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Sudan University of Science and Technology

College of Graduate Studies

Detection, Molecular and Genetic Characterization of Peste des Petits Ruminants Virus (PPRV) from Sheep, Goats and Gazelles in the Sudan

الكشف ودراسة الخصائص الجزيئية والجينية لفيروس طاعون المجترات الصغيرة

من الضإن والماعز والغزلان في السودان

A thesis submitted to the College of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Veterinary Medicine (M.V.M.) in Microbiology (Virology)

By

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Declaration of the Status of Thesis

By Student

The work described in this master degree thesis was carried out at the Virology Department Laboratory, Central Veterinary Research Laboratories (CVRL), Soba and the Department of Pathology, Parasitology and Microbiology at the College of Veterinary Medicine, Sudan University of Science & Technology from February 2016 to February 2020 under the supervision of Dr. Nussieba Ahmed Osman Elhag.
The experimental work is original and the thesis has not been submitted partially or fully to any other University.

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Dr. Nussieba Ahmed Osman Elhag
Supervisor
February, 2020

Dedication

To my lovely mother & father

To my husband and children

To my dears sisters & brother

To all my friends & colleagues

With love and respect

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First and foremost, my heartfelt thanks to almighty Allah for giving me the strength and willpower to complete this work.

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Abstract

The present study was designed to identify, isolate and characterize PPRV currently circulating in small ruminants “sheep and goats” and gazelles in the Sudan. A total of 359 whole blood and tissue samples of sheep and goats collected from ten different States of the Sudan were screened for the presence of PPRV antigen by an IC-ELISA assay. The results showed that PPRV antigen could be detected in 180/359 samples with an overall antigenic prevalence of 50.1%. On the species basis, higher antigenic-prevalence was obtained from goats samples (63.4%, 45/71) compared to sheep samples (46.9%, 135/288).

During 2015 to 2018, many suspected PPR outbreaks were occurred among sheep and goats in many localities within different States of the Sudan. These PPR suspected outbreaks were recognized mostly by the appearance of the typical clinical signs of the disease in small ruminants and were associated with a higher morbidity and mortality rates. Of the total 276 antigen samples from sheep and goats collected from suspected PPR outbreaks, 160/276 samples were found positive by an IC-ELISA, with an overall antigenic indicence of 58%. Considering the animal species, of the total 223 antigen samples collected from sheep, 123/216 (56.9%) whole blood samples and 4/7 (57.1%) lung were found positive for PPRV antigen. Of the total 53 antigen samples collected from goats, 31/50 (62%) whole blood samples and 2/3 (66.7%) lungs were found positive for PPRV antigen. Moreover, of the five States under study, the highest antigenic incidence was demonstrated in Western Kurdufan State (81.2%) while the least incidence was present in Elgedarif State (50%).

To investigate the presence of PPRV in sheep and goats in slaughter houses, screening of the collected 83 lung samples from sheep (65) and goats (18) by an IC-ELISA revealed that 20/83 samples were positive with an overall antigenic prevalence of 24.1%. Considering the animal species, higher overall antigenic prevalence (66.7%, 12/18) was detected among goats compared to sheep (12.3%, 8/65). Within States under study, results indicated that the highest antigenic-prevalence was demonstrated in Northern State (80%) while no incidence (0%) was present in Kassala State.

From the several attempts, PPRV isolation was successful only from two field samples in Vero cells inoculated with 10% lung homogenate. These two PPRV isolates designated as “PPRV/tc/Sudan/Khartoum/Bahri/2015 isolate” and

"PPRV/tc/Sudan/Gedarif/2015 isolate" were originated from goat lung from Bahri (Elfaki hashim), Khartoum State and from sheep lung from Elgedarif State, respectively. Typical PPRV cytopathic effect (cpe) consisting of cell rounding, detachment of the cells from the monolayer sheet, destruction of the monolayer sheet and syncytia formation appeared starting from day 15 post infection (p.i.) and completed at 27 d.p.i. Subsequently, PPRV isolates were identified using IC-ELISA and RT-PCR assays.

To confirm the positive results obtained by the IC-ELISA assay, 32 samples selected from sheep (27) and goats (5) were further analyzed using PPRV N-gene based RT-PCR assay. All of the analyzed samples from sheep and goats yielded bands of around 351 bp corresponding to the partial N-gene. The 351 nt partial PPRV N-gene nucleotide sequences of nine sheep and goat samples, which were positive when analyzed by RT-PCR, indicated that the PPRV obtained were clustered genetically with Lineage IV. PPR viruses sequenced in this study designated as "PPRV/Sudan/Khartoum/2015; PPRV/Sudan/Gedarif/2015; PPRV/Sudan/Gezira/Kab-Elgidad/2016; PPRV/Sudan/River-Nile/Garie/2016; PPRV/Sudan/Northern/Dongola/2017; PPRV/Sudan/Red-Sea/Port-Sudan/2017; PPRV/Sudan/White-Nile/Kosti/2017; PPRV/Sudan/Western-Kurdufan/Abuzabad/2017; PPRV/Sudan/Northern-Kurdufan/El-Obeid/2017" were deposited in the NCBI GenBank database under accession numbers MK371448.1-MK371456.1, respectively. Subsequently, sequence comparison and phylogenetic analysis confirmed that the nine PPRV strains from sheep and goats in the Sudan belonged to the PPRV lineage IV genotype and shared the closest sequence identity with PPRV strains originating from North African countries.

During 2016 and 2017, specimens were collected from six apparently healthy semi-captive and captive Dorcas gazelles "*Gazella dorcas*" from three areas in Khartoum State. In May 2017, many free-ranging Dorcas gazelles with suspected signs of PPR were reported in Dinder National Park. PPRV antigen and nucleic acid were detected in specimens from gazelles by an IC-ELISA and RT-PCR assays. One PPRV isolate designated as "PPRV/Gazelle/tc/Sudan/DNP2017" was recovered in Vero cells, from a lung sample of one gazelle from Dinder National Park outbreak. The partial PPRV N-gene nucleotide sequences of three gazelle samples, which were positive when analyzed by RT-PCR, was determined. The N-gene (351 nt) partial nucleotide sequences of three PPRV designated as "PPRV/Gazelle/Sudan/Khartoum2016-1" and "PPRV/Gazelle/Sudan/Khartoum2016-2" were originated from two whole blood

samples from Soba East and Elazhari, Khartoum State, respectively, and "PPRV/Gazelle/Sudan/DNP2017" was originated from one lung sample from a gazelle with suspected PPR from Dinder National Park were deposited in the NCBI GenBank database under accession numbers MG992016.1-MG992018.1, respectively. Further, genetic characterization clustered all PPRV from gazelles into lineage IV genotype. Antibodies developed against PPRV nucleoprotein were detected in sera from surviving gazelles from Dinder National Park outbreak using a C-ELISA assay. Based on the apparent clinical signs, higher fatalities and laboratory investigations the involvement of PPRV was confirmed. The present study demonstrates that gazelles are a potential wild small ruminant host for PPRV and may influence the epidemiology of PPR in the Sudan. This is the first report describes PPRV infection among gazelles in the Sudan and in Africa.

ملخص البحث

الدراسة الحالية اجريت للتعرف علي والعزل والتمييز الجزيئي لفيروس طاعون المجترات الصغيرة والمنتشرة حالياً في المجترات الصغيرة "الضلأن والماعز" والغزلان في السودان. تم جمع 359 عينة دم كامل وعينات انسجة من الضأن و الماعز من عشرة ولايات مختلفة من السودان ، ثم تم الفحص عن وجود انتجين فيروس طاعون المجترات الصغيرة بإختبار المقايسة المناعية IC-ELISA. اثبتت النتائج وجود انتجين فيروس طاعون المجترات الصغيرة في 180 عينة من مجموع 359 عينه بمعدل انتشار عام انتجيني 50.1%. عند الأخذ في الإعتبار نوع الحيوان فإن اعلي معدل انتشار انتجيني تم الحصول عليه من عينات الماعز (63.4%, 45/71) مقارنة بعينات الضأن (46.9%, 135/288).

خلال الفترة بين 2015-2018م ظهرت العديد من الاوبئة المشتبه بانها نتيجة للإصابة بفيروس طاعون المجترات الصغيرة بين قطعان الضأن والماعز في محليات عديدة من ولايات السودان المختلفة. هذه الاوبئه اشتبه بانها ناتجة عن مرض طاعون المجترات الصغيره وتم التعرف عليها غالباً بظهور الاعراض المرضية المميزة للمرض في المجترات الصغيرة مصحوبه بنسبة اصابة ووفيات عاليه. من إجمالي 276 عينة مستضد من الضأن والماعز تم جمعها من الاوبئة المشتبه أنها ناتجة بسبب مرض طاعون المجترات الصغيرة ، فإن 160 عينة من مجموع 276 عينة وجدت موجبة بإختبار المقايسة المناعية IC-ELISA وبمعدل حدوث عام هو 58%.

عند الأخذ في الإعتبار نوع الحيوان ، فإن من مجموع 223 عينة مستضد جمعت من الضأن ، كانت هناك 123 عينة من مجموع 216 عينة دم كامل (56.9%) و4 عينات من مجموع 7 عينات نسيج رئوي (57.1%) وجدت موجبة لمستضد فيروس طاعون المجترات الصغيرة. بينما من مجموع 53 عينة مستضد جمعت من الماعز فإن 31 عينة من مجموع 50 عينة دم كامل (62%) وعينتان من مجموع ثلاثة عينات نسيج رئوي (66.7%) وجدت موجبة لمستضد فيروس طاعون المجترات الصغيرة. بالإضافة فمن الخمس ولايات التي اجريت فيها الدراسه ، فإن اعلي معدل حدوث للمستضد كان في ولاية غرب كردفان (81.2%) بينما أقل معدل حدوث كان في ولاية القضارف (50%).

للتقصي عن وجود فيروس طاعون المجترات الصغيرة في الضأن و الماعز بالمسالخ ، تم جمع 83 عينة نسيج رئوي من الضأن (65) ومن الماعز (18) وتم اختبارها باستخدام إختبار المقايسة المناعيه والتي كشفت ان 20 عينة من مجموع 83 عينة نسيج رئوي كانت موجبه بنسبة معدل انتشار انتجيني عام 24.1%. مع الأخذ في الإعتبار نوع الحيوان، فإن اعلي معدل انتشار انتيجيني عام (66.7%, 18/12) وقد وجد في الماعز مقارنة بالضأن (12.3%, 65/8). من ضمن الولايات التي اجريت فيها الدراسة ، وضحت النتائج أن اعلي معدل انتشار انتيجيني كانت في ولاية شمال كردفان (80%) بينما لم يسجل اي معدل حدوث للمرض بولاية كسلا (0%).

من المحاولات الكثيرة ، تم بنجاح عزل فيروس طاعون المجترات الصغيرة من عينتين حقلية فقط في خلايا فيرو Vero المحقونة ب 10% من المستخلص النسيجي المحضر من عينات الرئة. عزلتي الفيروس تم تسميتها "PPRV/tc/Sudan/Khartoum/Bahri/2015 isolate" و "PPRV/tc/Sudan/Gedarif/2015 isolate" ومنشأها اصلاً من رئة ماعز من بحري (الفكي هاشم) ، ولاية الخرطوم ومن رئة ضأن من ولاية القضارف علي التوالي. الأثر الإمراضي لفيروس طاعون المجترات الصغيرة في الخلايا شمل إستدارة الخلايا ،

انسلاخ الخلايا من علي سطح الطبقة وحيدة الخلايا ، تدمير الطبقه وتكوين خلايا متعددة الانوية ظهرت ابتداء من اليوم الخامس عشر واكتملت في اليوم السابع والعشرين بعد الحقن. تم التاكد بأن العزلات هي فيروس طاعون المجترات الصغيرة وذلك بإستخدام إختبار المقايسة المناعية IC-ELISA وإختبار تفاعل البلمرة التسلسلي RT-PCR.

