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Original article

Optimization of the Polymerase Chain Reaction for Detecting Sheep Pox Virus in Alzawiyah City, Libya

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Abstract
Accurate and rapid diagnosis of SPV is essential to control the rapid spread of disease in Libya. This study was designed to optimize and develop a PCR assay for sheep pox virus identification in sheep farms in Alzawiyah City, Libya. A total of 120 oral swab samples were collected as follows: clinically suspected sheep pox (n=67), clinically suspected Contagious Ecthyma (n=18), and healthy sheep (n=35). The collected samples were subjected to DNA extraction followed by a polymerase chain reaction targeting the P32 gene with specific primers. All 67 clinically suspected sheep pox samples were found positive for SPV and yielded expected amplicon sizes of 390 bp. All the clinically suspected Contagious Ecthyma (CE) samples and healthy samples were negative. The result of the current P32 gene-based PCR assay demonstrated good sensitivity and specificity and could be used for molecular diagnosis of sheep pox disease.
Keywords. Sheep Pox Virus, P32 Gene, PCR, Alzawiyah City, Libya.

Introduction

Sheep pox disease is the severest and most contagious viral disease in sheep [1]. Clinically, Sheeppox virus (SPV) can be recognized by fever, anorexia, depression, the development of lung lesions, the appearance of pox lesions on areas free of wool, and the swelling of superficial lymph nodes. [2]. SPV is a serious skin disease of sheep. SPV belongs to the Poxviridae family's Capripoxvirus (CaPV) genus. Members of this genus, which also includes viruses that cause lumpy skin disease and goat pox, infect sheep, goats, and cattle and cause an economically significant disease (LSDV) [3,4]. The sheep poxvirus is the most important poxvirus of animals, listed in group A diseases of OIE [5]. It can cause severe production losses because of r damage to sheep's wool and hides, reduced milk yield, higher abortion rates, lower gain of weight, and high mortality rate [6-8]. Even though the disease was eradicated in many countries, it is still reported from different parts of the world, including North Africa, the Middle East, West Asia, India, and China [8,9]. In Libya, the disease generally has an enzootic appearance. It threatens the development of the agricultural sector, causing economic losses related to the mortality of lambs and a fall in reproduction and production in adults [10]. Diagnosis of SPV is usually based on highly characteristic clinical signs, isolation of the virus, virus-neutralization serological assays [11,12], and Polymerase Chain Reaction (PCR) assays [13,11].
Conventional virological and serological assays for identifying SPV are time-consuming, laborious, and most of them are of low specificity [14]. However, molecular methods, including PCR assays, are potential tools and could be used as a replacement or complementary tests of conventional laboratory techniques for the detection of SPV. They are proven to be reliable, sensitive, rapid, and specific methods that have been routinely used for the detection and characterization of many viruses in the world, including capripoxvirus [15,16,12]. In addition, Rapid, specific, and sensitive tests are necessary for the identification of the SPV because timely detection of SPV in field samples is crucial for successful SPV control and lowering the potentially severe economic damage that might arise from an epidemic. [17]. The objective of this study was to create a quick and sensitive method for detecting SPV in field samples within a short amount of time, making it practical and efficient.

Methods

Samples

The study was conducted at the Genetic Engineering Department of the Libyan Biotechnology Research Center (BTRC) in Tripoli, Libya. The current study collected a total of 120 oral swabs, with 67 taken from suspected cases of sheep pox, 18 from suspected cases of sheep Contagious Ecthyma (CE), and 35 from healthy sheep (negative control) in various flocks.
The specimens were gathered from sheep herds in Alzawiyah City, Libya, between May 2013 and April 2014. The collection of oral swab samples was carried out using buccal swab tubes provided by Isohelix, a company based in the United Kingdom. These samples were subsequently transported to the Genetic Engineering

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Laboratory at the Libyan Biotechnology Research Center (BTRC) in Tripoli, Libya. Upon arrival, the samples were stored at a temperature of 4°C until they were ready for use.

