9	This study
N49C	2023	Palestine	Cattle	7 12 9	This study
N58C	2023	Palestine	Cattle	7 12 9	This study
N60C	2023	Palestine	Cattle	7 12 9	This study
N61S	2023	Palestine	Sheep	7 12 9	This study
N62C	2023	Palestine	Cattle	7 12 9	This study
N69C	2023	Palestine	Cattle	7 12 9	This study
N72C	2023	Palestine	Cattle	7 10 9	This study
N75C	2023	Palestine	Cattle	7 12 9	This study
N86C	2023	Palestine	Cattle	7 12 9	This study
N92C	2023	Palestine	Cattle	7 12 9	This study
N113C	2023	Palestine	Cattle	7 10 9	This study
N119C	2023	Palestine	Cattle	7 12 9	This study
ITA-14-15	2014	Italy	Cattle	6 18 8	Regidor-Cerrillo et al .,2020
ITA-14-7	2014	Italy	Cattle	6 22 10	Regidor-Cerrillo et al .,2020

ITA-14-8	2014	Italy	Cattle	6 19 10	Regidor-Cerrillo et al .,2020
ITA-14-12	2014	Italy	Cattle	6 14 9	Regidor-Cerrillo et al .,2020
ITA-14-16	2014	Italy	Cattle	6 21 9	Regidor-Cerrillo et al .,2020
ITA-14-17	2014	Italy	Cattle	6 14 11	Regidor-Cerrillo et al .,2020
ITA-14-18	2014	Italy	Cattle	6 21 8	Regidor-Cerrillo et al .,2020
ITA-14-19	2014	Italy	Cattle	6 23 11	Regidor-Cerrillo et al .,2020
ITA-14-20	2014	Italy	Cattle	6 14 8	Regidor-Cerrillo et al .,2020
ITA-15-25	2015	Italy	Cattle	6 15 8	Regidor-Cerrillo et al .,2020
ITA-15-26	2015	Italy	Cattle	5 18 9	Regidor-Cerrillo et al .,2020
ITA-15-27	2015	Italy	Cattle	6 14 9	Regidor-Cerrillo et al .,2020
ITA-15-31	2015	Italy	Cattle	6 15 9	Regidor-Cerrillo et al .,2020
ITA-15-33	2015	Italy	Cattle	6 14 9	Regidor-Cerrillo et al .,2020
ITA-15-34	2015	Italy	Cattle	7 19 9	Regidor-Cerrillo et al .,2020
ITA-15-35	2015	Italy	Cattle	6 14 9	Regidor-Cerrillo et al .,2020
ITA-16-36	2016	Italy	Cattle	6 22 10	Regidor-Cerrillo et al .,2020
ITA-16-37	2016	Italy	Cattle	6 26 11	Regidor-Cerrillo et al .,2020
ITA-16-38	2016	Italy	Cattle	6 20 10	Regidor-Cerrillo et al .,2020
ITA-16-39	2016	Italy	Cattle	6 18 9	Regidor-Cerrillo et al .,2020
ITA-16-40	2016	Italy	Cattle	6 23 11	Regidor-Cerrillo et al .,2020
ITA-14-10	2014	Italy	Cattle	6 22 11	Regidor-Cerrillo et al .,2020
ITA-13-2	2013	Italy	Cattle	6 21 11	Regidor-Cerrillo et al .,2020
ITA-13-3	2013	Italy	Cattle	7 17 9	Regidor-Cerrillo et al .,2020
ITA-13-4	2013	Italy	Cattle	7 25 10	Regidor-Cerrillo et al .,2020
ITA-14-21	2014	Italy	cattle	7 12 10	Regidor-Cerrillo et al .,2020

ITA-14-22	2014	Italy	Cattle	9 23 8	Regidor-Cerrillo et al .,2020
ITA-15-24	2015	Italy	Cattle	6 23 10	Regidor-Cerrillo et al .,2020
ITA-15-29	2015	Italy	Cattle	6 14 9	Regidor-Cerrillo et al .,2020
ITA-15-30	2015	Italy	Cattle	6 18 8	Regidor-Cerrillo et al .,2020
ITA-16-41	2015	Italy	Goat	7 24 9	Regidor-Cerrillo et al .,2020
SP-03-NcSp1H	2003	Spain	Cattle	6 21 10	Regidor-Cerrillo et al.,2008
SP-03-NcSp2H	2003	Spain	Cattle	6 13 9	Regidor-Cerrillo et al.,2008
SP-03-NcSp3H	2003	Spain	Cattle	6 14 9	Regidor-Cerrillo et al.,2008
SP-03-NcSp4H	2003	Spain	Cattle	6 14 9	Regidor-Cerrillo et al.,2008
SP-03-NcSp5H	2003	Spain	Cattle	6 16 8	Regidor-Cerrillo et al.,2008
SP-03-NcSp6	2003	Spain	Cattle	6 13 9	Regidor-Cerrillo et al.,2008
SP-03-NcSp7	2003	Spain	Cattle	6 23 10	Regidor-Cerrillo et al.,2008
SP-03-NcSp8	2003	Spain	Cattle	6 18 10	Regidor-Cerrillo et al.,2008
SP-03-NcSp9	2003	Spain	Cattle	6 21 10	Regidor-Cerrillo et al.,2008
SP-06-NcSp10	2006	Spain	Cattle	6 21 10	Regidor-Cerrillo et al.,2008
SP-05-1	2005	Spain	Cattle	6 14 9	Pedraza-Diaz et al.,2009
SP-03-2	2003	Spain	Cattle	6 14 9	Pedraza-Diaz et al.,2009
SP-05-5	2005	Spain	Cattle	6 14 9	Pedraza-Diaz et al.,2009
SP-05-6	2005	Spain	Cattle	6 14 8	Pedraza-Diaz et al.,2009

SP-03-7	2003	Spain	Cattle	6 13 9	Pedraza-Diaz et al.,2009
SP-10-8	2010	Spain	Sheep	6 14 8	Moreno et al .,2012
SP-08-10	2008	Spain	Cattle	6 14 9	Regidor-Cerrillo et al.,2013
SP-09-12	2009	Spain	Cattle	6 14 9	Regidor-Cerrillo et al.,2013
SP-00-17	2000	Spain	Cattle	6 14 9	Pedraza-Diaz et al.,2009
SP-01-19	2001	Spain	Cattle	7 13 8	Pedraza-Diaz et al.,2009
SP-02-20	2002	Spain	Cattle	6 13 8	Pedraza-Diaz et al.,2009
GER-00-1	2000	Germany	Cattle	6 22 10	Regidor-Cerrillo et al .,2013
GER-08-2	2008	Germany	Cattle	6 14 8	Regidor-Cerrillo et al .,2013
GER-08-3	2008	Germany	Cattle	6 13 9	Regidor-Cerrillo et al .,2013
GER-08-4	2008	Germany	Cattle	6 15 8	Regidor-Cerrillo et al .,2013
GER-09-5	2009	Germany	Cattle	6 19 10	Regidor-Cerrillo et al .,2013
GER-05-6	2005	Germany	Cattle	6 15 8	Regidor-Cerrillo et al .,2013
GER-05-7	2005	Germany	Cattle	7 17 9	Regidor-Cerrillo et al .,2013
GER-09-8	2009	Germany	Cattle	6 14 8	Regidor-Cerrillo et al .,2013