لتأكيد النتائج الموجبة التي تم الحصول عليها بإختبار المقايسة المناعية ، تم إختيار 32 عينة منها 27 عينة من الضأن و5 عينات من الماعز والتي تم تحليلها باستخدام إختبار تفاعل البلمرة المتسلسل العكسي والذي يعتمد علي البروتين النووي لفيروس طاعون المجترات الصغيرة. كل العينات التي تم تحليلها من الضأن والماعز انتجت تسلسل نيوكليوتيدات مكون من 351 نيوكليوتيدة والتي كانت مطابقة لجزئية الجين المعني.

تسلسل النوكليوتيدات المكون من 351 نيوكليوتيدة لعدد 9 عينات من الضأن والماعز التي كانت موجبة عند إختبارها بإختبار تفاعل البلمرة المتسلسل العكسي دل علي ان فيروس طاعون المجترات الصغيرة مصنفة جينيا مع المجموعة الجينية الرابعة للفيروس. فيروسات طاعون المجترات الصغيرة والتي تم إجراء التسلسل لها في هذه الدراسة وسميت "PPRV/Sudan/Khartoum/2015; PPRV/Sudan/Gedarif/2015; PPRV/Sudan/Gezira/Kab-Elgidad/2016; PPRV/Sudan/River-Nile/Garie/2016; PPRV/Sudan/Northern/Dongola/2017; PPRV/Sudan/Red-Sea/Port-Sudan/2017; PPRV/Sudan/White-Nile/Kosti/2017; PPRV/Sudan/Western-Kurdufan/Abuzabad/2017; PPRV/Sudan/Northern-Kurdufan/El-Obeid/2017" قد تم ايداعها في بنك الجينات العالمي تحت الأرقام MK371448.1 - MK371456.1 علي التوالي. مقارنة تحليل التسلسل الجيني وتحليل النشوء والتطور اثبتا أن تسعة فيروسات من الضأن والماعز في السودان تنتمي للمجموعة الجينية الرابعة للفيروس وتشارك هوية التسلسل لفيروس طاعون المجترات الصغيرة ذات المنشأ التابع لدول شمال افريقيا.

خلال الفتره بين 2016-2017م ، تم جمع عينات من 6 غزلان من فصيلة الدوركاس "*Gazella dorcas*" وهي سليمة ظاهرياً وكانت أسيرة كلياً او جزئياً من 3 مناطق في ولاية الخرطوم. في مايو 2017م فإن عدد من الإصابات محتمل أنها نتيجة لفيروس طاعون المجترات الصغيرة تم رصدها في العديد غزلان الدوركاس الطليقة في حظيره الدندر الوطنية. تم الكشف عن وجود الانتجين والحمض النووي لفيروس طاعون المجيرات الصغيرة بإستخدام إختباري المقايسة المناعية والبلمرة المتسلسلة العكسية. ثم تم عزل الفيروس في خلايا فيرو من عينة رئة واحده من غزالة مصابة بالمرض من وباء حظيرة الدندر الوطنية وسميت "PPRV/Gazelle/tc/Sudan/DNP2017". تم تحديد تسلسل النوكليوتيدات الجزئى للجين النووي لفيروس طاعون المجترات الصغيرة لـ3 عينات غزلان والتي كانت موجبة بإختبار تفاعل البلمرة المتسلسل العكسي وتمت تسميتها "PPRV/Gazelle/Sudan/Khartoum2016-1" و "PPRV/Gazelle/Sudan/Khartoum2016-2" ومنشأها اصلاً من عينتي دم كامل من الغزلان من سوبا شرق والازهري ، ولاية الخرطوم علي التوالي ، و "PPRV/Gazelle/Sudan/DNP2017" ومنشأها اصلاً من عينة رئة من غزالة من وباء المرض في حديقة الدندر الوطنية ، تم إيداع التسلسل الجيني في بنك الجينات تحت أرقام MG992016.1-MG992018.1 علي التوالي. مقارنة تحليل النشوء والتطور صنفت كل فيروسات طاعون المجترات الصغيرة من الغزلان في المجموعة الجينية الرابعة للفيروس. الأجسام المضادة للفيروس تم كشفها في امصال الغزلان الحية من وباء المرض في حديقة الدندر الوطنية بإستخدام إختبار المقايسة المناعية C-

ELISA. إعتماداً علي ظهور الاعراض السريرية ، نسبة الوفيات العالية والتقصي المعملي اكد أن فيروس طاعون المجترات الصغيرة هو المسبب لهذا الوباء. الدراسة الحالية اثبتت أن الغزلان هي المجترات البرية الصغيرة المحتملة كعائل لفيروس طاعون المجترات الصغيرة ووالتي قد تاثر علي وبائيات مرض طاعون المجترات الصغيرة في السودان. هذا البحث يمثل أول تقرير يصف أصابة الغزلان بفيروس طاعون المجترات الصغيرة في السودان وفي أفريقيا.

Introduction

Peste des petits ruminants (PPR) is an acute, contagious and transboundary viral disease. It affects mainly sheep and goats, occasionally wild small ruminants and in a few occasions camel, cattle and buffaloes (Parida *et al.,* 2015; OIE, 2019). The disease is mostly fatal causing high morbidity rates which may reach 100% and mortality ranges from 50% up to 90% (Abubakar *et al*., 2012). PPR is considered as one of the notifiable diseases to the World Organization for Animal Health (OIE). Due to the higher rates of disease incidence, fatalities, and losses in small ruminants, PPR is generally considered a major constraint for small ruminant production in developing countries as the case in the Sudan (OIE, 2019).
PPR was firstly described in Côte d'Ivoire in East Africa (Gargadennec and Lalanne, 1942). It occurred in most of Africa, the Middle East, Asia, Turkey from Europe and more recently has also been reported from Bulgaria (Banyard *et al.,* 2010: Parida *et al.,* 2016; OIE-WAHIS, 2018; OIE, 2019).
The disease is caused by peste des petits ruminants virus (PPRV) which belongs to the *Small Ruminant Morbillivirus (SRMV)* species, *Morbillivirus* genus of the family *Paramyxoviridae*, order *Mononegavirales* (Gibbs *et al.,* 1979; Maes *et al.,* 2019). PPRV has a single serotype and viral strains were genetically classified into four lineages (I, II, III and IV) based on a partial sequence of the fusion (F) gene (Forsyth and Barrett, 1995; Ozkul *et al.,* 2002) or the nucleoprotein (N) gene (Couacy-Hymann *et al.,* 2002; Kwiatek *et al.,* 2007).
Sheep and goats are the main natural acutely infected hosts of PPRV (Lefevre and Diallo, 1990; Parida *et al.,* 2015). Camels can be infected by PPRV (Roger *et al*., 2000; Khalafalla *et al.,* 2010). PPRV infection of wildlife with a clinical disease were reported in Dorcas gazelle, Thomson's gazelle, gemsbok, ibex, white-tailed deer, wild sheep, wild goats and other small wild ruminant species (Abu Elzein *et al.,* 2004; Kinne *et al.,* 2010; Aziz-ul-Rahman *et al.,* 2018).
Clinically, the disease is characterized by fever, anorexia, serous and mucopurulent oculo-nasal discharges, erosion on the mouth which develop to necrotic stomatitis with gingivitis, conjunctivitis, diarrhea and bronchopneumonia is a frequent complication. The course of the disease may be per acute, acute or chronic (Roeder and Obi, 1999; OIE, 2019). Diagnosis of PPR is based mainly on clinical signs and laboratory

investigations. These diagnostic techniques are based on detection of PPRV antigen, viral nucleic acid and serological tests for detection of PPRV antibodies (OIE, 2019).

In the Sudan, PPR was known since 1971 where outbreaks of a Rinderpest-like disease, in sheep and goats, were reported in Elgedarif in Eastern Sudan (Elhag Ali, 1973). Since then, outbreaks of the disease were reported in Sinnar during the years 1971 to 1972 and in Mieliq in 1972 (Elhag Ali, 1973). At that time the disease was wrongly diagnosed as Rinderpest (RP) but many years later it is confirmed to be PPR (Elhag Ali and Taylor, 1984). Subsequently, outbreaks of the disease were reported continuously during the years. For control of PPR in small ruminants in the field, the live-attenuated cell culture PPRV Nigeria 75/1 vaccine has been used in the Sudan since the beginning of 2000s (Fadol and El Hussein, 2004).

Since its first outbreak and up to present, PPR was found to be one of the most important diseases affecting small ruminants in many States in the Sudan. Due to the continuous outbreaks occurred annually, the Sudan currently became one of PPR endemic countries. Therefore, the current situation of the disease and the lineages circulating in the field should be updated to aid in the control of the disease in the meantime and in the eradication program in the future.

Objectives

The present study was designed to achieve the following objectives:

1- To identify and isolate PPRV strains currently circulating in small ruminants in the Sudan.

2- To firstly isolate and identify PPRV strains infecting gazelles in the Sudan.

3- To perform molecular and genetic characterization of the Sudanese PPR viruses in order to update information about viral lineage (s) that is (are) currently circulating in the country.

Chapter I
Literature Review

1.1. Definition of peste des petits ruminants (PPR):

Peste des petits ruminants (PPR) is described as a severe fast spreading disease of mainly domestic small ruminants and occasionally wild small ruminants that threatens the food security and sustainable livelihood of farmers across Africa, the Middle East and Asia (Ozmen *et al*., 2009; Libeau *et al.,* 2014; Parida *et al.,* 2016; OIE, 2019). PPR is characterized by the sudden onset of depression, fever, discharges from the eyes and nose, sore in the mouth, disturbed breathing and cough, foul smelling diarrhea and death (Roeder and Obi, 1999; OIE, 2019).

1.2. Synonyms of PPR:

Peste des petits ruminants (PPR) has various synonyms including kata which is used locally in Western Nigeria for a pseudo-rinderpest of goats (Whitney *et al.,* 1967). Also PPR was named as psuedo-rinderpest, pneumoenteritis complex, stomatitis-pneumoenteritis syndrome and stomatitis-pneumoenteritis complex (Nduaka and Ihemelandu, 1973; Braide, 1981; Merck, 2010), goat plague, pest of small ruminants, rinderpest-like disease or syndrome pseudo-rinderpest of small ruminants (Johnson and Ritchie, 1968; Durtnell and Eid, 1973), contagious pustular stomatitis and contagious vulvovaginitis (Wosu, 1994). The disease was given a French name peste des petits ruminants because of its clinical, pathological and immunological similarities with rinderpest (Gibbs *et al*., 1979).

1.3. Economic importance of PPR:

Owing to the highly contagious nature and the rapid spread of the disease, PPR has major economic consequences for sheep and goat farming. Around 800 million animals are estimated to be susceptible in countries which report the presence of the disease (Taylor, 1984; Lefevre and Diallo, 1990). PPR represents one of the most economically important animal diseases in areas that rely on small ruminants. Outbreaks of the disease tend to be associated with contact of immuno-naïve animals with animals from endemic areas. In addition to occurring in extensive-migratory populations, PPR can occur in villages and urban settings though the number of animals is usually too small to maintain the virus in these situations (Abubakar *et al*., 2012). Morbidity rate in

susceptible populations can reach 90-100%. Mortality rates vary among susceptible animals but can reach 50-100% in more severe instances. Both morbidity and mortality rates are lower in endemic areas and in adult animals when compared to young ones (Abubakar *et al.*, 2012). The presence of PPR in developing countries can have a serious impact on livestock production and can limit local trade and export. Economic losses are due to loss of production, death, abortion and cost of controlling the disease (Banyard *et al.,* 2010).

