

Detection of bovine parainfluenza type 3 virus in Iraq

Aida Bara Allawe and Anton Sabri Al-bana

College of Veterinary Medicine/University of Baghdad

Summary

This study is considered the first study in our country to isolate bovine parainfluenza type 3 virus (BPIV-3) from naturally infected calves. BPIV-3 virus was isolated from nasal swabs collected from calves with respiratory disease. The virus was isolated and titrated in secondary embryonic bovine kidney (SEBK) cell culture; other cell cultures (secondary sheep testis and chicken embryos fibroblast cell cultures) were used for propagation of isolated virus. Examination of infected cell culture revealed giant cell formation and intracytoplasmic inclusions. The isolated virus agglutinated guinea pig's erythrocytes and produces clear hemadsorption of guinea pig's erythrocyte in infected SEBK cell culture. Virus titer was 2×10^2 TCID₅₀/0.1ml in the sixth passage and became $2 \times 10^{4.5}$ TCID₅₀/0.1ml in the ninth passage. The new isolated virus was identified as BPIV-3 by hemagglutination inhibition test, serum neutralization test and indirect immunofluorescent technique, by using specific monoclonal and polyclonal antibody. The virus was sensitive to ether. High titer of BPIV-3 antibodies was detected in sera of infected animals during convalescent period by ELISA test.

التحري عن الاصابه بفايروس نظير الأنفلونزا البقري نوع 3 (bovine parainfluenza 3virus) في العراق

عائدة برع علاوي و أنطوان صبري البنا

كلية الطب البيطري /جامعة بغداد

الخلاصة

في هذه الدراسة تم ولأول مره في القطر عزل فايروس نظير الانفلونزا البقري نوع 3 (bovine parainfluenza type 3 virus) من المسحات الانفيه التي تم جمعها من العجول المصابة بإعراض تنفسيه في خلايا كلية جنين البقر الثانويه (secondary embryonic bovine kidney cell culture) وكما استخدمت خلايا خصية الحملان الثانويه (secondary sheep testis cell culture) والخلايا الليفيه لاجنة الدجاج (chicken embryos fibroblast cell cultures) لتنمية الفايروس المعزول. كما اظهر الفايروس المعزول عند حفته في خلايا الزرع النسيجي تأثيرا مرضيا واضحا ومميزا اذ تم ملاحظة الخلايا العملاقة مع تكوين الأجسام الاشماليه في سايتوبلازم الخليه المصابه. كما لزن الفايروس المعزول خلايا الدم الخاصه بخنازير غينيا ولوحظت ظاهرة الامتزاز الدموي في خلايا كلية جنين البقر الثانويه. وقد كان معيار الفايروس المعزول 2×10^2 TCID₅₀/0.1ml في التمرير السادس وارتفع إلى $2 \times 10^{4.5}$ TCID₅₀/0.1ml في التمرير التاسع. تم تشخيص الفايروس بالاعتماد على اثباط التلازن الدموي. التعادل المصلي و اختبار التآلق المناعي غير المباشر باستخدام أمصال مرجعية مضادة للفايروس احاديه ومتعدده النسيله. واتضح ايضا ان الفايروس المعزول حساس للايثر. كما تم تشخيص الأجسام المضادة للفايروس المعزول في أمصال الحيوانات التي عزل منها الفايروس باستخدام اختبار الاليزا غير المباشر.

Introduction

Bovine parainfluenza type 3 (BPIV-3) virus, is a paramyxovirus, was first associated with Bovine respiratory disease complex BRDC in 1959(1) and has since been isolated from cattle and sheep with respiratory disease in many countries (2).

Bovine parainfluenza type 3 virus (BPIV-3) is an enveloped, non-segmented, negative-sense RNA virus within the genus Respirovirus, member of Paramyxoviridae family, antigenically related to human parainfluenza virus type 3 (HPIV-3) (3).

