DETECTION OF AUJESZKY'S (PSEUDORABIES) DISEASE VIRUS IN WILD BOARS AND DOMESTIC LOCAL ANIMALS BY POLYMERASE CHAIN REACTION (PCR)

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ABSTRACT

Aujeszky's disease of swine is caused by a herpesvirus that was been classified within the subfamily *Alphaherepevirinae* of the family *Herpesviridae*. The disease characterized by causing respiratory and nervous signs in domestic swine and wild boars. The neurological signs were similar to that of rabies, accordingly it was also known as pseudorabies. The virus can be transmitted to other domestic animals causing the same neurological signs in addition to mad itch. In Iraq there was no study concerning the swine as there was no swine industry. Recently, wild boars appeared in increasing number in Iraq and along the rivers of Tigris, Euphrates, and Diyala. To point out the possibility of presence of pseudorabies virus (PrV) in wild boars and local domestic ruminants, this study was designed. Accordingly, 100 blood samples were collected from cows (51), sheep (34), and killed wild boars (15). Furthermore, tissues from cervical lymph nodes trigeminal ganglion and tonsils also were collected from wild boars. All samples were processed, DNA extracted, and subjected to PCR using specific primers for glycoprotein gene (gII). The results showed that all the tested samples were negative for PrV. The DNA of the virus was detected by PCR in the cervical lymph nodes and blood 24 to 48 hrs post experimental inoculation of mice with killed vaccine of Aujeszky's disease virus. Further wide epidemiological study might be required to be sure that animals in Iraq are free of PrV.

Key words: Aujesky's disease, Pseudorabies, Polymerase Chain Reaction.

INTRODUCTION

Aujeszky's disease is caused by *Suid herpesvius*-1, and also known as pseudo-rabies virus (PrV). Pigs in which subclinical and latent infections can occur, are the natural host of the virus (Pomeranz *et al*., 2005 ; Muller *et al*., 2011). Other animal species also susceptible to infection, including bovine, sheep, and hunting dogs (Cay and Letellier, 2009 ; Muller *et al*., 2011), and cats (Moreno *et al*., 2015). The disease in these animals is usually fatal (Mettenleiter, 2000). Sheep, which are

highly susceptible, may acquire the infection following direct contact with pigs or when sharing the same airspace (Mettenleiter, 2000). Dogs and cats become infected when they feed on pig carcasses (Paenseart and Kluge 1990 ; Cay and Letellier, 2009 ; Moreno *et al*., 2015). The clinical sings in infected pigs appeared as respiratory and nervous signs (Gerdts *et al*., 2000 ; MacLachlan and Dubovi 2011). Respiratory signs included, sneezing, coughing, nasal discharge and dyspnea. Nervous signs appeared as incoordination, tremors, paddling and convulsions (Gerdts *et al*., 2000). Disease in other domestic animals occurs sporadically and is characterized by neurological signs resembling those of rabies (Cay and Letellier, 2009). Intense pruritis (‘mad itch’) leading to self-mutilation is a feature of the disease, particularly in ruminants. The clinical course is short with most affected animals dying with a few days (Quinn *et al*., 2011).

Infection is endemic in the pig populations of most countries. The virus is shed in oral-nasal secretions, milk and semen (Quinn *et al*., 2011). Transmission usually occurs by nose-to-nose contact or by aerosol. Wind-borne transmission over distance of a few kilometers has been recorded (Boadella, 2011).

Many reports mentioned the occurrence of the disease in wild swine (boar) in most countries dealing with swine industries (Muller *et al*., 2010 ; Muller *et al*., 2011 ; Boadella *et al*., 2012 ; Moerno *et al*., 2015). Wild boars become a source of infection for domestic animals (Lari *et al*., 2006 ; Pannwitz *et al*., 2011). The causative is a herpes virus that was classified with subfamily *herpesvirinae*, of the family *herpesviridae*.(Pomeranz *et al*., 2005 ; MacLachlan and Dubovi, 2011).