1.4. History of the disease:

Historically, PPR was primary associated with French-speaking West African countries and was reported for the first time in the Ivory Coast (Code D'Ivoire) in 1940 (Gargadennic and Lalanne, 1942). Afterwards, it was quickly recognized in other French West African colonies. PPR was observed and identified in Senegal in 1955 (Mornet *et al.,* 1956; Bourdin, 1973). The early work showed that the causative virus was related to rinderpest virus (RPV) with adapted pathogenicity to goats and sheep (Mornet *et al.,* 1956). A syndrome which closely resembled rinderpest, known locally as "Kata" and characterized by high mortality, was described in Western African Nigeria dwarf goats (Whitney *et al.,* 1967).

A syndrome which closely resembled rinderpest, known locally as "Kata" and characterized by high mortality, was described in Western African Nigeria dwarf goats (Whitney *et al.,* 1967).

The first outbreak of the disease in sheep and goats in north-east African countries was in Gedarif in Eastern Sudan (Elhag Ali, 1973) which was firstly diagnosed as rinderpest and later confirmed to be PPR (Elhag Ali and Taylor, 1984).

PPR outbreak was first reported in 2008 in Northern parts of Tanzania (Swai *et al.*, 2009). In Iran, a clinically, pathologically and serologically documented outbreak of PPR was first reported in 1995 (Radostits *et al.*, 2006). In India the disease was first reported in 1987 (Shaila *et al.*, 1989) and in 2000 in Iraq (Barhoom *et al.*, 2000).

In Oman, PPRV was isolated for the first time from a collection of wild animals in a zoological garden (Furley *et al.*, 1987). Taylor *et al.* (1990) stated that the disease seems to be endemic in Oman. PPR was reported in Lebanon in 1987 (Lefevre *et al.*, 1991).

The disease was suspected in Saudi Arabia in sheep (Asmar *et al.*, 1980) and also in gazelles (Hafez *et al.,* 1987). However, successful PPR virus isolation was performed from diseased goats (Abu Elzein *et al.*, 1990). During 2002 disease outbreak was

reported in a mixed flock of sheep and goats in Al-Hasa province, Hofuf in the eastern region of Saudi Arabia (Housawi *et al*., 2004). The first confirmation of PPRV in Pakistan was in 1994 (Hussain *et al*., 1998).

In Tunisia, the first positive serological detection of PPRV was in 2006 (Ayari-Fakhfakh *et al.,* 2011). Nowadays, PPR is well known not only in the whole of Africa but also in Asia and the Middle East. In Turkey, the first detection of PPR was in 1992, in south-east Anatolia, and it was detected using serological techniques (Guler *et al.,* 2014). Recently the disease reached Europe in 2016 (Parida *et al*., 2016).

1.5. Causative agent:

1.5.1. Virus classification:

Peste des petits ruminants is caused by peste des petits ruminants virus (PPRV) which belongs to the *Small Ruminant Morbillivirus (SRMV)* species, *Morbillivirus* genus of the family *Paramyxoviridae*, order *Mononegavirales* (Gibbs *et al.,* 1979; Maes *et al.,* 2019).

The *Paramyxoviridae* family is subdivided into seven genera namely *Aquaparamyxovirus, Avulavirus, Ferlavirus, Henipavirus, Morbillivirus, Respirovirus* and *Rubulavirus.* The *Morbillivirus* genus contains several species which are closely related to PPR, these viruses include: *Canine Morbillivirus, Cetacean Morbillivirus, Feline Morbillivirus, Measles Morbillivirus, Small Ruminant Morbillivirus, Phocine Morbillivirus, Rinderpest Morbillivirus* (Maes *et al.,* 2019).

1.5.2. Virus properties: virion size and morphology:

Peste des petits ruminants (PPR) virions are enveloped, pleomorphic particles containing a genome of single-stranded RNA that is enclosed in a ribonucleoprotein (RNP), and core (Munir *et al*., 2013). The Virions are covered with large peplomers, and contain a herringbone-shaped helically symmetrical nucleo-capsid, the diameter of which ranging between 150-300 nm (Murphy *et al.,* 1999).

The genome is a negative-sense single-stranded RNA about 15,948 nucleotides long with negative polarity (Chard *et al.,* 2008), which is considered the longest of all of the morbillivirus members (Bailey *et al*., 2005). The genes are arranged in the order of 3′ N–P/C/V–M–F–H–L 5′ and separated by inter-genic region (Diallo, 1990; Bailey *et al*., 2005).

1.5.3. Genome organization and viral proteins:

The genome of PPRV consists of a single linear molecule of negative-sense, single-stranded RNA with 15,498 Kb size (Bailey *et al.,* 2005; Mahapatra *et al.,* 2006). The PPRV genome encodes six genes, each is responsible for transcription of a single protein, in the following order: the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the hemagglutinin (H) and the large RNA polymerase (L). The P gene also encodes for two additional non-structural proteins, C and V (Figure 1) (Mahapatra *et al.,* 2006). The genome was found to be consistent with the rule-of-six where the open reading frames (ORFs) that encoded the eight proteins characteristic of morbilliviruses were identified. At the nucleotide (nt) level, the full length genome of PPRV was most similar to that of rinderpest virus (RPV) (Bailey *et al.,* 2005).

The nucleocapsid protein (N) is a major viral protein. It folds and protects the RNA genome against degradation by RNase (Barrett *et al.,* 1993) and plays an important role in the replication of PPRV (Servan de Almeida *et al*., 2007). The N protein is the most collected protein in infected cells and is antigenically most preserved between morbilliviruses (Libeau *et al.,* 1995). The N protein does not induce protective immunity against the virus, but it is the most immunogenic among PPRV proteins (Munir *et al.,* 2013).

The phosphoprotein (P) is the second protein involved in the ribonucleo-capsid complex and is very susceptible to proteolysis (Diallo, 2003). The P protein of PPRV is the longest among morbilliviruses and is the very important element of the viral L-polymerase complex which is suggested as a key determinant of cross-species morbillivirus pathogenicity (Yoneda *et al.,* 2004).

The matrix (M) protein is considered one of the smallest proteins among the structural proteins of the morbilliviruses (Diallo, 2003). The M protein of paramyxoviruses forms an inner coat to the viral envelope and serves as a link between the surface viral glycoproteins (F and H) and the ribonucleo-protein core. Based on its position, M appears to play a central role in the formation of the new virions which are liberated from the infected cell by budding (Mahapatra *et al*., 2006).

The two viral glycoproteins, the fusion (F) and the haemagglutinin (H) are embedded in the viral lipid bi-layer and protrude as spikes on the viral surface. The neutralizing antibodies produced by the infected host are directed against these proteins (Lefèvre and Diallo, 1990).

The first viral glycoprotein is the fusion (F) protein, which is one of the highly preserved and projects as spikes on the viral surface (Diallo, 2003). The F gene is the very conserved among PPRV strains, and it encodes for a protein of 546 amino acids long with a predicted molecular weight of 59.137 KDa (Dhar *et al.*, 2006). In morbilliviruses, the F protein, mediates viral penetration into mammalian cells by fusing the viral and cellular membranes at the cell surface leading to release of the viral genome into the host cell (Moll *et al.*, 2002).

The haemagglutinin (H) is the second viral glycoprotein. It serves for attaching the virus to the host cell. The H protein is encoding a protein of 609 amino acids long (Diallo, 2003; Dhar *et al.*, 2006). The H protein of PPRV, on the other hand, is the most diverse among all the Morbillivirus members which reflects its role in species specificity. If this is the case, the H proteins of RP and PPR virus may have significant potential for differentiation of infected from vaccinated animals (DIVA) strategies. Since the H protein determines the cell tropism, most of the protective host immune response is raised against it (Renukaradhya *et al.,* 2002).

The large (L) protein is considered as the largest protein in PPRV virions and is the least abundant (Flanagan *et al.,* 2000). It is third viral protein constitute the ribo-nucleoprotein structure (Murphy *et al.,* 1999). There are different enzymatic activities associated with the L protein including: RNA polymerase, mRNA capping, methylation, polyadenylation and protein kinase (Banerjee, 1987; Diallo, 2003). The L protein acts as RNA-dependent RNA-polymerase (Munir *et al.*, 2012). The association of the P to the N and L proteins is linked to viral cycle control, transcription and translation regulation (Munir *et al.,* 2012).

C and V proteins are the viral non-structural proteins which are encoded by the P gene open reading frame (ORF) by different mechanisms (Mahapatra *et al.*, 2003). C protein is the smallest viral protein that originates in the infected cells. The length of the V protein of PPRV is greatly variable among morbilliviruses. In contrast to the C protein, the V protein undergoes phosphorylation (Blom *et al.,* 1999).

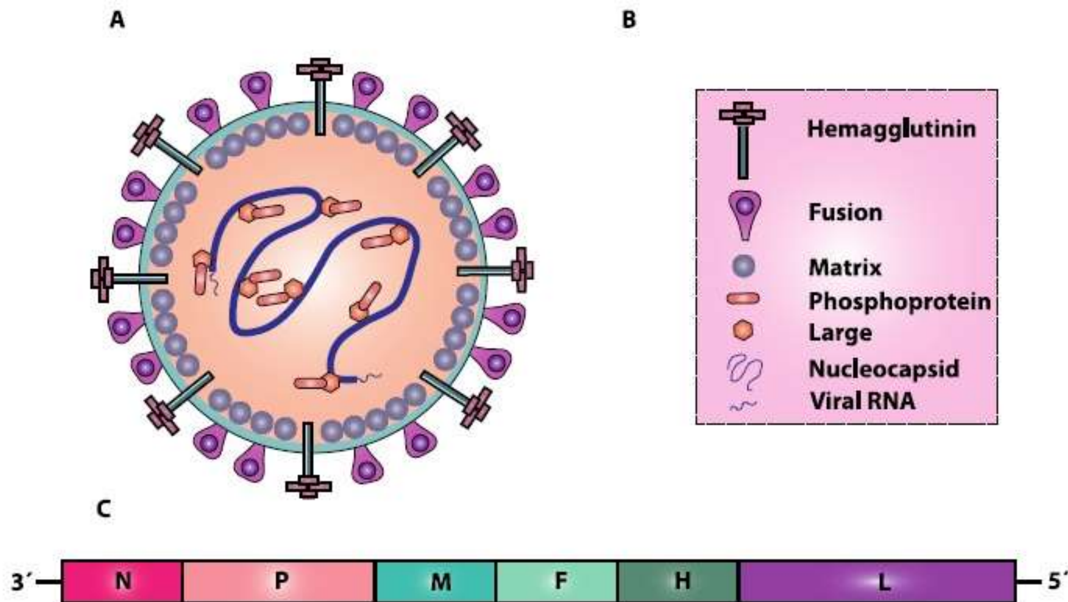

Figure 1. A schematic illustration of peste des petits ruminants virus structure. The arrangement of the viral proteins is shown in the structure (A). The names of the viral proteins present in the virus structure are shown in the box (B). The viral genome organization and arrangement of the different viral proteins in the genome are shown (C) (Munir *et al.,* 2012).