Bovine parainfluenza virus type 3 is an important pathogen for animals but some variants can cause disease in humans. In cattle it is associated to the clinical picture known as "shipping fever" (4). BPIV-3 has also been associated with abortion in cattle (5; 6). However the precise relationship of this virus to BRDC is still uncertain. Although Parainfluenza virus has been associated with sever outbreaks of both naturally occurring and experimentally

induced BRDC (7). In some reports exposure to BPIV-3 virus did not produce symptoms in the vast majority of animals (8; 9). BPIV-3 is of sufficient pathogenicity to cause severe respiratory disease in some instances (7), whereas in other studies the main effect of BPIV-3 may interfere with immune function, allowing concurrent or subsequent infections by other viruses and bacteria (10; 11). In general, BPIV-3 causes enzootic pneumonia and shipping fever in calves while in older animals the infection is subclinical form (12). The association of BPIV-3 with BRDC has been attempted through a number of laboratory tests. The most commonly reported technique is demonstration of the induction of serum antibodies to BPIV-3 viral antigens (13; 14), another means of implications are isolation of virus from tissues or body fluids of cattle with respiratory disease. An additional technique for associating viruses with disease is direct microscopic demonstration of the virus in affected tissues using immunohistochemistry (15).

In Iraq BRDC is a major health problem of cattle. It causes considerable financial losses in cattle and it is an important welfare problem of calves, several viral etiological agents of BRDC were studied and detected in our country like BRSV (16), IBR (17) and BVD (18) but bovine parainfluenza 3 virus still, has not been studied also we have no information about presence of this virus in calves with respiratory infection **therefore in this study, we investigated the presence of bovine parainfluenza virus type 3 in Iraq and the virus was demonstrated by using different diagnostic methods.**

Materials and Methods

1-Collection of samples

Sixty seven (67) nasal swabs were collected from calves with respiratory tract infection by using sterile Cotton swabs. The nasal swabs were dipped in test tubes containing 2ml of cooled transport media.

The nasal swabs collected from different areas which include:

- 1- Al-Naser dairy cattle farm station, Al-Sawara city.
- 2- Fallouja dairy cattle farms at Al- Anbar province.
- 3- Jebella dairy cattle farm station at Babel province.

Blood samples were collected from Jugular vein of calves with respiratory infection.

2-Reference antiserum:

- 1- Purified monoclonal antibodies anti PI3 prepared in mouse specific for Indirect Immunofluorescence Antibody Technique (IFAT), (ID-VET innovative diagnostics/France).
- 2- Goat anti-mouse IgG Conjugated with (FITC), (US Biological Laboratories, USA.).
- 3- Standard Bovine PI3 antiserum from ELISA kit (Cypress Diagnostics/Belgium).

3-Cell culture used for virus isolation and propagation

1. Secondary embryonic bovine kidney (SEBK) cell culture prepared in virology Laboratory / College of veterinary medicine/University of Baghdad (19).
2. Chicken embryo fibroblast cell culture prepared in Virology Laboratory / College of veterinary medicine/University of Baghdad (19).
3. Primary Sheep testis supplied by Al-Kindy Company for production of veterinary vaccines and drugs.

4-Virus isolation

The isolation was carried out by using SEBK cell culture which was inoculated by nasal swabs collected from calves with respiratory infection (20). Three successive passages were carried out for this isolation in the same cell culture and cellular changes were recorded daily. Chicken embryo fibroblast cell culture and Secondary sheep testes cell culture were used for virus propagation.

5- Virus Titration: the isolated virus harvested from inoculated SEBK cell culture after six passages was titrated in the same cell culture (21), then titer of virus was calculated (22).

6-Inclusion bodies' detection: The test was made by using Hematoxylin and Eosin staining (28).

7- Ether sensitivity: ether sensitivity was performed by using ether 20 % (26).

8-Heamadsorption test: The test was conducted by using 0.5% guinea pig RBCs (23).

9-Heamagglutination test: micro method was performed by using 0.5% guinea pig RBCs(24).

10- Serological identification of BPIV-3:

1-Heamagglutination inhibition test: B- micro method was performed by using 0.5% guinea pig RBCs, standard antiserum for BPIV-3 and 4 HA units of isolated virus (tenth passage in SEBK cell culture) (24).

2-Serum neutralization test: Beta neutralization procedure was applied by using standard positive serum for BPIV-3 and 100 TCID₅₀ of isolated virus ($2 \times 10^{4.5}$ TCID₅₀/0.1ml) were used (25).

3-Indirect fluorescent antibody technique (IFAT):

IFAT was performed by using SEBK cell culture was prepared in Leighton tubes, monoclonal anti BPIV-3 immune serum, and Goat Anti-mouse IgG –FITC conjugate (27).

