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Article

Serological Detection, Isolation and Molecular Confirmation of Parainfluenza Virus-3 in Camels, Iraq

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ABSTRACT

The objectives of this study were to detect and isolate the Parainfluenza-3 virus (PIV-3) in camels with naturally developed respiratory illness and to determine the titer of the isolates using the virus titration. Therefore, an overall 100 nasal swabs and jugular vein blood samples were collected from diseased camels in four districts in Wasit province (Iraq) from December (2019) to March (2020). The swabs were subjected to six subsequent passages on bovine kidney cell culture (BKCC) to isolate the virus and to confirm infection by molecular PCR assay. Fever (≤40°C), abundant runny nasal discharge, ocular discharge, coughing, depression, increased respiratory rate, abnormal breath sounds, and mainly wheezing are the most observed clinical signs. Positive findings were involved 24% by ELISA and 37% by RT-PCR. The age group from 1-2 years old showed a high infection rate, while the lower level was in the 4-6 years old group. Regarding season, the infection rate was high in winter compared to spring. Sheik Saad city appeared to have a higher infection rate than other districts. The positive samples inoculated into the Bovine kidney cell culture (BKCC) revealed the cytopathic effects (CPE) after three successive passages, which appeared as clumping and rounding with the progression of infection time at the 4th passage. Elongation and giant cell formation were shown in some isolates after the 5th and 6th passages until they reached complete detachments of the cells from the cell sheet. The titer of viral tissue culture infective dose (TCID50) of the 3rd passage was determined in BKCC cells at 10–3/0.05 ml, and the high titer was shown at the 5th and the 6th passages equal to 10-5/ 0.05 ml. In conclusion, PIV-3 is widespread among camels infected with respiratory illness; therefore, studies are necessary to detect the prevalence rate among camels in other Iraqi regions.

Keywords: PIV-3, Fusion protein gene, Hemagglutination protein gene, ELISA, PCR

INTRODUCTION

Although the etiology of most respiratory diseases has not been determined to date, camel respiratory disease outbreaks have been linked to a wide range of viral, fungal, bacterial, and parasitic microorganisms [1, 2]. Parainfluenza virus-3 (PIV-3), influenza A and B, adenovirus, respiratory syncytial virus (RSV) and infectious bovine rhinotracheitis (IBR) are some of the viruses that cause respiratory infections in camels [3, 4]. Bovine PIV-3 (BPIV-3) is a non-segmented, single-stranded, negative-sense, enveloped RNA virus known as bovine retrovirus-3 [5]. The BPIV-3 belongs to the Respirovirus genus and is categorized as a member of the Orthoparamyxovirinae subfamily of the Paramyxoviridae family in the order Mononegavirales [6-8]. This virus infects ungulates, causing significant respiratory illnesses, as it can cause diseases on its own or in combination with other pathogens such as viruses, bacteria, and Mycoplasma [9]. When the strains of long-distance transport weaken an animal's immune system and increase its vulnerability to infection, BPIV-3 appears as part of the mixed infection. The disease known as 'shipping fever' or the bovine respiratory disease complex (BRDC) may be developed as a result [10].

BPIV-3 is a factor in BRDC, a global cattle disease that results in severe economic losses in stocker and feedlot operations [11]. Recent research has connected particular viruses such as BPIV-3, BHV-1, or BRSV for both severe and fatal BRDC cases in cattle [12-14]. BPIV-3 virus was first isolated from nasal swabs of calves with symptoms such as lack of appetite, coughing, nasal discharges, various respiratory indications, fever, lacrimation, and conjunctivitis in the United States in 1959 [15]. Since then, BPIV-3 has been reported in both asymptomatic and clinically sick cattle worldwide, with a significant incidence of BPIV-3-specific antibodies, particularly in beef and dairy herds and in different areas in Asia, Europe, and North and South America [16].

They cause rhinitis, pharyngitis, laryngitis, tracheobronchitis, bronchiolitis, and pneumonia by reproducing in the respiratory tract's epithelial cells. Mucilage of the nose and throat are affected early on during PIV infection [17]. PIVs can spread directly by human-animal contact or by large droplet spread. In several countries, severe symptoms, high mortality rates and inability to make a definitive diagnosis are common [18]. Infection in camels can result in obvious economic losses in production in most countries due to deaths, lower productivity, and higher medical costs [19].