1.5.4. Viral replication:

Paramyxoviruses replicate mainly within the cytoplasm (Figure 2) (Murphy *et al.,* 1999). The first interaction of the host and pathogen is mediated by receptor-binding, PPRV interacts with the host cell membrane through the haemagglutinin (H) protein and sialic acid receptors (Munir *et al.,* 2013). However, other receptors are also exist for PPRV like signaling lymphocyte activation molecule (SLAM) receptor or CD150 which proved as important for viral growth and replication (Pawar *et al*., 2008; Adombi *et al.,* 2011). This virus-host interaction, followed by F protein-mediated fusion, leads to release of the nucleocapsid from the viral envelope into the cytoplasm. The large (L) protein then works as an RNA-dependent RNA-polymerase (RdRp) and initiates the transcription of messenger RNAs (mRNAs) into the cytoplasm. As in all paramyxoviruses, RdRp binds to the genome promoter, which is a stretch of the nucleotide before the nucleocapsid (N) open reading frame (ORF) that initiates transcription in a ‘‘stop-start’’ fashion. There is a series of transcription attenuations across each gene junction, a natural justification for the protein quantity required for the

viral replication. The mRNA for the N protein, being at the N-terminus, is most abundantly transcribed and is required most, while the L protein, which is required only in a small amount, is the least transcribed due to its far away position from the genome promoters (Munir *et al.,* 2013). Briefly, the P protein regulates transcription and replication and assembly of the N protein to nucleocapsids, the M proteins mediate viral assembly, and the H protein facilitates the budding process. Viral genome copies are formed from the minigenome through replicative intermediates. The role of the C and V proteins in PPRV is still not clear. It is believed that these proteins have abilities to abrogate the cellular interferon (IFN-a/b) responses and hence contribute in the PPRV virulence (Figure 2) (Munir *et al.,* 2013).

Finally, when all the structural components of the virus, including viral glycoproteins and viral RNPs, have assembled at selected sites on the membranes where virions bud, then pinch off to achieve particle release allowing the transmission of infections to new cells and hosts (Figure 2) (Harrison *et al*., 2010).

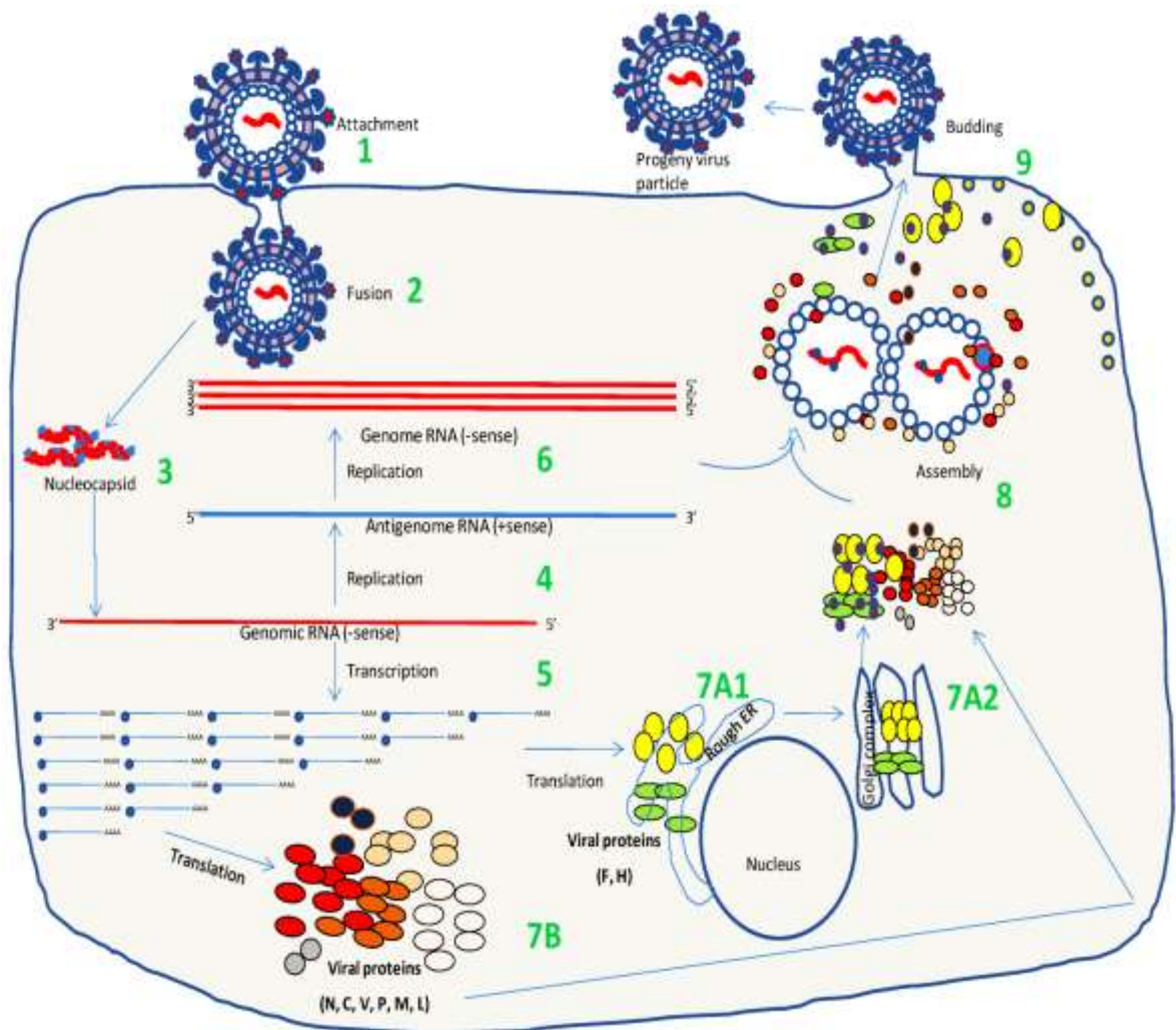

Figure 2. Replication cycle of PPRV. (1) Attachment of the virus to host cell receptors (SLAM) via its H protein. (2) Fusion with the plasma membrane via the F protein. (3) Release of the viral genome into the cytoplasm. (4) Genome replication by the virus-encoded RdRp of the RNPs. (5) mRNA synthesis by the virus-encoded RdRp in the ‘start-stop’ mode (a mechanism of controlling the amount of individual protein being produced). (6) Synthesis of the full-length positive sense RNA (antigenome RNA or complementary RNA, cRNA). (7) Synthesis of the viral proteins: F and H on RER (7A1) and translocate across Golgi complex (7A2), where post-translational modifications take place. Other viral proteins (N, P, C, V, M, L) are synthesized on ribosomes (7B). (8) Assembly of the progeny virions and (9) Budding of the progeny virions at the plasma membrane (Kumar *et al.,* 2014).

1.6. Epidemiology of the disease:

1.6.1. Geographic distribution:

PPR was first described in the Ivory Coast (Côte d'Ivoire) in West Africa (Gargadennec and Lananne, 1942). Since then, the disease has spread far beyond its origin in Western Africa. Currently, PPR occurs in most African countries (except Southern Africa), in the Arabian Peninsula, throughout most of the Middle East, in nearly all Asian countries and in few European countries including Turkey, Georgia and Bulgaria (Figure 3) (Lefevre and Diallo, 1990; Banyard *et al.,* 2010; Parida *et al.,* 2016; OIE-WAHIS, 2018).

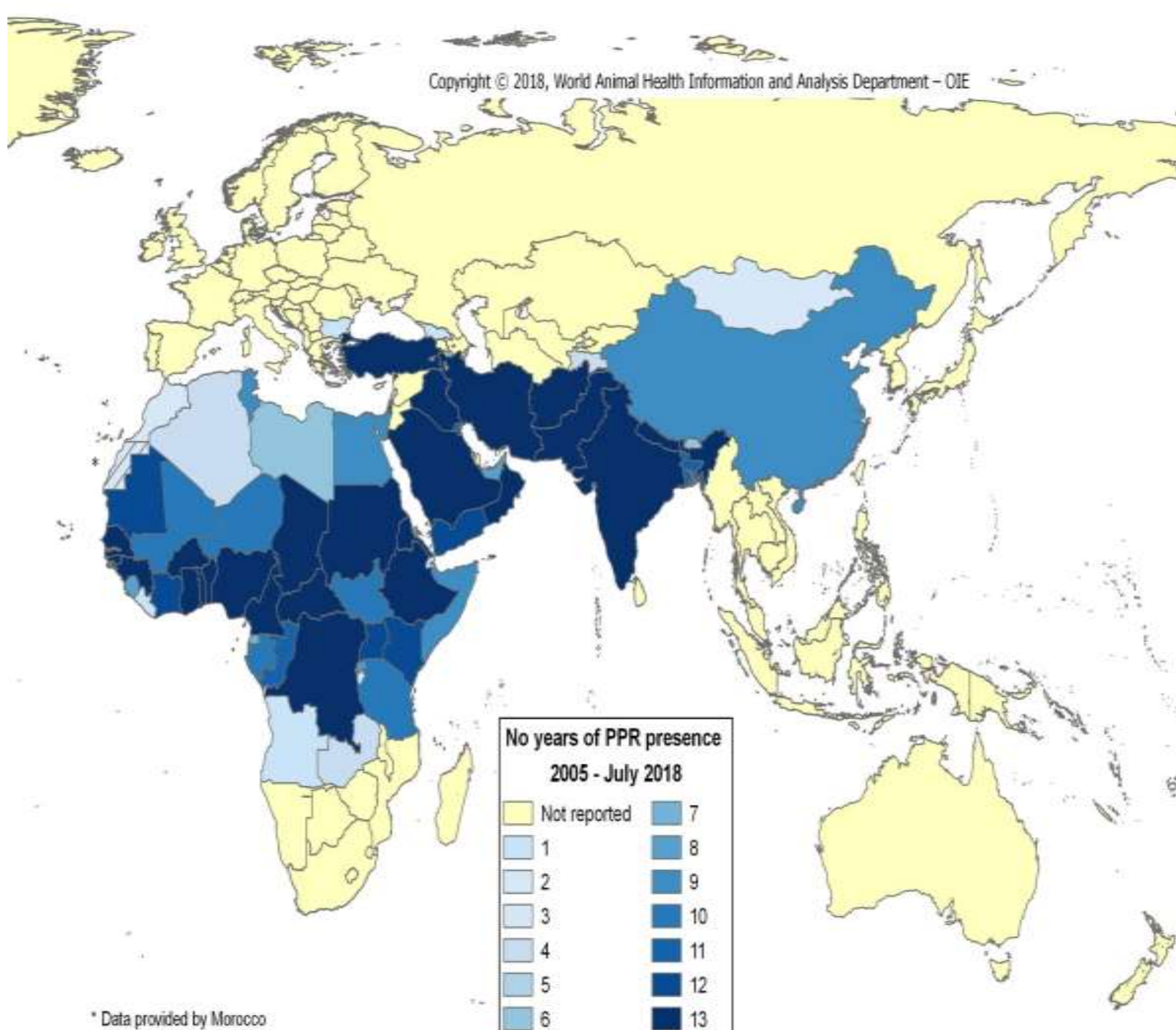

Figure 3. Geographic distribution of PPR. Map based on the information received through six-monthly reports up to July 2018. World Organization for Animal Health (OIE, 2018). https://www.oie.int/en/animal-health-in-the-world/ppr-portal/distribution/

1.6.2. Lineage identification of PPRV:

Based on partial sequences of the fusion (F) (Forsyth and Barrett, 1995; Ozkul *et al.,* 2002) and nucleocapsid (N) (Couacy-Hymann *et al.,* 2002; Kwiatek *et al.,* 2007) genes. PPRV can be classified into four distinct lineages (I, II, III, IV) (Shaila *et al.,* 1996; Dhar *et al.,* 2002; Munir *et al.,* 2013). PPRV strains belonging to lineages I and II are exclusively originated in West African Countries. Lineage III strains are restricted to the Middle East (Yemen, Qatar and Oman) and East Africa. Lineage IV is considered a new lineage comprising newly emerging viruses, and is most prevalent in Asian countries. The geographical source of the new lineage IV virus is unknown although it is most closely related to the African lineages (Shaila *et al.,* 1996; Munir *et al.,* 2013). Molecular typing provided evidence of the recent spread of PPRV lineage IV in North and Middle Africa (Kwiatek *et al.,* 2011).