11-Indirect ELISA test for the detection of BPIV-3 antibodies (Cypress diagnostica/Belgium): Sera were collected from calves with respiratory infection were examined by ELISA test during acute and convalescent period.

Results

1-Isolation of BPIV-3 in cell culture:

Secondary embryonic bovine kidney(SEBK) cell culture inoculated with nasal swabs were collected from infected calves with respiratory infection at Al-Naser dairy cattle farm station showed no cellular changes after two blind passages, However the 3th passage showed cytopathic effects (CPEs) after 5 days Post infection (P.I.) characterized by opaque foci of cells and lead eventually to cell rounding and degeneration of floating cells in cell culture medium (Fig.1) but no cellular changes were noticed in control SEBK cell culture (Fig.2).

A further passage of harvested virus, CPE became more pronounced and was detected after (48 – 72) hr. P.I., mainly after 8 passages with formation of small and large syncytia (Fig.3) but no cellular changes were noticed in control SEBK cell culture (Fig.4).

Primary chicken embryo fibroblast cell culture inoculated with the 8th passage of SEBK cell culture virus showed CPEs started after 3 days P.I. (Fig.5) , but no cellular changes were noticed in control Primary chicken embryo fibroblast cell culture (Fig.6).

Similarly secondary sheep testis cells inoculated with the 8th passage of SEBK cell culture virus showed CPEs started after 2 days P.I.(Fig.7) but no cellular changes were noticed in control secondary sheep testes cells.

(Fig.8).Both types of infected cell culture showed focal cell rounding and formation of syncytia.

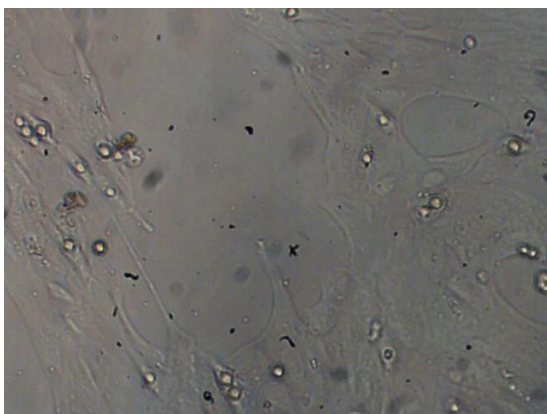


Figure 1

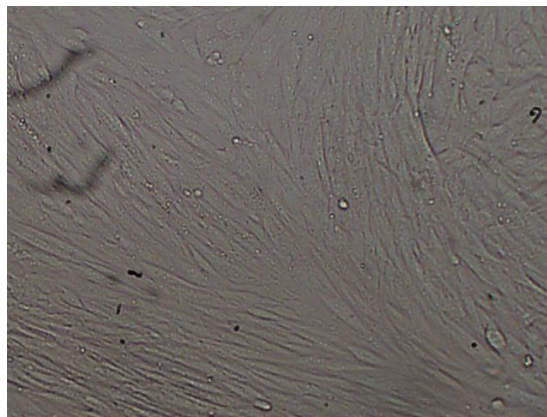


Figure 2

Figure 1: infected SEBK cells with BPIV-3(4 passages), after 7 days of virus inoculation, opaque foci of cells, focal rounding of cells with small and large syncytia formation. 100X

Figure 2: normal SEBKcells 100X.