Although BPIV-3 is most commonly detected in cattle and small ruminants, infections in buffalo, camelids, horses, pigs, dogs, and primates have been described, as well as cross-species infections in humans [20-23]. Viruses, on the other hand, do not last very long in the environment. Since the virus can be easily transmitted from one animal to another [24, 25], it is reasonable to assume that they are highly contagious. In addition to being a major animal pathogen, BPIV-3 has been linked to human disease. By detecting antibodies, RNA, and virus isolation, this study was designed to determine the occurrence of PIV3 infection in camels in Wasit province due to the high morbidity rate of serious respiratory infection in herds of camel, especially young animals.

MATERIALS AND METHODS

Ethical approval

The Scientific Committee licensed this study in the College of Veterinary Medicine (University of Wasit, Wasit, Iraq). Additionally, all agreements for safe and humane animal handling were obtained from owners.

Specimen collection and study areas

The present diagnostic study was conducted between December 2019 and March 2020. Four districts were chosen specifically for their ease of access, camel population, and safety. Camels in random selections of herds were examined for respiratory signs, and owners provided information about their health state. Infected camels would exhibit respiratory symptoms such as coughing, dyspnea, nasal discharge and an increased respiratory rate (18-20 per minute). Study animals were categorized into age groups (6 months – 6 years), both sexes and from various areas in Wasit province. Using sterile cotton swabs, 100 nasal swabs were taken from camels with a respiratory tract illness that never received antimicrobial treatment and under strict sterilization cooled conditions to be used later for isolation of PIV-3 and molecular examination by polymerase chain reaction (PCR). Nasal swabs were inserted in 2 ml of chilled transport media in test tubes. Blood samples (5 ml) were drawn from the jugular vein of study animals using the disposable syringe, centrifuged (5000 rpm for 10 min), and sera were kept frozen into labeled Eppendorf tubes to be subjected later for serological examination by enzyme-linked immunosorbent assay (ELISA).

Serology by ELISA

Indirect ELISA kits for detecting antibodies (IgG) to PIV3 were used following the manufacturer's instructions (Svanovir, Sweden*)*. The assay has shown excellent specificity in various scientific studies and field investigations. This was useful in management practices because it separates exposed and non-exposed individuals, preventing disease introduction and spreading in ruminant populations.

Isolation of the virus

Isolation was carried out throughout the primary and secondary bovine kidney cell culture (BKCC). These cells were prepared from embryonic bovine kidney cell culture at a private laboratory using highly aseptic methods described previously [26].

Isolation of virus on cell culture

A laminar airflow class II cabinet was used for virus isolation. To grow the (BKCC), we used a minimal essential medium (MEM) with phosphate buffer saline (PBS) and fetal bovine serum (FBS) at 10% (FBS). Antibiotic medium (penicillin, streptomycin, and amphotericin B solution) was mixed with 1 ml of the sample, and the falcon flask was inoculated with 1.5 ml of specimen suspension after growth media from single-layer cells were discarded. At 37ºC for 60 minutes, the jars were cultivated with the infectious virus stock on the prepared cells for adsorption, and 10 ml of maintenance media (2% serum) was added to be incubated at 37ºC. Perceptions of the inoculated samples were made daily using an inverted microscope to check for tainting or growth. The media was changed after two days for growth-negative samples and rehashed at regular intervals. Every day, a microscope was used to check for viral growth, and it was left to develop for a week. Reading and interpreting the cultures were carried out according to the standard manifestations of the growing virus. Titrations were performed as previously done [27] but with 96 well plates cultured in a 5% CO_2 environment. Tissue culture 50 % infectious doses were used to calculate titrations (TCID50). After five passages, the isolated virus from the infected BKCC culture was determined by titration in the same cell culture [28], and the virus titer was measured [29].