DNA extraction and PCR

It was implemented at the Libyan Biotechnology Research Center (BTRC) in Tripoli, Libya, specifically in the Genetic Engineering Laboratory. DNA was extracted from the samples utilizing the DNeasy Tissue Kit (Promega Company). The oral swab samples were mixed with a 50% phosphate-buffered saline and then transformed into a 10% suspension. Following this, a sample suspension of 200 µl was subjected to incubation in a water bath alongside 20µl of proteinase K and 400 µl of lysis buffer at a temperature of 56 °C for 30 minutes. After the incubation period, the specimen underwent washing and centrifugation in accordance with the guidelines provided by the manufacturer. The DNA was extracted using 100 µl of the elution buffer supplied in the kit and then stored at a temperature of -20°C. The PCR reactions utilized specific primers, B68 and B69, which were developed based on the methodology outlined in the study conducted by Hein et al. in 1999. The PCR primers had the following sequences and were synthesized by (MWG-biotech, Germany): B68: 5' - CTA AAA TTA GAG AGC TAT ACT TCT T- 3' B69: 5' - CGA TTT CCA TAA ACT AAA GTA-3'. The primers amplified a 390 bp fragment of the P32 gene of SPV.

PCR amplification and gel electrophoresis

The polymerase chain reaction (PCR) was executed in PCR tubes using 50 µl of amplification mixture for each reaction, comprised of 25 µl GoTaq master mixture, 1.25 µl forward primer, 1.25µl reverse primer, 19.5 µl of RNase-free water, and 3 µl template DNA. Subsequently, the tubes were promptly sealed to prevent any potential contamination and subjected to a brief centrifugation to consolidate the primary mixture at the base of the reaction tubes. Subsequently, the consolidated mixture was transferred to the PCR automated thermal cycler (GeneAmp® PCR System 9700) by Applied Biosystem, USA.

The amplification process began with an initial denaturation stage at 94°C for 5 minutes, after which 35 cycles were performed: denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension step at 72°C for 7 minutes using a thermal cycler. The amplified products underwent analysis through electrophoresis on a 1% agarose gel, following the protocol described by Mangana-Vougiouka et al. (1999), with the addition of 0.5 g/ml ethidium bromide. Visualization was carried out under ultraviolet (UV) light, using 6 µl of duplex PCR products, and electrophoresis was conducted for 80 minutes at a constant 120 V.

Following visualization, the resulting image was documented using a gel documentation system (Biometra GmbH), and the data was subsequently analyzed through computer software. The limit of detection of the PCR assay was evaluated by amplifying 10-fold serially diluted purified SPV DNA (10^{-1} to 10^{-5} copies per µl). DNA was extracted from diluted virus suspension as templates and examined by PCR assay. The sensitivity was defined as the lowest dilution of viral DNA giving an amplification signal**.** The concentration of the purified viral DNA was not measured because of the lack of resources needed in our laboratory. The specificity of the PCR reaction was determined by amplifying the minimum detectable amount of genomic DNA (1µl) extracted from SPV in the presence of the maximum amount of genomic DNA (7µl) extracted from apparently healthy (negative control) and in the presence of the maximum amount of genomic DNA (7µl) extracted from clinical Contagious Ecthyma (CE).

Results

Sheep were subject to both physical and clinical examination during the process of sample collection. The investigation revealed that the typical clinical manifestations seen in cases of suspected Sheep pox disease presented as skin lesions, featuring pustules, papules, and scabs primarily located on the hairless regions, including the tail and perineum, as well as the nose, eyes, and lips, resulting in discomfort and pain during feeding. In suspected cases of CE, clinical manifestations include the presence of papules and pustules on the lips, with some cases exhibiting the formation of scabs at the corners of the mouth. The objective of this study was to refine the PCR assay used for detecting and identifying SPV by focusing on a 390 bp segment of the P32 gene. The most effective assay parameters have been outlined in the Methods section above. The primers, quantity of DNA templates, annealing temperature, and duration of PCR steps were fine-tuned to produce the desired result, which was the evident presence of 390 bp bands in gel electrophoresis upon visualization in a 1% agarose gel using ethidium bromide staining, as confirmed by the DNA marker (Figure 1).