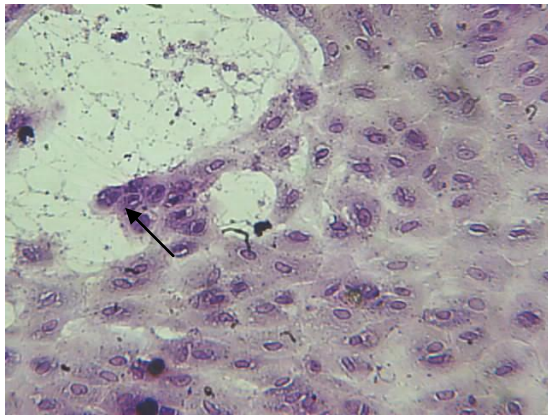


Figure 3

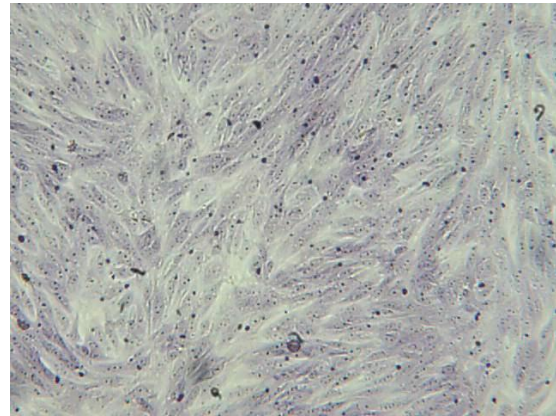


Figure 4

Figure 3: infected SEBK cells with BPIV-3 (8 passages), after 3 days of virus inoculation opaque foci of cells, focal rounding of cells with small and large syncytia formation (→), the syncytia increase in number and size. 100X

Figure 4: normal SEBK cell culture 100X.

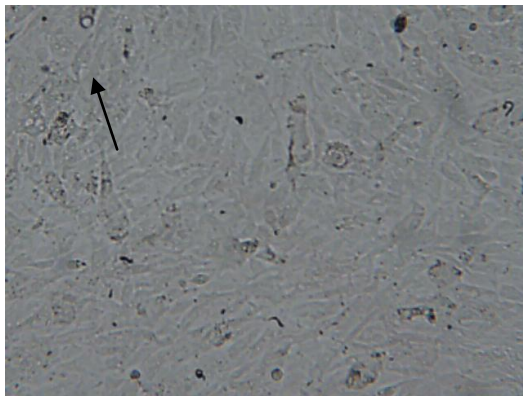


Figure 5

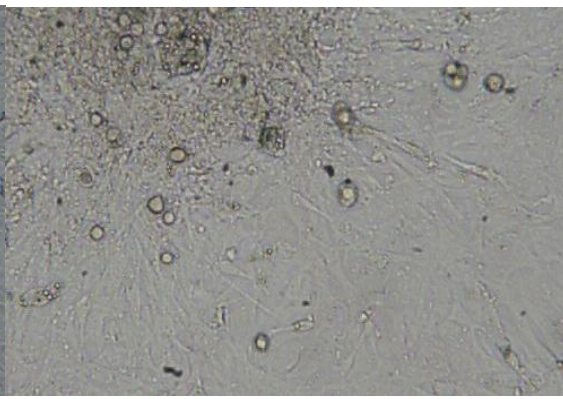


Figure 6

Figure 5: Infected Chicken embryo fibroblast cell cultures 100X after 5 days of virus inoculation (3 passages), focal rounding of cells with formation of giant cells

Figure 6: Normal Chicken embryo fibroblast cell cultures 100X

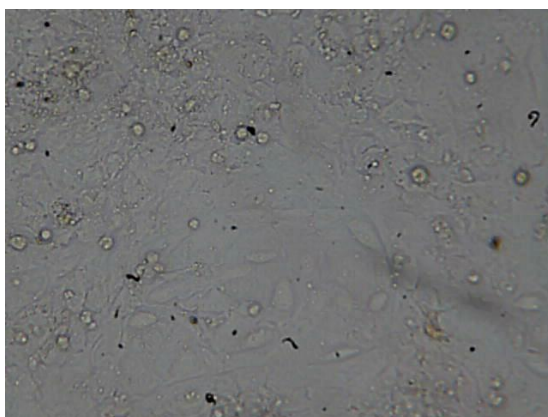


Figure 7

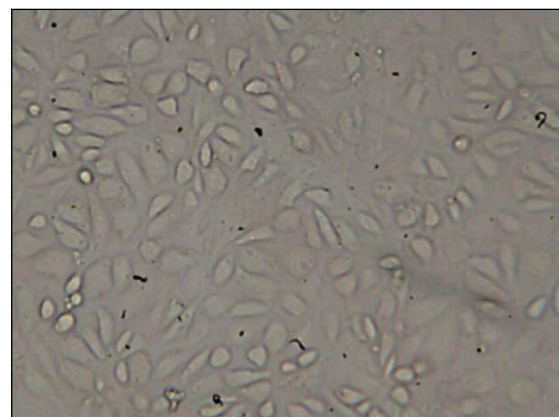


Figure 8

Figure 7: Infected secondary sheep testis cell cultures 100X after 5 days of virus inoculation (3 passages), focal rounding of cells with formation of giant cells

Figure 8: normal secondary sheep testis cell cultures 100X.