In Iraq, wild boars are increasing in their population, especially among villages along main revers (Tigris, Euphrates and Diyala). These boars accidently, come in contact with domestic animals of farmers, like cattle and sheep. Furthermore, farmers and due that harms of such boars adapted to hunt them. This will offer swine meat for their domestic dogs and cats when they fed on swine carcasses. The above-mentioned data, led as to speculation on the possibility of wild boars infection in Iraq, and also the possible transmission of such disease to domestic animals in this country. Until the time of preparation of this manuscript, there is no data about the infection of domestic animals with Aujeszky’s disease in Iraq. Accordingly, this study was designed to check some of ruminants for Aujeszky’s disease in some areas that possibly in contact with wild boars along the river of Diyala (Al-Mugdadia and Abu-Saida) and Tigris (Al-khalis, Jadidat Al-Shat and Al-Suwairah).

MATERIALS AND METHODS

SITE OF STUDY

Laboratory and experimental studies were carried out in the laboratory of molecular biology, and laboratory of virology and immunology, College of Veterinary Medicine, University of Diyala, IRAQ.

COLLECTION OF SAMPLES

The study was carried out from August 2016 to November 2017. Blood samples were collected from 51 cows, 34 sheep and 15 killed wild boars of different ages (Figure 1 and 2). Furthermore, tissue samples from lymph nodes, tonsils and trigeminal ganglion of 15 killed boars were collected, labeled and kept in -30 ºC until use. These animals were distributed in different villages of different local towns of Diyala province and Al-Rahmania village of Al-Suwairah town of Wasit province (Table 1). 10 ml of blood from each animal were collected from Jugular vein using 5 ml size EDTA glass tubes.

Figure 1. A hunted wild boar in Al-Mugdadia, Diyala province. Two dogs attacked the killed boar

Figure 2. Live wild boar at the time of hunting. It was attacked by two white dogs at Al-Mugdadia, Diyala province

Table 1. The number of animals included in this study according to their species and locality

Animal species	Number of animals according to locality					Total number (%)
	Al-Mugdadiah	Abu-Saida	Al-Khalis	Jadidat Al-shat	Al-Rahmania	
Cow	16	11	9	8	7	51%
Sheep	6	5	7	12	4	34%
Wild boars	4	3	2	1	5	15%
Total	26	19	18	21	16	100%

PROCESSING OF SAMPLES

Collected blood samples with EDTA of each sample were centrifuged at 1300-1500 rpm for 5 minutes using refrigerated centrifuge (Eppendorf 5810R). The upper layer of plasma and the buffy coat of cells were collected by micropipette and sterile tips. The collected samples were kept in sterile Eppendorf tubes, labeled, and kept at -30 ºC until use.

MOUSE INOCULATION

Killed viral vaccine against Aujeszky's disease was used to follow the fate of pseudorabies virus (PrV) antigens by PCR. This killed vaccine (Aujesky's disease killed vaccine, NYJ.G strain OMP antigen) was kindly provided by CAVAC, South Korea.

Forty white BALB/c mice were divided into two groups equally, A (20) and B (20) mice. Group A was inoculated intra-dermally with 200 microliter of the killed vaccine. Group B mice were inoculated intra-dermally with 200 microliter with sterile normal saline. Group A was totally separated from group B, the mice observed daily, and 24 hours post-inoculation (PI).Two mice from each group was killed daily for 10 days PI. Tissue samples were collected from cervical lymph-nodes and tonsils. Tissue samples for each type were pooled together for a particular time of inoculation, homogenized at 1600 rpm, transferred into sterile Eppendorf tube, and kept at -30 ºC until use. Furthermore, blood samples (plasma and buffy coat) were collected from experimentally inoculated and control mice, for each time and subjected to DNA extraction.

PROCESSING OF BLOOD SAMPLES, SWINE TONSILS AND MOUSE TISSUES FOR PCR

Each collected plasma and buffy coat sample were used for DNA extraction. The extraction was followed by the use of DNA–Sorb-B extraction kit (Sacace Biotechnologies, SrI, 44 Scalabrini str., 22100 Combo, Italy) and according to the manufacturer protocol. Frozen swine and mouse tissues were removed from freezer, thawed at room temperature and used for DNA extraction for PCR as above-mentioned.