To ascertain the most suitable temperature for amplification in the PCR assay, a series of temperatures ranging from 47°C to 52 °C were experimented with. The findings indicated that specific primers can be used to amplify the targeted SPV genes at all of the annealing temperatures mentioned. The most intense amplified products were identified at a temperature of 52°C (Figure 1). During the optimization of primer concentration, it was determined that the optimal amplification of the targeted genes occurred when each primer was at a concentration of 1.25µl. The findings indicated that the amplification of the targeted genes could be achieved after 30 to 40 reaction cycles using the optimized primer concentration of 1.25µl at 52 °C. However, it was observed that the amplified products were clearer at 35 cycles compared to other cycle conditions (refer to Figure 1). All 67 samples, which were suspected of sheep pox infections, were confirmed

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to be positive for SPV as indicated by the presence of a 390 bp amplicon of the P32 gene in gel electrophoresis (Figure 2).

The findings demonstrated that the clinical signs observed in suspected cases of sheep pox were indicative of the presence of the sheep pox virus. Contrastingly, PCR-specific primers failed to produce any amplified product in any of the 35 samples of suspected CE and 18 samples of apparently healthy sheep, as demonstrated in Figure 2. The results indicated that the limit of detection, determined through 10-fold serial dilutions of genomic DNA infected with SPV, was able to detect as low as a 10-5 dilution in the gel electrophoresis, with an expected amplicon size of 390 bp (Figure 3A). The PCR achieved specificity in detecting 1µl of genomic DNA infected with SPV in the presence of 7 µl of negative control (apparently healthy). An identical result was noted when 1µl of genomic DNA infected with SPV was compared to 7 µl of genomic DNA infected with CE. Furthermore, no detectable amplified products were found in the 7 µl genomic DNA samples from individuals classified as apparently healthy (negative control) and those infected with Contagious Ecthyma (CE) (refer to Figure 3B). The findings demonstrated the recognition of SPV and the distinction between SPV and CE virus in clinical samples (see Figure 4). Based on the findings, it can be concluded that the PCR method demonstrated swift and precise performance, along with outstanding sensitivity and specificity.