2-Titration of the isolated virus: The titer of the isolated BPIV-3 in infected SEBK cells was 2×10^2 TCID₅₀/0.1ml in passage 6; however the virus titer increased with further passages to reach its maximum titer $2 \times 10^{4.5}$ TCID₅₀/0.1ml in 9th passage. Table (1).

Table (1): Titration of isolated BPIV-3 in SEBK cells.

Passage number	Virus titer TCID ₅₀ /0.1ml
6	2×10 ²
7	2×10 ^{2.6}
8	2×10 ³
9	2×10 ⁴

3-Inclusion bodies' detection: Intracytoplasmic inclusion bodies' were observed, in the cytoplasm and giant cells formation were also observed in 2nd days following inoculation of SEBK cell culture, In the cytoplasm inclusion bodies' increase in size and number until the 5 days P.I.. Intracytoplasmic inclusion bodies 'were surrounded by clear halo zone as seen in Figure (9, 10):

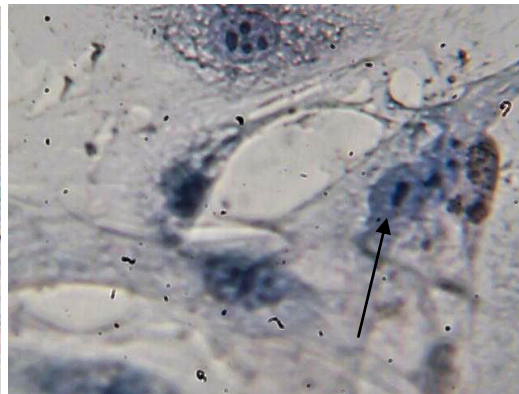
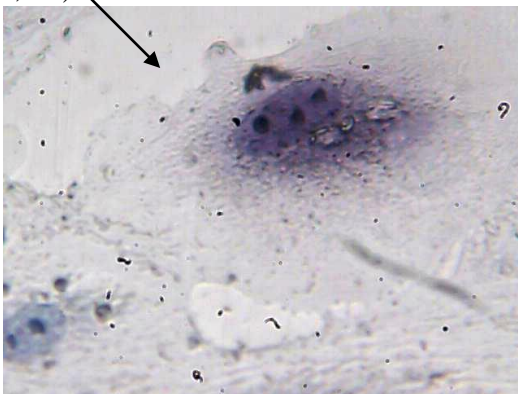


Figure 9

Figure 10

Figure (9, 10): Intracytoplasmic inclusion bodies (—) after 72 hours post infection 400X.

4- Ether sensitivity:

Isolated virus was sensitive to ether; no infectivity could be detected in inoculated SEBK cell culture following virus treatment with 20% diethyl ether in comparison to control virus which was treated with normal saline.

5-Haemadsorption test: infected SEBK cells with isolated virus (8 passages) showed positive adsorption of guinea pig RBCs around the infected cells Figure (11) in comparison to non infected SEBK cell culture (control) Figure (12):

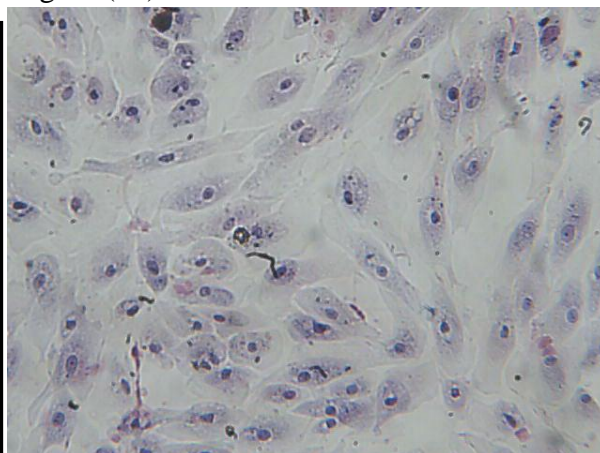
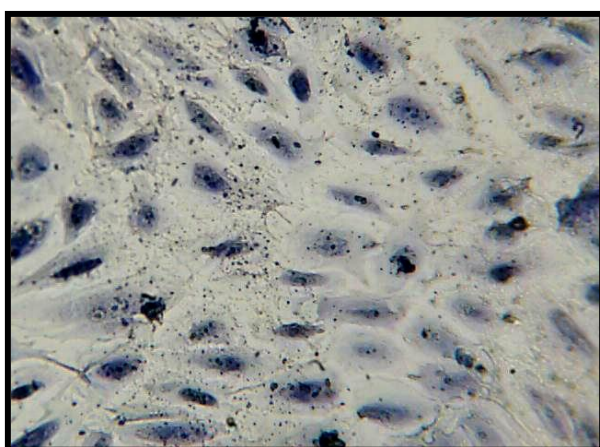