AMPLIFICATION BY PCR

DNA extracted from plasma- buffy coat, tonsils tissue samples of boars, and tissues from experimentally inoculated mice were used in this experiment. 15 µl from each DNA sample was mixed with PCR mixture Gene Amp® in 0.5 ml PCR Eppendorf tube. The mixture was composed of 16 µl of nucleotides (dATP, dGTP, dCTP and dTTP), 1 µl Taq polymerase (5 U μl^{-1}), 1µl primer1, 1 µl primer 2, 10µl PCR buffer (10x), and 56 µl sterile deionized distilled water. Primers were selected from the Pseudorabies virus (PrV) glycoprotein gene II (gII) which had 778 base pairs. These two primers were as: P1 (5'ATCTTGTGCAGAACTCCATG3') and P2 (5'TCATTGTACCGGATCATGTC3'). The two primers were designed according to BAMHI digestion map of the Rice strain (Rea *et al*., 1985). The location of gII gene was reported by Robbins *et al.,* (1987). The mixture with DNA sample was processed as 95 ºC for 5 minutes. This was followed by 40 PCR cycles using Eppendorf thermocycler (each cycle was programed as 95 ºC for 1 minute, 55 ºC for 1 minute, and 72 ºC for 2 minutes). This was followed by one cycle of long

extension of 72 ºC for 10 minutes. The cycler was then dropped to 4 ºC, and the PCR product was electrophoresed.

DETECTION OF AMPLIFIED DNA PRODUCTS

Electrophoresis of the PCR sample was carried out by the use of 25 µl of PCR product loaded in well of 1% agarose in 1 x TBE running buffer (0.025 M Tris, 0.192 M glycine, 0.1% w/v SDS). The gel was run at 95V for 2 hours using a 0.1% bromophenol blue as an indicator and ø X 174 DNA molecular weight marker (DNA ladder). Followed electrophoresis, the gel was stained with ethidium bromide (0.5 µg ml^{-1}) for 15 minutes, and DNA products visualized by UV illuminator and photographed.

RESULTS AND DISCUSSION

Samples were collected from suspected animals which were in close contact with wild boars. These animals were grassed near rivers of Diyala and Tigris when wild boars were reported to be appeared in certain times.

It is well known that PrV (Aujeszky') disease caused by a Suid herpesvirus-1. The disease was mainly associated with domestic swine, and can be transmitted to ruminants, whereas, man was refractory to Prv infection (MacLachlan and Dubovi, 2011). Recently many reports mentioned that the disease was reported increasingly in wild boars that might pointed a risk factor of transmission of the disease to domestic swine industry and ruminants that already eradicate the disease (Capua *et al*., 1997 ; Vengust *et al*., 2006 ; Sedlak *et al*., 2008).

In Iraq, there was no swine industry and no data were available on wild boars or PrV disease. Wild boars in Iraq were increased in areas surrounding the main rivers (Tigris, Euphrates and Diyala) as information was received from farmers in such areas. Accordingly, this study was designed.

PCR OF DNA PRODUCTS

All bovine blood samples showed negative results for PCR. The same result was noticed for sheep samples, and 15 of killed wild boars. Only one blood sample from wild boar showed amplified DNA but it was less than 778 bp of PrV. It was about 500 bp when compared to positive control of the PCR kit (Figure 3).

Tissue samples of tonsils that collected from two killed wild boars, DNA extracted and subjected to PCR, also were negative for PrV.