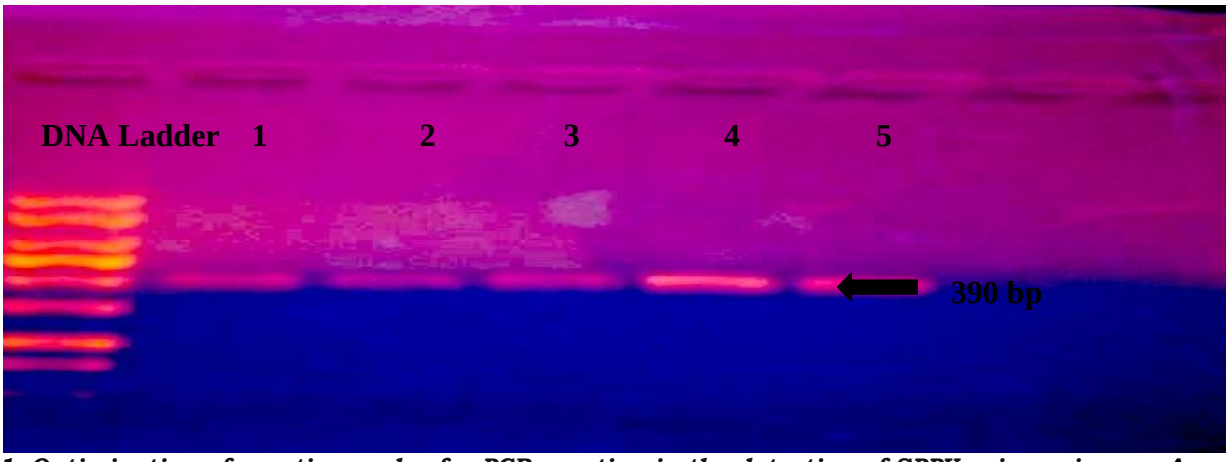

Figure 1. Optimization of reaction cycles for PCR reaction in the detection of SPPV using primers. Agarose gel electrophoresis showing the amplification of PCR products performed on 390 bp fragments-P32 gene of sheep pox virus by using a specific primer. Lanes 1–5: showing positive samples indicated to marker (molecular size 1000-bp) DNA ladder.

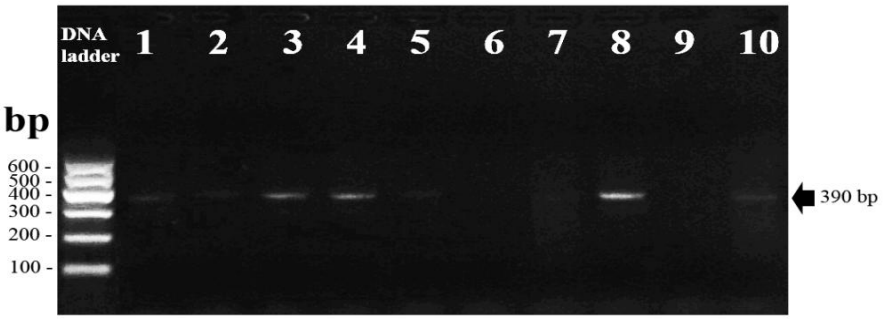

Figure 2. Identification of sheep poxvirus showing amplification (bands) at 390 bp from the P32 gene of sheep poxvirus. Lane M: 1000 bp marker. Lanes 1 –5: amplification (bands) performed on genomic DNA infected with SPV. Lane 6: no amplification at 390 bp of negative control performed on genomic DNA from apparently healthy. Lane 7: no amplification at 390 bp of negative control was performed on genomic DNA from CE. Lanes 8: no amplification of control performed on contents of PCR mixture without DNA template.

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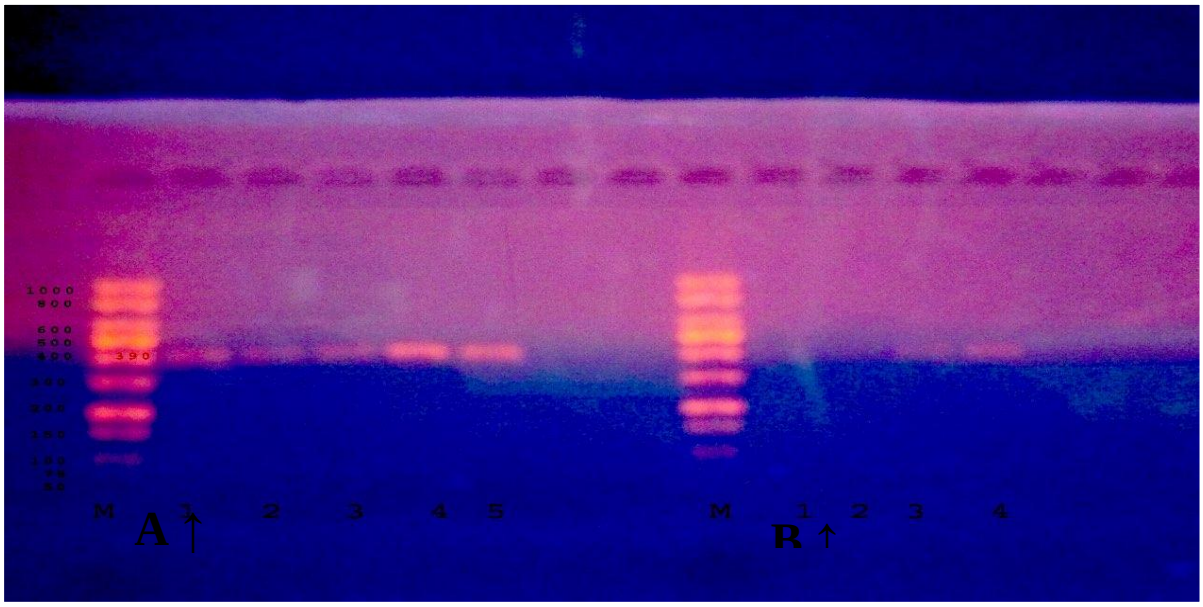