1.7. PPR in the Sudan:

In the Sudan, outbreaks of peste des petits ruminants (PPR) were first appeared as a rinderpest-like disease in sheep and goats in Elgadarif in Eastern Sudan (Elhag Ali, 1973). Following that, the disease was reported in Sinnar and Mieliq, Central Sudan during the years 1971 and 1972. At that time the disease was wrongly diagnosed as Rinderpest (RP) and many years later it is confirmed to be PPR (Elhag Ali and Taylor, 1984). PPRV was firstly isolated by Elhag Ali and the isolates were named “PPRV SUD 72/1” (Sinnar strain) and “PPRV SUD 72/2” (Mieliq strain) (Elhag Ali and Taylor, 1984). Subsequently, outbreaks of the disease were reported during the years 1989 and 1990 in El Hilalia Area, Gezira State with morbidity and mortality rates ranging from 10-66% and 3-37%, respectively. PPRV was isolated from El Hilalia outbreak and named “PPRV/VHL” (El Hassan *et al.,* 1994).

The prevalence of the disease in sheep and goats from Khartoum State during 1992-1994 was demonstrated using a sero-surveillance study (Zeidan, 1994). Further studies demonstrated the presence of PPRV from suspected outbreaks and sero-prevalence of the disease from many States of the country including Khartoum, Gezira, Northern State, River Nile, Blue Nile, White Nile, Sinnar, Gedarif, Kassala, Kordofan, Darfur, and Southern Sudan States (Haroun *et al.,* 2002; Osman *et al.,* 2008; 2009a; Saeed *et al.,* 2010; Abdalla *et al.* 2012; Enan *et al*., 2013; Ali *et al.,* 2014; Salih *et al*., 2014; Shuaib *et al.,* 2014; Saeed *et al.,* 2017; Osman *et al.,* 2018; Saeed *et al.,* 2018).

In 2019, serological detection demonstrated the presence of PPRV antibodies in sera of sheep and goats collected from suspected outbreaks in Kordofan States in the Western part of the country (Ahmed, 2019) and in White Nile State in Central Sudan (Ali Ahmed, 2019). The presence of PPRV antigen was demonstrated in camel lungs collected from Tambul slaughter house, Gezira State (Abdalla, 2019) and in sheep and goats lung samples collected from Al-Hasaheisa slaughter house, Gezira State (Alhussain, 2019).

Recently, higher sero-prevalence of PPRV antibodies had been reported among cattle populations in the Sudan (Ali *et al.,* 2019).

The first report of PPRV infection in camels in the Sudan which described a per-acute fatal respiratory disease of camels in Kassala State, Eastern Sudan was in 2004 (Khalafalla *et al.,* 2010). There were many studies of the disease in camels, PPR viruses were isolated from camels and molecular studies demonstrated the presence of PPRV lineage IV (Kwiatek *et al.,* 2011; Saeed *et al.,* 2014). The prevalence of PPRV in camels in different areas of the Sudan and its association with other respiratory viruses was investigated (Saeed *et al.,* 2015).

Recently, a study represented the first and only report describing PPRV infection in Dorcas gazelles (gazelle dorcas), and demonstrated that gazelles are a potential wild small ruminant host of PPRV and may influence the epidemiology of PPR in the Sudan (Asil *et al.,* 2019).

1.8. Host range:

Sheep and goats are the main natural acutely infected hosts of PPRV (Lefevere and Diallo, 1990; Parida *et al.,* 2015). Generally, sheep and goats are probably equally susceptible to the virus, however, goats show more severe clinical signs than sheep in the same environmental conditions with higher recovery rate in sheep (Khan *et al*., 2008; Munir *et al.,* 2009; Merck, 2010).

There are some reported cases of high mortality in sheep within mixed small ruminant flocks (Yesilbag *et al.,* 2005). In small ruminants, the severity of the disease depends on age, sex, breed and season (Munir *et al*., 2009; Meng *et al*., 2011; Salih *et al*., 2014).

Cattle are only subclinically infected (Merck, 2010; Lembo *et al*., 2013). The virus has been isolated from experimentally infected cattle, however, it is not excreting the virus and does not contribute in the transmission of PPRV (Sen *et al*., 2014; Parida *et al*., 2015).

Additionally, PPR has now been recognized as an emerging disease in camelids. The main sign of PPRV infection in camels is the respiratory syndrome as reported in Ethiopia and the Sudan (Roger *et al*., 2000; Khalafalla *et al.,* 2010).
PPRV infection of wildlife and a clinical disease were reported in Dorcas gazelle, Thomson's gazelle, gemsbok, ibex, white-tailed deer, wild sheep, wild goats and other small wild ruminant species (Abu Elzein *et al.,* 2004; Wohlsein and Saliki, 2006; Gur and Albayrak, 2010; Bao *et al.,* 2011; Hoffmann *et al.,* 2012; Aziz-ul-Rahman *et al.,* 2018).
Whether PPRV-infected sick buffaloes and camels are source of infection for small ruminants remains unclear. Other wild ruminants, including representatives of the Gazellinae, Tragelaphinae and Caprinae subfamilies, may express a serious illness and mortality. In specific conditions, wildlife may have played an important role in PPR epidemiology (Kinne *et al.,* 2010). In areas where PPRV has been present for a long time, there are evidences that PPRV might represent a threat for wildlife. As for rinderpest, wildlife is more likely to be a victim rather than a reservoir for PPRV (Couacy-Hymann *et al.,* 2005).
Pigs are dead-end hosts and do not transmit the disease to in contact susceptible pigs or goats. Domestic buffalo are susceptible to PPRV infection, although, there is no evidence for presence of a clinical diseases natural or experimental infection or that they transmit the disease to other susceptible animal species (Merck, 2010). Recently, PPRV was detected from Asiatic lion tissues which might be due to feeding of PPRV-infected animal (Balamurugan *et al.,* 2012). The presence of the PPRV genome in the nasal swabs of the dogs has been demonstrated (Ratta *et al.,* 2016).

1.9. Transmission of PPRV:

PPR is generally regarded as a seasonal disease in endemic areas of Africa with peak infections usually occurring during the dry, cool season (Abubakar *et al.,* 2009). Transmission of PPRV occurs mainly by aerosol and droplets from infected animals (Murphy *et al*., 1999; OIE, 2019). A close contact between the animals is essential to establish PPRV infection (Khan *et al*., 2008).
Animals affected by PPR shed the virus in exhaled air for approximately 10 days after the onset of fever. PPRV is shed in secretions and excretions from all body orifices including the mouth, eyes and nose, and in feces, semen, and urine. Sneezing and coughing by the infected animals can spread viral infection, while the transmission

between animals in the vicinity can occur through inhalation or through fomites (Munir, 2013). Infection may spread to offspring by feeding the infected milk from their dams, it is believed that the virus is present in the milk from 1-2 days before the signs appear and until 45 days after the complete recovery (Munir, 2013). PPRV infected animals started virus transmission before the onset of the clinical signs (Couacy-Hymann *et al.*, 2007). However, during the post-recovery state of the animal goats infected with PPRV can shed the virus in faeces for 11 weeks after the complete recovery (Ezeibe *et al.*, 2008).

Since PPRV is quickly inactivated in the environment, its transmission most often occurs by direct contact between infected and susceptible animals. However, indirect transmission through recently contaminated materials can occur and should be considered in the epidemiological models. The high virulence of PPRV has been demonstrated in cases of experimental and natural infections (Couacy-Hymann *et al.,* 2007).

1.10. Clinical signs:

The morbidity and mortality rates can reach 100 %, but it varies depending on the species infected, the age of the animals, the prevalence of secondary infectious agents and the PPRV lineage involved (Zahur *et al.,* 2009; Kivaria *et al.,* 2013; Chowdhury *et al.,* 2014).

Adult as well as young sheep and goats were equally affected but the symptoms were more severe in young animals (Elhag Ali, 1973).

The clinical disease resembles rinderpest in cattle and is usually acute that is characterized by pyrexia, anorexia, depression, serous ocular and nasal discharges, , inflammation of the gastrointestinal tract (GIT), diarrhoea, pneumonia, and erosive lesions on different mucous membranes particularly in the mouth. PPR can also occur in a subclinical form (Abubakar *et al.,* 2008; Wasee Ullah *et al.*, 2016; OIE, 2019). The incubation period of PPR is 4-6 days although it may range between 3 and 10 days. At the acute stage of the disease, animals showed pyrexia of up to 41°C that may last for 3-5 days; the animals become depressed, anorexic and develop a dry muzzle. Serous oculo-nasal discharges become progressively mucopurulent and may persist for around 14 days if death does not happen. Within 4 days of the onset of fever, the gums become hyperaemic, and erosive lesions develop in the oral cavity with excessive salivation. These lesions may become necrotic. Watery blood-stained diarrhoea is common in the

later stage. Pneumonia, coughing, pleural rales and abdominal breathing may also occur (OIE, 2019). Bronchopneumonia, revealed by productive cough and dyspnea, and diarrhea usually appears 3 days after the oral lesions. As a consequence of pneumonia and dehydration caused by diarrhea, severely affected animals may die within 5-10 days after the onset of the clinical signs (Diallo, 2006). The affected animals showed signs of severe dehydration and their hind quarters were soiled with diarrhea material. The mouth lesions were found in all the affected animals with red raw areas on the inner side of the lips, lower gums, and necrosis on the dorsal surface of the tongue (Wasee Ullah *et al.,* 2016). Abortions are often observed during PPR outbreaks, caused by PPRV alone or in combination with other pathogens (Abubakar *et al.,* 2008).

PPRV experimentally infected goats developed clinical signs similar to those of the naturally infected animals (Osman *et al.,* 2009b).

1.11. Post-mortem lesions:

At necropsy, the lesions caused by PPRV were very similar to those observed in cattle affected with rinderpest (OIE, 2019). External postmortem examination of recently dead animals revealed sunken eyes, rough and dry skin, and the hind quarter soiled with diarrhoea material (Wasee Ullah *et al.,* 2016). All dead animals were found emaciated and severely dehydrated (Chauhan *et al.,* 2011). Erosive lesions may extend from the mouth to the reticulo-rumen junction. Characteristic linear red areas of congestion or haemorrhage may occur along the longitudinal mucosal folds of the large intestine and rectum known as zebra stripes (OIE, 2019).

Autopsy showed necrotic and ulcerative lesions in the mouth, stomach and intestine, erosive lesions on the lips and around the nose. The nostrils showed mucopurulent discharges and congested mucus membranes. The buccal cavity revealed erosive stomatitis appeared as erosive and ulcerative lesion on the mouth. Additionally, there is congestion, red hepatisation, raised patches of emphysema in the lung, heamorrhage and froth exudates in the trachea (Chauhan *et al.,* 2011).

The lungs were patchy pneumonic. The liver was slightly yellowish in colour and the gall bladder was distended with the bile (Chauhan *et al.,* 2011). Congested and necrotic lesions appeared on the liver and kidney and were only seen in goats (Al-Dubaib, 2009).