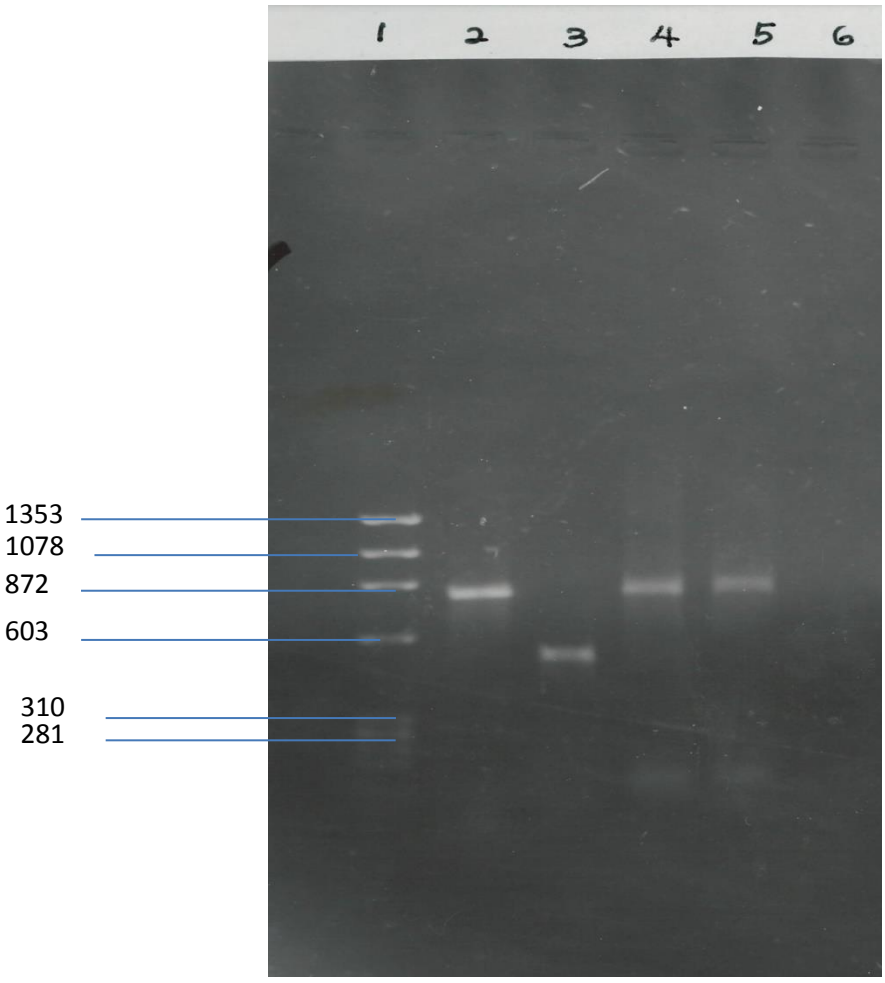

Figure 3. Amplification of PrV from samples showed, Lane 1 is the bacteriophage ø X 174 as DNA ladder. Lane 2 is positive control of PrV PCR kit. Lane 3 PCR of unexpected fragment from buffy coat sample of wild boar. Lane 4 PCR of lymph node of mice 24 PI. Lane 5 PCR of PrV killed vaccine. The products are electrophoresed in 1% agarose at 90 volts for 2 hours

PCR negative results of present study gave an indication on the possibility of absent of active virus in blood samples of such animals. Furthermore, PrV virus causes latency in trigeminal ganglion, neurons, and tonsils of infected animals (Mettenleiter, 2000 ; Romero *et al*., 2003 ; MacLachlan and Dubovi, 2011), and this may explained the absence of viral DNA in such samples. Furthermore, infection of animals rather than pigs was ended with death (MacLachlan and Dubovi, 2011). Negative results also reported with blood samples, trigeminal ganglion and tonsils tissues collected from hunted wild boars of present study, and this can be attributed to same above-mentioned latency. The PCR positive results of one blood sample from wild boar might be attributed to non-specific matching of

primers (Eckert and Kunkel, 1992), or presence of a herpes related sequence latently available some were in such sample.

Tissue samples collected from cervical lymph nodes of experimentally inoculated mice with PrV killed vaccine, showed positive PCR product 24 hours PI while samples collected from trigeminal ganglion and tonsils were negative for PCR. Furthermore, blood samples were positive for PrV (Figure 4). After 48 hrs PI, the same results were observed. Moreover, 72 hrs PI, until the 10th day of the experiment all blood and tissue samples from killed mice were negative for PCR. Furthermore, all tissue and blood samples that collected from control mice at 24 hrs to 240 (10th day) PI, were negative for PCR.