Figure 3. A: Sensitivity of the PCR assay applied to 10-fold serial dilutions of the genomic DNA infected with sheep pox virus (SPV). Lane M: 1000 bp ladder DNA marker. Lanes 1 to 5 amplification from 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, of genomic DNA infected with SPV, respectively. B: Specificity of the PCR. Lane M: 1000 bp ladder DNA marker. Lane 1 No amplification from 7 µl of genomic DNA of negative control. Lane 2: No amplification from 7µl of genomic DNA infected with CE. Lane 3: amplification from 1µl of genomic DNA infected with the SPV in the presence of 7µl genomic DNA infected with the CE. Lane 4: Amplification from 1µl of genomic DNA infected with the sheep poxvirus in the presence of 7µl genomic DNA of negative control.

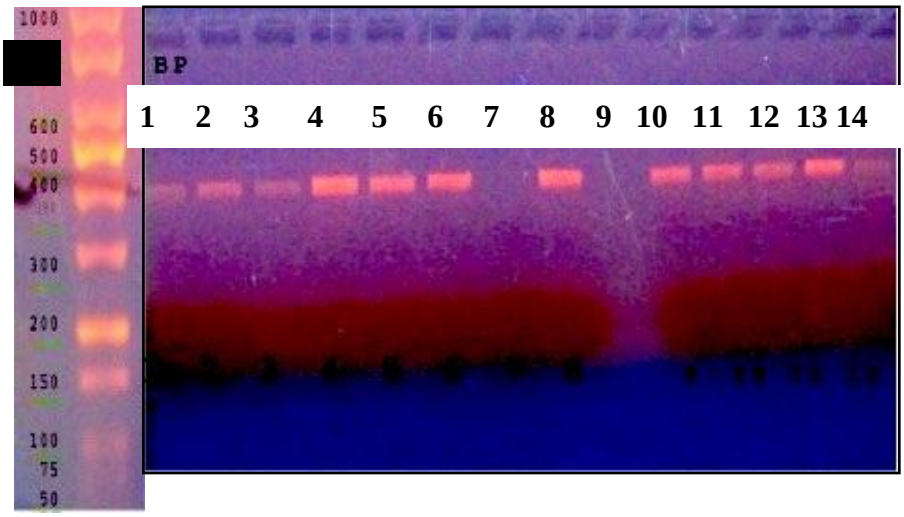

Figure 4. Identification and differentiation of sheep poxvirus from contagious ecthyma virus in clinical samples. Lane M contains a 1000 bp ladder DNA marker. Lanes 1 to 6 depict the amplification process carried out on genomic DNA that has been infected with SPV. Genomic DNA infected with the CE virus did not undergo amplification for Lane 7 and Lane 9. Amplification was conducted on genomic DNA that had been infected with SPV, and the results were observed in Lane 8. Lanes 10 to 13 depict the results of amplification conducted on genomic DNA that has been infected with SPV.

Discussion

Sheep pox (SPP) is the result of an infection with the sheep pox virus (SPV), also known as SPPV. In regions where it is prevalent, Small Ruminant Pox (SPP) has a significant economic influence on the production systems of small ruminants, resulting in decreased productivity, mortality, harm to skins and hides, and imposing limitations on international trade [18]. The disease is classified as one of the economically significant animal diseases that require immediate notification to the World Organization for Animal Health (OIE). The successful diagnosis of capripox virus infection can be achieved through the use of traditional laboratory methods such as cultural isolation and electron microscopy, as well as newer techniques including polymerase chain reaction (PCR) [19,16].