Molecular examination by PCR

Viral RNA was recovered from all swabs and culture samples according to the manufacturer guidelines using the extraction manual Kit (Bioneer, Korea). Reverse transcription in one-step RT/PCR kit components at 50°C for 30 minutes. RT-PCR was used to amplify the predicted size of 400 bp for the Fusion (F) protein gene sequence of BPIV-3 and 164 bp for the HA protein gene (Table 1).

The PCR findings were evaluated using a 2% agarose gel stained with Ethidium bromide. Briefly, 10 µl of products were mixed with loading buffer and loaded into wells in a prepared gel, which was then electrophoresed for 90 minutes at 100 volts in parallel with a DNA 100 bp molecular weight ladder in electrophoresis equipment using TAE buffer. UV light was employed to see the suspected bands, and a DNA molecular weight ladder standard was used to assess their size previously explained [30]. The size of the studied amplicons was expected to be 400 and 164 bp [31, 32].

Gene	Step	Temp	Time	No. of cycles	Expected Size
HA	Initial denaturation	94ºC	15 min	1	164 bp
	Denaturation Annealing Extension	94ºC 45ºC 72°C	30 sec 30 sec 30 sec	40	150-200 bp
	Final extension	72°C	10 min	1	
RT/PCR kit components at 50°C		30 min			
F	Initial denaturation	95°C	10 min	1	400 bp
	Denaturation Annealing Extension	94°C 51°C 72°C	45 sec 45 sec 1 min	40	
	Final extension	72°C	10 min	1	
HA primers (F): 5'- TGTGCATGGTGAGTTCGCA-3' (R):5'-ATTCAGCATCACGTGCCACTG-3'				Noori et al. [32]	
F gene primers (F): 5′-CATTGAATTCATACTCAGCAC-3′ (R): 5′-AGATTGTCGCATTT(AG)CCTC-3′				Lyon et al. [31]	

Table 1. PCR program of selected genes

Statistical analysis

The Chi-squared statistical test was performed to examine the potential relationship between collected data and study outcomes. All tests were carried out at a significance level of $p<0.05$.

RESULTS

Clinical findings

Fever (≤40°C), abundant runny nasal discharge, ocular discharge, coughing, depression, increased respiratory rate, abnormal breath sounds, mostly wheezing are the most observed clinical signs. Sera and nasal swab samples were checked to detect specific antibodies and genes using indirect ELISA and RT-PCR. The pos-

itive findings were involved 24 % by ELISA and 37% by RT-PCR. The age group from 1-2 years old showed a high infection rate, 28.2% and 46.8% in ELISA and PCR, respectively, while the lower level was in the 4-6 years old group, 17.5% and 30.4% in ELISA and PCR respectively (Table 1). Regarding the season incidence, the infection rate was high (43.2%) in the winter compared to spring appeared (28.6%) (Table 1). Sheik Saad City appeared to have a higher infection rate (51.5%) than other districts. All these results were statistically significant at P<0.05. All these results were statistically significant at P<0.05 (Table 2).

Variables	Category	Total No. of samples	No. (%) of positives by ELISA	No. (%) of positives by PCR
Districts	Sheik Saad	35	10 (28.6) ^{A}	18 (51.5) ^{A}
	Al-Numaniyah	20	4 (20.0) ^{B}	6 (30.0) ^{B}
	Badra	27	6 (22.3) ^{B}	9 (33.4) ^{B}
	Hay	18	4 (22.3) ^{B}	4 (22.3) ^{C}
Age groups	≤ 1 – 2 Years	32	9 (28.2) ^{A}	14 (46.8) ^{A}
	2 – 4 Years	45	11 (24.5) ^{A}	16 (35.5) ^{B}
	4 – 6 Years	23	4 (17.5) ^{B}	7 (30.4) ^{B}
Season	Winter	44	14 (32.0) ^{A}	19 (43.2) ^{A}
	Spring	21	3 (14.3) ^{C}	6 (28.6) ^{B}
	Autumn	26	5 (19.3) ^{B}	9 (34.7) ^{B}
	Summer	9	2 (22.3) ^{B}	3 (33.5) ^{B}

Table 2. Distribution of *PI3* infection at different variables. Different letters referred to significance (P<0.05)

Isolation of the virus in Bovine kidney cell culture (BKCC)