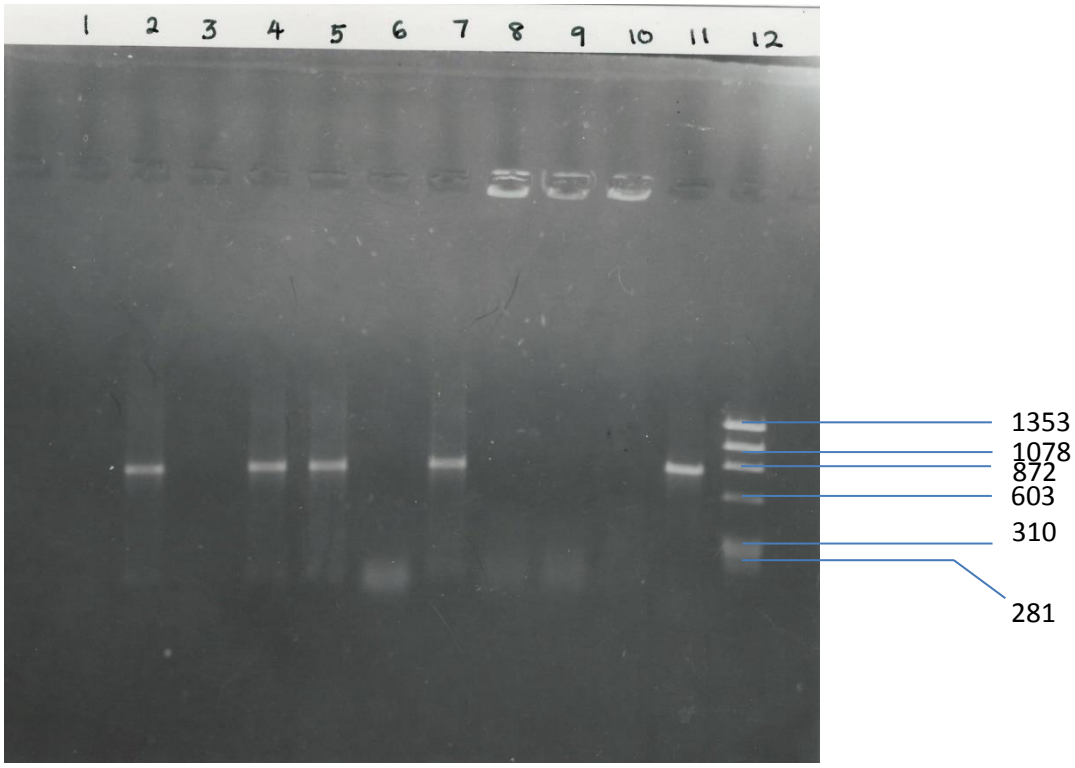

Figure 4. Amplification of PrV from blood samples and boar tonsils. The products were electrophoresed in 1% agarose at 90 volts for 2 hours. Lane 1 is negative PCR from buffy coat of bovine samples. Lane 2, positive PCR of Prv Killed vaccine. Lane 3 negative PCR of buffy coat sample from wild boar. Lanes 4 and 5 positive PCR samples are from lymph nodes of PrV inoculated mice. Lane 6 negative PCR sample from tonsils of PrV inoculated mice. Lane 7 PrV PCR positive buffy coat sample of inoculated mice 48 hours PI. Lanes 8, 9 and 10 negative PCR samples from trigeminal ganglion and tonsils of wild boars. Lane 11 PCR positive control of PrV kit. Lane 12 bacteriophage øX 174 as DNA ladder.

In this experiment we used white BALB/C mice which were inoculated with killed vaccine. This was used only to assess or evaluate of PCR kit and primers to detect viral genome of PrV. The DNA of the virus was detected by PCR 24 hrs PI in cervical lymph nodes. This is a normal drainage of antigen when intra-dermally inoculated in such mice (Day and Schultz, 2011). No viral DNA was detected in mouse tissues from tonsils and trigeminal ganglions. This was a real result on the vaccine that it was killed virus and no chance for PrV to multiply. Killed vaccines always ended fast as they were easily catabolized and neutralized by immune system (Tortora *et al*., 1992 ; Tizard, 2000 ; Day and Schultz, 2011; Quinn *et al*., 2011). We used killed vaccine in this study to prevent the possibility of the spread of the virus from laboratories to our animal industry. Furthermore, no clinical signs were observed on such inoculated mice. It had been reported that PrV caused severe nervous signs in experimentally inoculated mice (Damann *et al.*, 2006).