The body lymph nodes, particularly the mesenteric lymph nodes, were inflamed and swollen. There is severe enteritis with streaks of haemorrhages in the intestine, stunning and blunting of villi with necrosis at villous tips and erosion and infiltration of

inflammatory cells in the lamina propria with the presence of focal pale areas of necrosis. The heart was congested and contained white necrotic patches with prominent blood vessels. The spleen was enlarged, congested, oedematous and showed petechial to ecchymotic haemorrhages (Chauhan *et al.,* 2011).

1.12. Diagnosis of PPR:

1.12.1. The importance of the diagnosis:

All diagnostic methods of peste des petits ruminants, were applied for different purposes, such as confirming the diagnosis of clinical cases, determining the infection status for trade and/or movement, estimates of infection or degree of exposure prevalence/surveillance) or monitoring post-vaccination immune status (OIE, 2019). A series of diagnostic tools have been developed for detecting PPR virus, viral nucleic acid or antibodies develop against the virus.

1.12.2. Methods for virus isolation:

Techniques for isolation of PPRV cannot be used routinely because they are time-consuming. However, even when diagnosis of PPRV has been carried out by rapid diagnostic technique, the virus should always be isolated in tissue culture from field sample (OIE, 2019).

For successful PPRV isolation, it is vital to have samples from the early stages of the disease and the samples should be kept cold till it is transported to the laboratory (Lefevre and Diallo, 1990). Using lamb and goats kidney cells increased the sensitivity of virus isolation (Taylor, 1984). Virus isolation in cell culture can be attempted using different cell lines such as MDBK, BHK-21 and Vero (African green monkey kidney) cells that are widely and routinely used and have been the choice for isolation and propagation of PPRV (Rahman *et al.,* 2011).

It has been reported that B95a, an adherent cell line derived from Epstein-Barr virus transformed marmoset B-lymphoblastoid cells, is more sensitive for growth of PPRV lineage IV as compared to Vero cells (Bruning-Richardson *et al.,* 2011). The cytopathic effect (CPE) produce by PPRV develop within 5-19 days and consists of cell rounding, refractile cells and aggregation culminating in syncytia formation with eventual detachment of cell (Taylorand Abegunde, 1979). These syncytia are characterized by a circular arrangement of nuclei giving a clock face appearance (Hamdy *et al.,* 1976; Elhag Ali and Taylor 1984; Abu Elzein *et al.,* 1990). Moreover, the presence of intra-

cytoplasmic and intra-nuclear inclusions bodies had been reported (Murphy *et al.,* 1999).

Recently, derivatives of cell lines (Vero, CV1) expressing the morbillivirus signalling lymphocyte activation molecule (SLAM) or CD150 receptor, had been developed and enable isolation of field viruses in less than one week, without requirement for blind passages (OIE, 2019) These cell lines include a derivative of the monkey cell line CV1 expressing goat SLAM or dog SLAM (Adombi *et al.*, 2011).

1.12.3. Methods for antigen detection:

1.12.3.1. Agar gel immunodiffusion test (AGID):

Agar gel immunodiffusion (AGID) is a very simple, rapid and cheap test that can be performed for diagnosis of PPR in any laboratory and even in the field (OIE, 2019). The most suitable samples used are tissues like lymph nodes, spleen and the organs of the gastrointestinal tract (OIE, 2019). Agar Gel Immuno Diffusion (AGID) test are not used for routine diagnosis because it lack sensitivity when compared to other assays (Osman *et al*., 2008; Aslam *et al*., 2009). Positive samples produced clear lines of precipitation on AGPT which were seen after 24-48 hours of the onset of the test (Osman, 2005; Osman *et al.,* 2008).

1.12.3.2. Counter Immuno-electrophoresis (CIEP):

Counter immuno-electrophoresis (CIEP) is a useful for PPRV diagnosis. It is the more rapid version of the AGID test and results can be obtained within 2-3 hours (Majiyagbe *et al*., 1984). The reagents are the same as those used for AGID and it is 4 to 16 times more sensitive than the AGID for detection of PPR virus antigen. CIEP detected viral antigen in many samples when collected up to 14 days after the onset of fever. The presence of 1-3 precipitation lines between pairs of well viewed by intense light considered as a positive reaction (Forman *et al.,* 1983; Rossiter *et al*., 1985). Lines of precipitation could be seen after 30-45 min (Obi and Patrick, 1984).

1.12.3.3. Haemagglutination test (HA):

Haemagglutination test (HA) is a simple laboratory confirmatory test for diagnosis and monitoring of PPR with samples from live animals such as ocular and nasal discharges. The advantage of thi test is that it can diagnose positive cases of the disease and offers a quick and accurate technique for less sophisticated laboratories (Wosu, 1991; Osman;

2005). It had been found that the HA test was more sensitive than AGPT for detection of PPR antigen (Osman, 2005; Osman *et al.,* 2008). Haemagglutination tests (HA) tests can be used for routine screening purposes in control programmes due to its comparative sensitivity alongside being simple to perform and cheap to produce (Manoharan *et al*., 2005).

1.12.3.4. Immunofluorescent antibody test (IFAT):

Indirect immunofluorescence technique had been used to detect PPRV antigen in conjunctival epithelial cells of goats from suspected cases of the disease with 100% specificity (Sumption *et al.,* 1998).

1.12.3.5. ELISA for antigen detection: Immunocapture ELISA (IC-ELISA) and sandwich ELISA (S-ELISA):

Immunocapture enzyme-linked Immunosorbent assay (IC-ELISA) was performed using several anti N-monoclonal antibodies (MAbs) directed against non-overlapping antigenic domains on the nucleo-capsid (N) protein (Libeau *et al*., 1994). The assay, which is very sensitive, uses the MAbs to detect the virus N-protein in supernatants from infected cells and field specimens (Libeau *et al*., 1994).

A Sandwich ELISA (S-ELISA) assay using PPR specific monoclonal antibodies (clone 466) to an epitope of the nucleo-capsid protein had been developed (Singh *et al.,* 2004a). The test uses polyclonal sera to capture the antigen from clinical samples which were detected using PPR specific monoclonal antibody. The test showed 92.8% specificity and 88.9% sensitivity with the IC-ELISA (Singh *et al.,* 2004a).

1.12.4. Methods for RNA or genome detection:

1.12.4.1. Reverse transcriptase-polymerase chain reaction (RT-PCR):

Reverse transcription-polymerase chain reaction (RTPCR) is the method of choice for detecting nucleic acids of PPRV in clinical samples. It is highly sensitive with results obtained in 5 hours, including the RNA extraction. RT-PCRs have been reported for detection and differential diagnosis of RP and PPR viruses targeting the N and F genes (Balamurugan *et al*., 2006; Couacy-Hymann *et al*., 2002). RT-PCR is a very useful and sensitive tool for the detection of PPR virus in clinical samples and also for monitoring the growth of the inoculated virus in cell culture (Rahman *et al*., 2011).

A rapid and specific RT-PCR, based on the rapid purification of RNA on glass beads followed by the RT-PCR for amplification of a fragment in the 3' end of the RNA messenger that encodes the nucleo-capsid protein of the PPRV, had been developed (Couacy-Hymann *et al.,* 2002). The assay is 1000-fold more sensitive than the conventional titration technique in VERO cells.

An RT-PCR using purified viral RNA has been easily adopted for direct detection of PPRV in clinical field samples. A single tube one-step multiplex RT-PCR has been used to amplify fragments of N and M genes of PPRV with a high sensitivity than the two-step assay (Balamurugan *et al.,* 2006). The one-step RT-PCR has the advantages of being more sensitive, quicker and cheaper and it minimizes the chances of carry over contamination (Balamurugan *et al.,* 2006).

1.12.5. Methods for serological diagnosis:

For demonstration of PPRV antibodies in serum samples, many diagnostic tests have been used including virus neutralization test (VNT), agar gel diffusion test (AGDT), counter immuno-electrophoresis (CIEP), haemagglutination inhibition test (HI) and recently the blocking and competitive ELISA assays (OIE, 2019).

1.12.5.1. Agar gel diffusion test (AGDT):

AGDT was used for the detection of antibodies against PPRV in sera of the affected goats (Durojaiye, 1982). It provides a rapid serological diagnostic tool for PPR and is considered useful for field diagnosis of PPR. Precipitating antibodies were detected in sera obtained in the acute phase of the disease and also in sera obtained at convalescence (Durojaiye, 1982).

1.12.5.2. Virus neutralisation test (VNT):

The virus neutralisation test (VNT) is sensitive and specific, however, it is time-consuming and expensive. The standard neutralisation test is carried out in primary lamb kidney cells or Vero cells. VNT is the most reliable test for detection of morbillivirus and PPRV antibodies (Rossitter and Taylor, 1994; El-Yuguda *et al.,* 2013; Boshra *et al.,* 2015).

1.12.5.3. Counter immuno-electrophoresis (CIEP):

CIEP has been applied successfully for detection of PPRV antibodies. The rapidity, simplicity and sensitivity of the CIEP made it a suitable technique in serological studies of PPR (Majiyagbe *et al.,* 1984; Osman *et al.,* 2009a). Despite its low specificity CIEP can be a useful indicative screening test for PPRV antibodies in flocks that neither been vaccinated nor otherwise exposed to PPR virus. CIEP, like the HI test, could be a useful screening test where it is not possible to use C-ELISA (Osman *et al.,* 2009a).

1.12.5.4. Haemagglutination inhibition test (HI):

Haemagglutination inhibition test is a simple and rapid serological method for PPRV antibodies detection. HI technique elucidated the problem of the cross-reactivity which exists in the diagnosis of PPR and RP (Wosu and Ezeibe, 1992).

1.12.5.5. ELISA for antibody detection: Competitive Enzyme-Linked Immunosorbent Assay (C-ELISA) and Blocking ELISA (B-ELISA):

Competitive and blocking ELISAs, based on monoclonal antibodies specific for N-protein (Libeau *et al*., 1995) and H-protein (Singh *et al*., 2004b) had been developed for detection of PPRV antibodies in animal sera. These tests either used gradient purified PPR virus or recombinant expressed antigens. In the N-protein C-ELISA, the serum antibodies and the MAbs compete on specific epitopes on the nucleoprotein obtained from recombinant baculovirus. It has been documented that there is no cross reaction in the N-protein C-ELISA with a high level of competition up to 45% observed among the negative sera (Libeau *et al*., 1995).

Both blocking ELISA and C-ELISA detect anti-H antibodies that based on the competition between anti-H monoclonal antibodies (MAbs) and serum antibodies (Saliki *et al.,* 1993). The sensitivity and specificity of the H-protein blocking ELISA were found to be 90.4% and 98.9%, respectively. A competitive ELISA assay that uses monoclonal antibody to a neutralizing epitopes of the haemagglutinin protein of PPRV had been developed for detection of antibodies to PPRV sera of sheep and goats (Singh *et al.,* 2004b). The competitive ELISA is the most suitable choice for PPRV diagnosis as it is sensitive, specific, reliable, and has a high diagnostic specificity (99.8%) and sensitivity (90.5%) (Khan *et al.,* 2008; Abubakar *et al.,* 2009; Aziz-ul-Rahman *et al.,* 2016).

1.13. Treatment:

Since PPR is a viral disease, there is no specific treatment. However, to reduce the severity of the disease, affected animals were treated by administration of antibiotics to prevent secondary bacterial infections and anti-diarrhoeal medicines with supportive therapy such as B-complex and Dextrose saline has been practiced for 5-7 days. Treatment and management of clinical cases of PPR or in the event of outbreaks in sheep and goats is necessary in order to minimize the economic losses to farmers (Balamurugan *et al.,* 2014).