One of the positive samples in ELISA and PCR was inoculated in the Bovine kidney cell culture (BKCC) and checked daily for determining specific cytopathic effects (CPE). After three successive passages, the CPE appeared clumping and rounding with the progression of infection time at the 4th passage (*48-hour post-inoculation*). Some of the cells showed detachments from the monolayer. These cells may show elongation and giant cell formation after the 5th and 6th passages until they reach complete detachments of the cells from the cell sheet (Figure 1). The titer of viral tissue culture infective dose (TCID50) of the third passage was determined in BKCC cells. The titer of the virus was 10^{-3}/0.05 ml. The high titer of the virus was shown at the 5th and 6th passages, equal to 10^{-5}/ 0.05ml, indicating that the virus has a good growth tendency in this type of cell culture.

Detection of virus genes

The RT-PCR technique was used with two primers designed from the conserved sites of the PI-3 viruses' fusion protein gene (F gene) and hemagglutination protein gene (HA gene). Because of the conservation of this region of the genome among PI-3 viruses, the primers were chosen there. Camels can also be infected with the BPI-3 virus. As a result, these primers may be able to maintain the virus's identity. The predicted amplicons were 400bp for the F gene and 164bp for the HA gene (Figure 2).

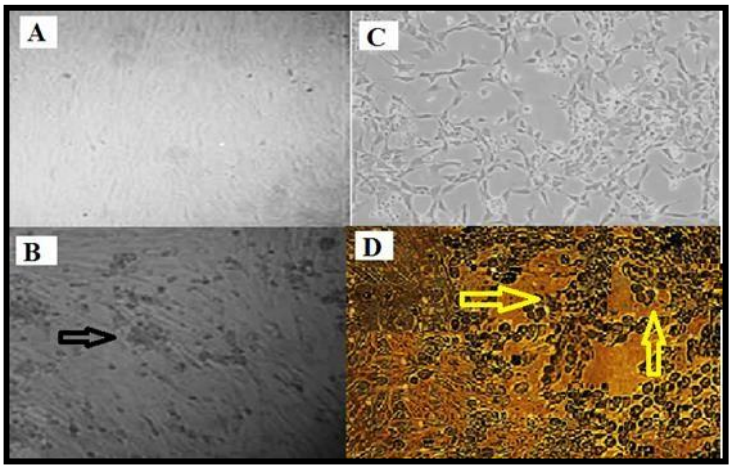

Figure 1. Cell culture results of PI3 effects on BKCC (40X). (A): Control (not inoculated); (B): Third passage after 72 hr. show moderate aggregation and rounding (Black arrow); (C): Fourth passage after 48 hr. show detachment of the cells and elongation; (D): Fifth passage after 48 hr. shows clumping and detachment of cells (yellow arrows)

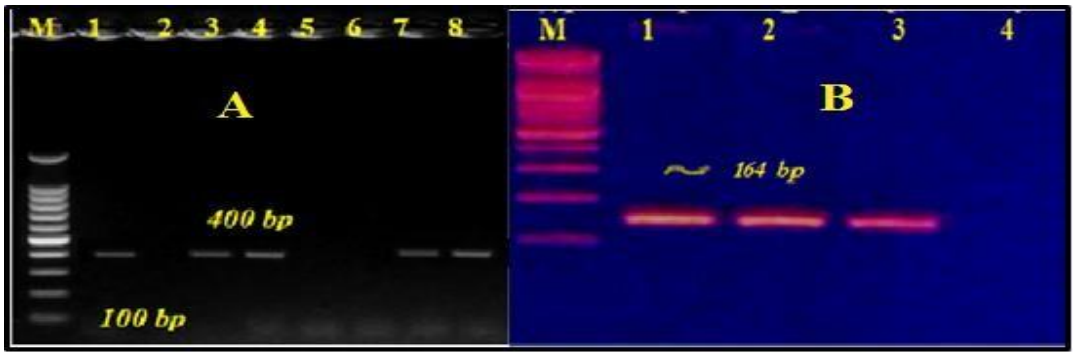

Figure 2. A. Ethidium bromide-stained gel electrophoresis for PCR amplification for F genome detection of PIV3 400 bp: lane M 100 bp marker, lane 1 PIV3 positive control, lanes 3- 4-7-8 camel PIV3 positive specimens, lanes 2-5-6 camel PIV3 negative specimens. B. Results of HA gene amplification (≈164 bp)as follows: lane M: Marker, lane (1): Positive control, lanes (2-3): Positive results, and lane (4): Negative control