Traditional laboratory methods for diagnosing the disease rely on clinical symptoms and serological tests such as virus neutralization, immune fluorescence, agar gel immune diffusion (AGID), enzyme-linked immune sorbent assay (ELISA), virus isolation, and electron microscopy [20]. The methods outlined are labor-intensive, challenging to implement, and present challenges about potential cross-reactivity with other viral strains [21]. Nevertheless, PCR has been effectively established for the specific identification of the

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capripox virus and is regarded as one of the most preferable alternatives to traditional methods due to its high sensitivity, specificity, and reproducibility [22,23,19,21].

Furthermore, the PCR method of diagnosing viral diseases through the amplification of genetic material (DNA or RNA) has established itself as the most reliable and efficient technique, particularly in the case of Capripox virus (CaPVs). PCR tests [9,24, 25] were conducted. PCR can also enhance the ability to respond to outbreaks, monitor and regulate the capripox virus in endemic areas, and investigate the epidemiology of capripox diseases, thus mitigating the potential economic harm associated with an outbreak [23,12,26-28,13,9,29-31]. This research aimed to optimize and develop a conventional PCR assay that targets a 390 bp fragment of the P32 gene to detect SPV in suspected clinical samples obtained from sheep in Al Zawiyah, Libya.

The assay successfully produced a specific 390 bp fragment of the P32 gene for all suspected sheep pox samples, marking the first molecular evidence of sheep pox detection in Libya, as far as our knowledge extends. These findings align with previous research conducted by [12,31-37]. Heine and colleagues According to a study conducted in 1999, it was found that the full-length P32 protein of capripox virus contains a transmembrane region located near the C-terminus. This protein is associated with the membrane and serves as a structural component present in all strains of the virus. Furthermore, it contains a significant antigenic determinant. The gene encoding this particular protein is situated in a highly preserved region of the capripox virus genome, as noted by [38]. Previous studies have utilized sequencing analysis of PCR products, demonstrating a significant homology of 100% with the sheep pox P32 gene found in GenBank, as reported by [38,39].

Sheep pox and Goat pox necessitate immediate and accurate laboratory verification due to their highly contagious and severe nature. The amplification of genetic material (DNA or RNA) via PCR has emerged as the preferred method for promptly diagnosing viral diseases such as CaPVs. It is considered the standard approach in molecular diagnostics. Numerous PCR-based tests have been documented for promptly diagnosing CaPVs, encompassing conventional and real-time PCR or qPCR as outlined by [40], although its use necessitates costly, high-precision equipment and specialized expertise for operation and data interpretation. To enhance the sensitivity of the aforementioned method, it was essential to optimize the parameters of primers and annealing temperature. Given these conditions, it is possible to detect the viral DNA at a dilution level of up to 10−5. This approach eliminates the need for subsequent manipulation after the polymerase chain reaction (PCR) as the melt curve data enables verification of amplification products, thereby reducing the likelihood of contamination. The amplification products were examined via melting curve analysis and no primer-dimers were detected. Given the aforementioned circumstances, it was feasible to determine a sensitivity of 10 copies per reaction mixture. A rapid diagnostic test for porcine sapelovirus utilizing various clinical sample types would be advantageous for the management of the disease. According to [23], understanding the pathogenesis of capripoxvirus indicates that skin lesions, as well as nasal and oral swabs, are the most effective samples for detecting and isolating the virus. According to [41], Capripox viruses demonstrate host-specificity, with the majority of isolates causing illness primarily in either sheep or goats. However, certain isolates have the potential to induce severe disease in both species. According to [13], the similarity in antigenic and virulent characteristics of SPV and GPV rendered it difficult for serological assays to differentiate between the two viruses. However, recent research conducted by [40], has demonstrated that the viruses have distinct phylogenetic characteristics and can be differentiated using molecular methods. According to our information, the virus in Libya has not been sequenced or phylogenetically characterized, despite the disease being recognized clinically for several years and outbreaks causing economic losses across the country (unpublished data, National Center for Animal Health, Libya). According to published data, sheep pox outbreaks can be attributed to various factors, including the rapid spread of the virus within the flock, the severe course of the disease, and the relatively high morbidity rate. These factors are believed to be the result of the introduction of a virulent strain of virus into a large, susceptible population, as well as the particular breed of sheep, climatic and management conditions (such as crowding), and the various modes of transmission of the virus. Biting flies, which were abundant on the farm, are also believed to be efficient mechanical vectors of the virus. These findings were reported by [41-43,45].