1.14. Immunity against PPRV:

Animals that recover from infection with the attenuated PPRV vaccine strains develope high levels of neutralizing antibodies as well as antigen-specific proliferating CD4+ T cells (Lund *et al.,* 2000), this solid immunity protects sheep and goats for several years or even for the rest of the host's life (Libeau *et al.*, 1992; Zahur *et al.,* 2014). This protection is independent of the PPRV lineage. This immunity is mainly neutralizing antibodies, although precipitating antibodies or haemagglutination-inhibiting antibodies may be developed (Lefevre and Diallo, 1990).

Suckling lambs acquire passive immunity via the colostrum from previously exposed or vaccinated dams. This immunity last for 3-4 months and antibodies were detectable until four months of age in virus neutralization test but only until 3 months in C-ELISA (Libeau *et al.,* 1992; Rahman *et al.,* 2011). Lambs and kids should be vaccinated at four to five months ages as recent studies indicate that lambs and kids are protected from PPRV until three and a half to four and a half months ages (Awa *et al.*, 2002).

Vaccination and infections with a morbillivirus leads to development of high quality antibodies. However protection by antibodies seems only to be possible with homologous virus since B cell epitopes are not totally conserved among the morbillivirus. Epitopes on N protein of B cell, which are produced after infection or vaccination with PPRV, can be divided into four antigenic domains, A-1, A-11, C-1 and C11 (Choi *et al.,* 2005). The B cell epitopes of HN have also been determined (Renukaradhya *et al.*, 2002). Goats and sheep develop both humoral and cell mediated immune response when vaccinated with a recombinant HN protein of PPRV (Sinnathamby *et al.*, 2001).

1.15. Control of PPR:

Effective implementation of control measures for PPR requires a quickly diagnosis of the disease in order to contain outbreaks and minimize the economic losses (Munir *et al.,* 2009). Control of PPR may be achieved using classical measures such as restricted movement of small ruminants from affected areas, quarantine of affected animals, elimination of contact fomites and restriction on the importation of small ruminants from infected areas, basic hygienic practices and biosecurity levels within and between flocks (Balamurugan *et al.,* 2010). Affected premises should be cleaned and disinfected with lipid solvent solutions of high or low pH, disinfectants and detergents (Anonymous, 2002). Control of PPR outbreaks require movement control (quarantine) combined with the use of focused ("ring") vaccination and prophylactic immunization in high-risk populations (Balamurugan *et al*., 2010).

The most effective way to control PPR is mass immunization of small ruminants using an effective vaccine. Therefore, several vaccines had been developed, all of which are cell culture-attenuated strains of natural PPRV (Sen *et al.,* 2010). The most famous is the homologous PPRV vaccine which was introduced in 1989 after attenuation of the Nigeria 75/1 strain by serial passages on Vero cells (Diallo *et al*., 1989). Recently, both homologous and recombinant vaccines have been developed. The other most commonly used vaccine is Sungri/96 strains which has been used extensively in Asia. Both vaccines protect animals against PPRV isolates of all lineages (Hodgson *et al.*, 2018). The Nigeria 75/1 PPRV vaccine strain has been successfully reproduced and used in the Sudan for control of the disease and to support goats and sheep trade (Fadol and El Hussein, 2004).

Chapter II
Materials and Methods

2.1. Materials:

2.1.1. Reference virus:

2.1.1.1. Peste des petits ruminants virus (PPRV):

PPRV Nigerian 75/1 vaccine strain which is a live attenuated cell culture vaccine (Diallo *et al.,* 1989), kindly supplied by the Viral Vaccine Production Department, Central Veterinary Research Laboratories (CVRL), Soba, Khartoum, Sudan. PPRV vaccine strain was classified according to sequence differences in the N-gene into PPRV lineage II.

2.1.2. Cell Culture:

2.1.2.1. Cells lines:

African green monkey kidney cells or Vero cell lines were used for PPRV isolation and propagation in this study. These cells were kindly provided by the Viral Vaccine Production Department, CVRL, Soba.

2.1.2.2. Cell Culture medium and reagents:

Reagent	Company
Glassgow Minimum Essential Medium (GMEM)	Sigma-Aldrich, USA
Lactalbumine Hydrolysate	Conda, Spain
Yeast Exstract	Oxoid, England
Tryptose Phosphate Broth	Oxoid, England
Trypsin	Fisher Scientific, USA
$NaHCO_3$	-
Penicillin/Streptomycin/Gentamycin	Biochrom GmbH, UK
Penicillin	Biochrom GmbH, UK
Streptomycin Sulphate	Biochrom GmbH, UK
Gentamycin	Biochrom GmbH, UK

Amphotercin B	Biochrom GmbH, UK

2.1.3. Kits:

Kit name	**Company**
ID Screen® PPR Antigen Capture Sandwich ELISA for the detection of PPR virus Reagents provided with the ELISA kit: 1- Microplates Coated with anti-PPRV-N antibody 2- Anti-PPRV-N Mab-HRP conjugate (10X) 3- Positive control, Freeze-dried. 4- Positive control reconstitution solvent 5- Negative control 6- Dilution Buffer 13 7- Dilution Buffer 19 8- Wash concentrate (20X) 9- Substrate Solution 10- Stop solution (0.5 M)	IDvet Innovative Diagnostics, France
Immunocapture Enzyme-Linked Immunosorbent Assay (IcELISA). It is a solid phase immunocapture ELISA (ICE) Reagents provided with the ELISA kit: 1- Capture antibody 2- Blocking buffer 3- Detecting antibody 4- Conjugate 5- Substrate Solution (OPD) + H_2O_2 6- Positive control 7- Negative control 8- Washing buffer 9- Stop solution	CIRAD EMVT, Montpellier, France, distributed by BDSL, UK
RNeasy ®Mini Kit (RNA Extraction from whole blood)	Qiagen, Germany

Reagents provided with the kit: Buffer RW1 Buffer RPE RNase free water	
QIAamp ®Viral RNA Mini Kit (RNA Extraction from Tissue) Reagents provided with the kit: Buffer AVL* Buffer AW1* (concentrate) Buffer AW2† (concentrate) Carrier RNA (poly A)	Qiagen, Germany
One-step RT-PCR Kit/ One-step Reverse-Transcription Polymerase Chain Reaction (PCR) Kit Reagents provided with the kit: Sterile water ultrapure PCR buffer 5X dNTPs Mix 10 Mm One-step RT-PCR Enzyme mix	Qiagen, Germany

2.1.4. PCR reagents and buffers:

Name	Company
RNase-free water	Qiagen, Germany
Qiagen One-step RT-PCR Buffer (5X conc.)	Qiagen, Germany
dNTPs mix (10Mm)	Qiagen, Germany
Qiagen One-Step RT-PCR Enzyme Mix	Qiagen, Germany
Universal Agarose	Bio & SELL, Germany
TAE-Buffer (10X conc.)	Bio & SELL, Germany
Ethidium Bromide	Vivantis, USA
6X Loading dye	Vivantis, USA
100 Kbp DNA Marker	Vivants, USA

2.1.5. Oligonucleotide primers:

Oligonucleotide primers used in this study targeting the 3'end of PPRV N-gene partial sequence were designed previously by Couacy-Hymann *et al.* (2002) and recommended by the World Organization for Animal Health (OIE, 2019). Oligonucleotide primers were supplied as freez-dried preparations and were dissolved in RNase-free water to a final concentration of 5 pmol/µl and were subsequently used for cDNA synthesis, PCR and sequencing.

Primer Name	Sequence 5' to 3'	Company
NP3 (Forward primer)	5'-TCT CGG AAA TCG CCT CAC AGA CTG-3'	Eurofins Genomics
NP4 (Reverse Primer)	5'-CCT CCT CCT GGT CCT CCA GAA TCT-3'	Eurofins Genomics

2.1.6. Chemicals and reagents:

Name	Company
PBS (Dry powder in foil pouches)	Thermo Fisher, USA
Ethanol	S d fine CHEM Limited (SDFCL), India
Trizol	-
Chloroform	BDH Laboratory Supplies, England

2.1.7. Apparatuses, equipment and instruments:

Name	Company
ELISA Washer	BioTek (Elx50), USA
ELISA Shaker	BioTek, USA
ELISA Reader	BioTek, USA
Computer & Printer	HP
Single & Multichannel Micropipettes	Fisher Scientific, USA
Trough	-

Thermocycler	Peqlab Biotechnology GmbH, Germany
Refrigerated Centrifuge	Sigma Laborzentrifugen, Germany
Mixer UZUSIO VTX 3000L	LMS Laboratory and Medical Supplies, UK
BDSL Biosafety Cabinet	-
Crumapcr-PCR Cabinet	Progen Scientific, UK
Sensitive Balance	Kern, UK
UV-Light trans illuminator	Peqlab, France
Microwave	Sharp, Thailand
Incubator	-
Inverted Microscope	Olympus, Japan
Water Bath	Polyscience, USA
Oven	Bio-Tech, India
Autoclave	-
Mortar and Pestle	-

2.1.8. Disposables:

Name	**Company**
25 cm^2, 75 cm^2 Cell culture flasks	Corning® Flask
Cell culture cluster, flat bottom: 6, 24, 48, 96 well plates	Corning Incorporated Costar®
Tips 50-1000 µl	PlasTiBRand, Germany
Filter Tips 20, 100, 300 µl	Eppendorf, USA
Syringes 1, 3, 10 ml	Changzhou Huichun Medical Equipment Co. Ltd., China
Eppendorf Tubes	Eppendorf
PCR Tubes	-
Parafilm Dispense PM-996	Pechiney Plastic Packaging
Syringes	Cangzhou Huichun Medical

	Equipment Co. Ltd., China
Vacutainers	BD Precisionglide®, England
Needles for Vacutainers	BD Precisionglide®, England
Tubes with EDTA for blood	VacuMed, Italy
Plain Tubes for Serum collection	Ningbo Mflab Medical Instruments Co. Ltd., China
Sealed Plastic Bags for Tissue Collection	-
Cotton	Jiangsu Province Jianerkang Medical Dressing Co. Ltd., China
Disinfectants	Royal Cosmetic Co. Ltd., Egypt
PBS buffer	Thermo Fisher, USA
Antibiotics	Biochrom GmbH, UK

2.1.9. Softwares and websites:

Microplate Reader Software (Gen 5 2.07), BioTek ELx800, USA.

https://www.google.com

Pubmed (https://www.ncbi.nlm.nih.gov/pubmed)

NCBI Genbank database (

BLASTn (http://blast.ncbi.nlm.nih.gov/)

Chromas version 2.6.4 (www.technelysium.com.au)

Fench TV version 1.4.0 - Geospiza Inc. (www.geospiza.com/finchtv)

CodonCode Aligner version 7.1.2 DEMO, CodonCode Corporation, LI-COR, Inc.

MEGA version 7.0.26 (Molecular Evolutionary Genetics Analysis) program (www.megasoftware.net/mega.html)

ClustalW multiple alignment program (http://www.ebi.ac.uk/Tools/msa/clustalw2/)

Maps of the Sudan (http://www.d-maps.com/carte.php?num_car=1310&lang=en)

2.2. Methods:

2.2.1. Samples collection:

2.2.1.1. Sample collection from sheep and goats:

Samples were collected from sheep and goats from ten different States of the Sudan during the period from May 2015 to May 2018 (Table 1 and 2; Figure 4). A total of 359 Samples were collected from sheep (288) and goats (71) (Table 1). Generally, samples which were collected from sheep and goats included 266 whole blood samples (216 from sheep and 50 from goats) and 93 lung samples (72 from sheep and 21 from goats) (Table 1).