Figure (11): haemadsorption test showing adsorption of guinea pig RBCs around the infected SEBK cells 100 x.

Figure (12): Non infected SEBK cell culture 100 x.

6: Heamagglutination test: The results of heamagglutination showed ability of isolated virus to agglutinate guinea pig RBCs it indicated positive result started with titer 4 HA unit in the tenth passage to 16 HA unit in the twelfth passage as explained in table (2).

Table (2): the results of heamagglutination test

Passage number In SEBK cell culture	titer of heamagglutination test/0.5%guinea pig RBCs
3rd passage	0
fourth passage	0
fifth passage	0
sixth passage	0
seventh passage	0
eighth passage	0
ninth passage	0
tenth passage	4
eleventh passage	8
twelfth passage	16

7-Serological identification of BPIV-3:

1- Heamagglutination inhibition test (HI): The standard positive serum for BPIV-3 gave positive result to inhibit the heamagglutination of 4 HA units of the isolated virus and the titer of the standard positive serum was 32.

2-Serum neutralization test: The standard positive serum for BPIV-3 neutralized 100 TCID 50 of Iraqi viral isolate with the titer of 4 by using SEBK cell culture.

3-Indirect fluorescent antibody technique (IFAT): BPIV-3 antigen was detected in infected SEBK cells by IFAT which was carried out after 48 and 72 hours Post infection, few fluorescing cells were noticed in passage 4, but further passage of the virus result in increasing number of fluorescing cells figure (13):

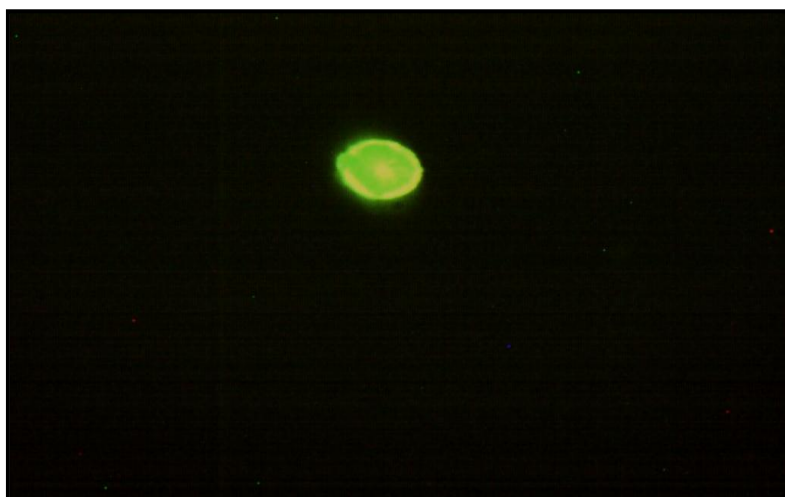


Figure 13: Result of IFAT showing positive result.

8- ELISA test for BPIV- 3 antibodies detection: Results of ELISA test showed positive detection of BPIV- 3 antibodies in serum samples of two calves suffered from respiratory tract infection as these calves were the same animals from which virus was isolated from their nasal swabs. These calves showed an optical density of 1.543 and 0.860 respectively in their

sera during acute phase then elevated to 1.856 and 1.429 during convalescent phase (after two weeks), (table 3):

Table (3): Optical Density (OD) of specific BPIV-3 in sera of infected animals

samples	Age of calf (days)	OD of samples (acute phase)	OD of serum sample (convalescent phase)*
1-serum 1	40	1.543	1.856
2-serum 2	30	0.860	1.429
3-kit positive serum	*NA	1.296	-
4-kit negative serum	*NA	0.118	-

* Convalescent phase: serum was taken after two weeks of the first serum.