Many studies used different serological test for identification of PrV in domestic swine and wild boars (Lari *et al.*, 2006 ; Vengust *et al.*, 2006 ; Ruiz-Fons *et al.*, 2008 ; Sedlak *et al.*, 2008 ; Pannwitz *et al.*, 2012 ; Meier *et al*., 2015 ; Milicevic *et al*., 2016). PCR technique was also used, but on samples collected from nervous tissues, trigeminal ganglions, and tonsils of pigs (Mettenleiter, T. C. 2000 ; Muller *et al.*, 2010 ; Moreno *et al.*, 2015). Their results showed that PrV was endemic in Europe.

CONCLUSIONS

Accordingly, and from above mentioned data and findings final conclusions can be speculated. PCR cannot be applied on blood samples from suspected animals as the virus was latently present in nervous tissues and tonsils, and can be used when the animal actively infected with the virus or when the virus somehow was reactivated. The PCR technique can be used to detect the virus in animal secretions of infected animals and in tissue culture when attempt was designed to isolate the PrV. Therefore, a wide epidemiological study must be applied on wild boars in Iraq to confirm the presence of the PrV and to avoid the possibility of virus transmission to our domestic animals.

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الكشف عن فيروس مرض اجسكي (السعار الكاذب) في الخنازير البرية والحيوانات الاليفة المحلية بوساطة اختبار التفاعل المتسلسل لانزيم البلمرة

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المستخلص

مرض اجسكي او السعار الكاذب يسببه فيروس هربز المصنف مع تحت العائلة هربز الفا (*Alphaherepevirinae*) العائدة الى عائلة فيروسات الهربز (*Herpesviridae*). يتميز المرض بانه يحدث علامات تنفسية وعصبية تشبه مرض السعار في الخنازير الاليفة والبرية وعلى هذا الاساس سمي بالسعار الكاذب. الفيروس يمكنه الانتقال الى الحيوانات الاليفة الاخرى محدثا نفس المرض والاعراض العصبية فضلا عن ما يسمى حكة جنون (Mad Itch). لاتتوفر معلومات في العراق حول المرض في الخنازير الاليفة لانها لاتربى ولكن حديثا ظهرت تقارير تشير الى انتشار الخنازير البرية في المزارع في العراق وعلى امتداد نهر دجلة ونهر الفرات ونهر ديالى. لاثبات احتمالية وجود فيروس مرض السعار الكاذب في الخنازير الوحشية والحيوانات المحلية الاليفة فقد تم تصميم هذه الدراسة، وعلى هذا الاساس تم جمع 100 عينة دم من 51 بقرة و34 نعجة و15 خنزيرا بريا فضلا عن انسجة من الغدد اللمفاوية واللوزتين والعقدة التوأمية للخنازيرالبرية المقتولة. تمت معاملة هذه العينات واستخلاص الحمض النووي الريبي اللاوكسجيني DNA منها وتعريضها الى اختبار التفاعل المتسلس لانزيم البلمرة وباستخدام بادءات (primers) خاصة بالجين gII. اظهرت النتائج ان جميع العينات المأخوذة من الخنازير البرية المقتولة او من الابقار والاغنام هي سالبة لوجود فيروس مرض السعار الكاذب، ولكن تم الكشف عن وجود الحمض النووي الريبي اللاوكسجيني بوساطة اختبار PCR في العقد اللمفوية الرقبية والدم بعد 24 الى 48 ساعة من حقن فئران تجريبيا بلقاح مقتول لفيروس اجسكي، وتقترح االدراسة اجراء فحوصات وبائية واسعة مصلية وبايولوجية جزيئية لاثبات خلو العراق من هذا المرض.

الكلمات المفتاحية: مرض اجسكي، السعار الكاذب، اختبار انزيم البلمرة المتسلسل.