DISCUSSION

One of the viruses that might cause respiratory infection was the PIV-3. Without developing complications, the condition is not dangerous because the infection usually lasts 3-4 days before completely recovering [6, 33]. PIV-3 infection, on the other hand, appears to predispose the host to secondary bacterial infection and has been linked to shipping fever. Moreover, the substantial active immunity that emerges from PIV-3 infection is transient and does not save the animal from subsequent infections or the risk of shipping fever if the animal is stressed. Fever, lack of appetite, depression, nasal discharge with sneezing and whistling sounds, and difficulty in respiration with mouth breathing accompanied by cough with a dry or moist presentation were among the clinical signs that emerged in the camels in the current study. These findings were consistent with previous researchers who reported similar results on the calves [19, 34-36].

Intisar *et al*. (2009) found the same clinical symptoms on camels in several Sudanese locations [21]. Because these tests had great sensitivity and specificity to achieve this aim, the current study focused on detecting PIV-3 antibodies by in-

direct ELISA and RT-PCR for the detection of certain viral genes such as the Fusion protein gene (F gene) and hemagglutinating gene (HA gene). Also, the results were supported by the findings of other researchers who proved the zero molecular detection of the virus through these techniques [31, 32, 37]. The outer envelope antigen, Hemagglutin-neuraminidase (HN), PIV-3 is a tetramer comprising two disulfide-connected dimers. This multifunctional molecule has three distinct and important functions in cell infection: receptor attachment, neuraminidase activity, and fusion-promoting activity [38].

In mammals, including humans, PIV-3 has been demonstrated to cause extensive respiratory illnesses and outbreaks and appears to play a small impact in developing clinically apparent respiratory illnesses in camels [21]. PIV-3 was first investigated in the 1960s using largely serological approaches [39, 40].

PIV-3 seroprevalence in camels varies by country and study population [21, 41, 42], though camel PIV-3 genomic sequences have never been published. Antibodies to PIV3 have been found in 5.6 % of seemingly healthy racing camels in the Arabian Gulf region [43]. The current results investigated the infection rate in camels: 24% by ELISA and 37% by RT-PCR. The results were grouped to include numerous risk factors such as age, season, and areas chosen in this study. These findings appeared to be corroborated by many works that utilized similar diagnostic approaches with similar results when investigating camels and other ruminants [21, 32, 35, 36]. The differences in chosen regions could be attributable to the province's geographical placement on the border.

Camels may be particularly susceptible to this illness because they cultured close to other susceptible ruminants, making infection transmission simpler, as reported by other studies [44-47]. Regarding the cell culture findings, isolation was accomplished using six passages in BKCC culture, yielding one isolate with clear CPE. The CPE of BPIV-3 in BKCC culture was visible as giant cells with syncytia formation in agreement with others [48]. CPEs demonstrated that the isolated virus caused severe cellular alterations in mammalian cell culture as sloughing and detachment of the cells from the monolayer sheet, similar to those observed in the bovine kidney by the T1 strain of the virus and in monkey kidney cell culture by earlier research. According to others, BKCC was suitable for isolating BPIV-3 [12, 44, 49, 50].

CONCLUSIONS

In conclusion, PIV-3 is widespread among camels infected with respiratory illness; therefore, studies are necessary to detect the prevalence rate among camels in other Iraqi regions. Indirect ELISA confirmed a high efficacy in detecting specific antibodies against PIV-3, while PCR assay demonstrated high sensitivity and specificity in detecting targeted genes.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Scientific Ethics Committees of the Department of Microbiology (College of Medicine, University of Wasit), Department of Biology (College of Science, University of Wasit) and Department of Internal and Preventive Veterinary Medicine (College of Veterinary Medicine, University of Wasit).

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Conflicts of Interest: The authors declare no conflict of interest.

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