*NA: not applicable.



Figure (14)

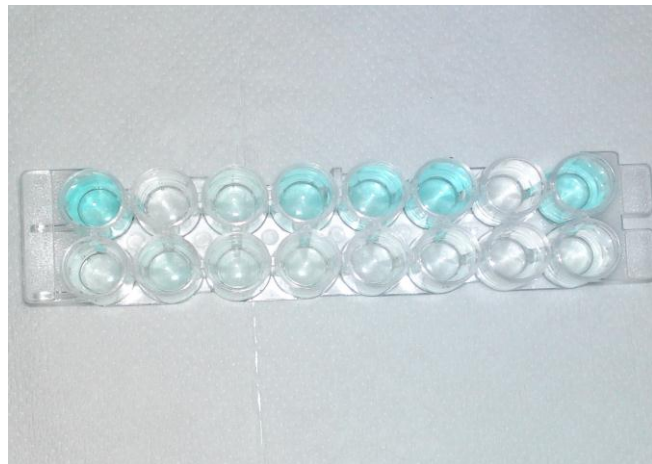


Figure (15)

Figure (14, 15): Positive results of ELISA test for sera of infected animals from which virus was isolated

Discussion

Bovine parainfluenza type 3 viruses are an important pathogen for cattle but some variants can cause disease in human. Laboratory detection of BPIV-3 is classically performed by virus isolation in cell culture. This study is the first in Iraq to record the presence of BPIV-3 in calves with respiratory tract infection by detection of bovine viral isolate by using different techniques beside detection of specific viral antibodies in infected calves with respiratory infection. Based on the results which were obtained, the isolation was achieved through three passages applied in SEBK cell culture giving rise to one isolate developed clear CPE; the CPE of BPIV-3 in SEBK cell culture was evident from 2 days post infection as giant cells, formation of syncytia with intracytoplasmic inclusions in agreement with others (29).

CPEs revealed that isolated virus produced sever cellular changes in mammalian cell culture which were similar to those reported by others studies (30), in bovine kidney by T1 strain of virus, and by other studies(31) for BPIV-3 in monkey kidney cell culture. Based on results were obtained; the SEBK cell culture was suitable for isolation of BPIV-3 in agreement with others (32),(33). The secondary sheep testis and chicken embryo fibroblast

were suitable for propagation of BPIV-3 virus in agreement with other studies (32) , (33).The new isolated virus produced hemadsorption with 0.5% G.pig RBCs at 25 °C and agglutinate G.pig RBCs at 37 °C due to the virus has HN spikes as other bovine parainfluenza viruses in agreement with others(34).Virus passages revealed low titer of HA units of hemagglutination test due to high amount of virions needed to give one HA unit in agreement with other studies(35). The infectivity of isolated virus was destroyed by ether, this finding agreed with earlier studies which have shown that BPIV-3 is sensitive to ether (34).Serological testes (HI and neutralization test) revealed that the isolated virus was BPIV-3 by using standard positive serum for BPIV-3 in agreement with others(31) .IFAT is very specific assay for detection of BPIV-3 in agreement with other studies which have shown that the FAT is the most commonly assay used for specific testing of BPIV-3 and FAT is sensitive and reliable method for detection of BPIV-3, and its sensitivity is reported to be at least 3 times greater than hemadsorption (15) , .In a number of other studies both FAT and HA test have been used and in all cases, FAT has achieved greater sensitivity than HA test (36). Based on the results small intracytoplasmic eosinophilic granular inclusions were observed, and surrounded by clear halo similar results previously reported (37). ELISA test appears to be very sensitive to detect specific BPIV-3 antibodies in sera of infected calves, this finding agreed with earlier studies which have shown that ELISA test was more sensitive than HI test because it permitted the detection of both antibodies specific for Hemagglutinin glycoprotein and neutralizing antibodies which are specific for other glycoproteins in compare with HI test which permitted the detection of only antibodies specific for Hemagglutinin glycoprotein, the titer s obtained by ELISA test 4 to 64 times higher than HI(38).

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