Sheep pox is a prevailing issue in Libya, with frequent outbreaks causing significant economic hardship for the sheep industry, as highlighted by [34]. To date, the primary preventative measures employed in Libya for the reduction of SPV infections have been surveillance and annual vaccination. Vaccination is deemed to be the most cost-effective and sustainable method of disease control. Despite the drawbacks associated with inactivated vaccines, such as short-term immunity and the need for large quantities of antigen for vaccination of the vast livestock population, live vaccines are considered the optimal long-term solution for the control of capripox infections [40]. Live attenuated vaccines have been commonly used worldwide for the immunization of small ruminants [46]. However, it has been noted that there is limited epidemiological data on sheep pox in Libya over the past two decades, with gaps in reporting for the years 2005-2006 and 2010-2016 due to internal political issues.

The identification of sheep pox outbreaks in Libya is primarily based on clinical symptoms, and despite extensive control efforts by the Libyan veterinary services, the disease persists in an enzootic form due to

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the consistent and substantial number of annual sheep pox cases. (Data not yet published by the National Center for Animal Health in Libya.) Preventing capripox diseases is essential in sheep farms to enhance and safeguard small ruminant productivity. The main objective of this study, from a preventive-medicine perspective, was to support Libyan veterinary services in reducing the prevalence of sheep pox and minimizing economic losses through the efficient use of techniques such as PCR for routine diagnosis.

Conclusion

The study found that the PCR test for the P32 gene effectively detected the sheep pox virus in sheep clinical samples. The optimized PCR test quickly and accurately detects the sheep pox virus without requiring labor-intensive techniques or isolating the virus. In the future, this PCR test, along with gene sequencing studies, can be used to study Capri poxvirus infection in Libya and develop a disease control strategy, including mass vaccination programs.

Conflict of interest. Nil

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المستخلص

التشخيص الدقيق والسريع لفيروس الجدري البقري ضروري للسيطرة على الانتشار السريع للمرض في ليبيا. صُممت هذه الدراسة لتحسين وتطوير اختبار تفاعل البوليميراز المتسلسل لتحديد فيروس الجدري البقري في مزارع الأغنام في مدينة الزاوية، ليبيا. تم جمع ما مجموعه 120 عينة مسحة فموية على النحو التالي: جدري الأغنام المشتبه به سريريًا (ن = 67)، والإكثيما المعدية المشتبه بها سريريًا (ن = 18)، والأغنام السليمة (ن = 35). خضعت العينات المجمعة لاستخراج الحمض النووي متبوعًا بتفاعل البوليميراز المتسلسل الذي يستهدف جين P32 باستخدام بادئات محددة. تم العثور على جميع عينات جدري الأغنام المشتبه بها سريريًا والبالغ عددها 67 إيجابية لفيروس الجدري البقري وأسفرت عن أحجام أمبليكون متوقعة تبلغ 390 زوجًا قاعديًا. كانت جميع عينات الإكثيما المعدية المشتبه بها سريريًا والعينات السليمة سلبية. أظهرت نتيجة اختبار تفاعل البوليميراز المتسلسل الحالي القائم على جين P32 حساسية وخصوصية جيدتين ويمكن استخدامها للتشخيص الجزيئي لمرض الجدري البقري.

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