A Total of 276 (223 sheep and 53 goats) antigen samples were collected from suspected PPR occurred in individual cases as well as suspected outbreaks occurred in herds. These samples were collected from five different States namely Khartoum, Gezira, River Nile, Elgedarif and Western Kurdufan States (Table 2 and 3; Figure 5). In addition 83 lung samples from sheep (65) and goats (18) were collected from slaughter houses from 5 different States of the country namely Northern, White Nile, Northern Kurdufan, Kassala and Red Sea States (Table 2 and 4; Figure 6).

Table 1. Total number of samples collected from sheep and goats from the Sudan.

	Type and Number of Samples		Total No. of Samples
Animal Species	Whole Blood	Lung	
Sheep	216	72	288
Goats	50	21	71
Total	266	93	359

Table 2. Samples collected from sheep and goats from PPR suspected outbreaks and slaughter houses from different States of the Sudan.

Locations of Sample Collection	Total No. of Samples	Type and Number of Samples					
		Sheep			Goats		
		Total No.	Whole Blood	Lung	Total No.	Whole Blood	Lung
Khartoum State	142	109	106	3	33	30	3
Gezira State	82	66	66	-	16	16	-
White Nile State	25	25	-	25	-	-	-
Northern State	20	5	-	5	15	-	15
River Nile State	32	28	28	-	4	4	-
Kassala State	5	2	-	2	3	-	3
Red Sea State	12	12	-	12	-	-	-
Elgedarif State	4	4	-	4	-	-	-
Northern Kurdufan State	21	21	-	21	-	-	-
Western Kurdufan State	16	16	16	-	-	-	-
Total	359	288	216	72	71	50	21

Figure 4. Locations of samples collected from sheep and goats from PPR suspected outbreaks and slaughter houses from different States of the Sudan.

2.2.1.1.1. Sample collection from sheep and goats from PPR suspected outbreaks:

Samples (276) from sheep (223) and goats (53) were collected from suspected PPR individual cases and suspected PPR outbreaks from five different States of the Sudan. From middle Sudan samples were collected from Khartoum State (142 samples, 136 whole blood and 6 lung, 109 sheep and 33 goats) and Gezira State (82 whole blood samples, 66 sheep and 16 goats) (Table 2 and 3; Figure 5). From Northern Sudan samples were collected from River Nile State (32 whole blood samples, 28 sheep and 4 goats) (Table 2 and 3; Figure 5). Samples collected from Eastern Sudan area were from Elgedarif State (4 lung, 4 sheep); while samples from Western Sudan were collected from Western Kurdufan State (16 whole blood samples, 16 sheep) (Table 2 and 3; Figure 5). These animals had no previous vaccination history against PPR.

Table 3. Samples collected from sheep and goats from PPR suspected outbreaks from different States of the Sudan.

Locations of Sample Collection	Total No. of Samples	Type and Number of Samples					
		Sheep			Goats		
		Total No.	Whole Blood	Lung	Total No.	Whole Blood	Lung
Khartoum State	142	109	106	3	33	30	3
Gezira State	82	66	66	-	16	16	-
River Nile State	32	28	28	-	4	4	-
Elgedarif State	4	4	-	4	-	-	-
Western Kurdufan State	16	16	16	-	-	-	-
Total	276	223	216	7	53	50	3

Figure 5. Locations of samples collected from sheep and goats from PPR suspected outbreaks from different States of the Sudan.

2.2.1.1.2. Sample collection from sheep and goats from slaughter houses:

Additionally, lung samples (83), with signs of pneumonia and congestion, from sheep (65) and goats (18) were collected from slaughter houses from Northern State in Northern Sudan (20 lungs samples, 5 sheep and 15 goats); White Nile State in middle of the Sudan (25 lungs, 25 sheep), Northern Kurdufan State in Western Sudan (21 lungs, 21 sheep), and from Kassala State (5 lungs, 2 sheep and 3 goats) and Red Sea State (12 lungs, 12 sheep) in Eastern Sudan (Table 2 and 4; Figure 6). Samples were submitted to the Central Veterinary Research Laboratory (CVRL), Soba, Khartoum.

Table 4. Lung samples collected from sheep and goats from slaughter houses located in different States of the Sudan.

Locations of slaughter house	Total No. of Lung Samples	Animal Species	
		Sheep	Goats
White Nile State	25	25	-
Northern State	20	5	15
Kassala State	5	2	3
Red Sea State	12	12	-
Northern Kurdufan State	21	21	-
Total	83	65	18

Figure 6. Locations of samples collected from sheep and goats from slaughter houses located in different States of the Sudan.

2.2.1.1.3. Sample collection from gazelles:

Samples were collected from Dorcas gazelles "*Gazella dorcas*" from two different States of the Sudan from the period from November 2016 to May 2017 (Table 5; Figure 7). Two whole blood samples, from two apparently healthy semi-captive Dorcas gazelles *"Gazella dorcas"* from Soba East, Khartoum State, were submitted to the Central Veterinary Research Laboratory (CVRL), Soba, Khartoum, in November 2016. Another two whole blood samples, from two apparently healthy semi-captive Dorcas gazelles from Elazhari, Khartoum State, were also submitted to the CVRL laboratory in December 2016. Those gazelles were submitted for screening for PPR viral infection before export (Table 5; Figure 7). Samples including nasal swabs, whole blood and sera, from two apparently healthy captive Dorcas gazelles, were collected from Kuku Animal Zoo, West Nile, Khartoum-North, in April 2017 (Table 5; Figure 7).

In May 2017, many free-ranging Dorcas gazelles with suspected signs of PPR were reported in Dinder National Park, located between Sinnar and Gedarif States in South-Eastern Sudan. Infected gazelles showed typical clinical signs of PPR accompanied by fatalities. One lung tissue was collected from a dead gazelle whereas two whole blood and four serum samples were collected from surviving gazelles. Samples were immediately submitted to the CVRL laboratory, Soba, Khartoum, Sudan (Table 5; Figure 7).

All animals from which samples had been collected had no previous vaccination history against PPR.

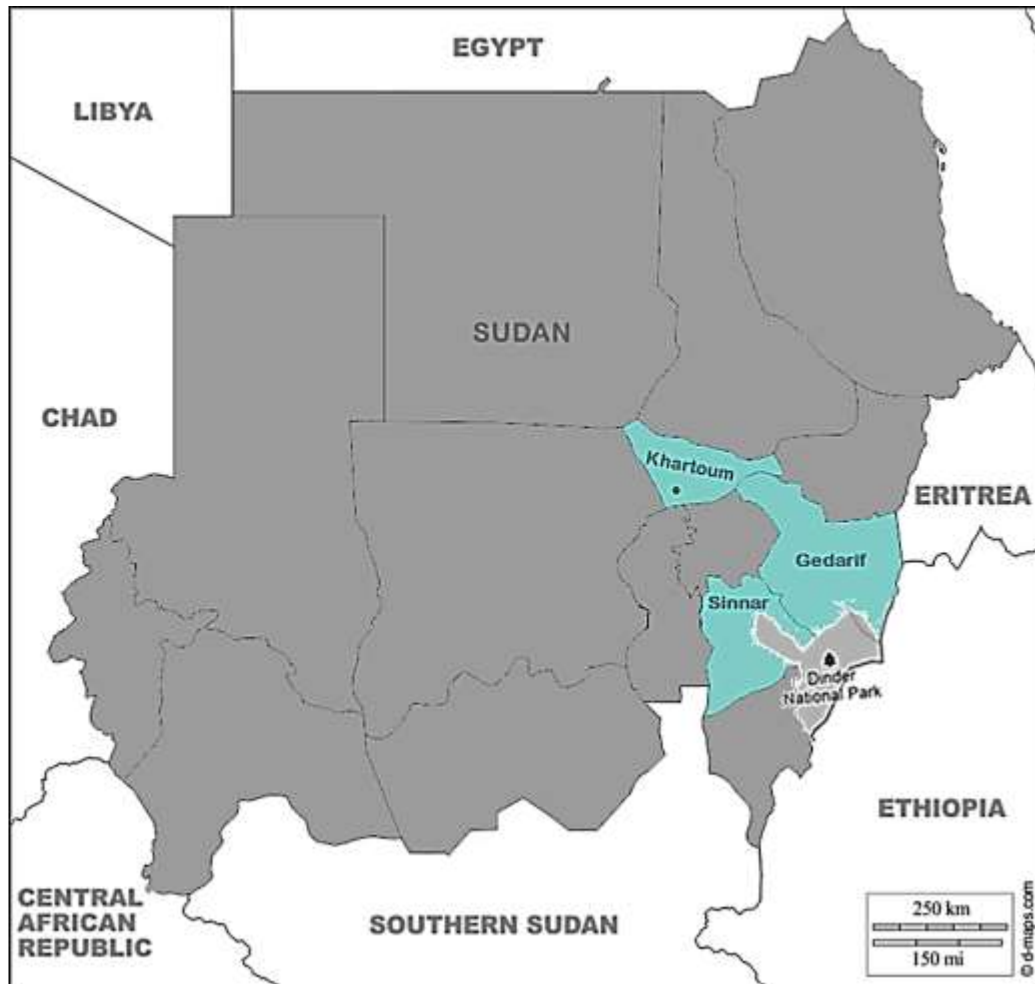

Figure 7. Map of the Sudan (dark Grey colour) and neighboring countries. Locations of PPR suspected cases and outbreak occurred in gazelles (*Gazelle Dorcas*) from two different States of the Sudan during 2016-2017 (Asil *et al.,* 2019). The map was retrieved from: "http://www.d-maps.com/carte.php?num_car=1310&lang=en" with some modifications.

Table 5. Samples collected from apparently healthy gazelles and a PPR suspected outbreak in gazelles (*Gazelle Dorcas*) from two different States of the Sudan.

Locations of Sample Collection	Type and Number of Samples				
	Total No. of Samples	Whole Blood	Nasal swab	Lung	Total No. of Sera
Soba East, Khartoum State	2	2	-	-	-
Elazhari, Khartoum State	2	2	-	-	-
West Nile, Kuku Zoo, Khartoum State	4	2	2	-	2
Dindir National Park, Sinnar and Elgedarif States	3	2	-	1	4
Total	11	8	2	1	6

2.2.2. Preparation of antigen samples:

2.2.2.1. Preparation of 10% tissue suspension:

A 10% tissue suspension was prepared from lung, lymph node and spleen tissues in phosphate buffer saline (PBS) containing antibiotics (Penicillin, Streptomycien, Gentamycen and Amphotericin B) by cutting the tissue into small pieces using scissors then grinding into mortar and pestle using sterile sand followed by centrifugation at 2000 rpm for 10 minutes, the supernatant was transferred into new sterile Eppendorf tubes and stored at -20°C till used.

2.2.2.2. Preparation of whole blood samples:

Whole blood samples were collected in vacutainers with anticoagulants and were kept at 4°C for a maximum of one week period, then buffy coat was separated from whole blood samples by centrifugation at 2000 rpm for 30 minutes and transferred into new sterile Eppendorf tubes and stored at -20°C till used.

2.2.2.3. Preparation of swab samples:

Swab samples in viral transport medium containing antibiotics were stored at 4°C, subjected to centrifugation at 4°C at 3000 rpm for 10 minutes and supernatants were separated from mucus and cellular debris into new sterile Eppendorf tubes and stored at -20°C till used.

2.2.2.4. Preparation of serum samples:

Blood samples were collected in vacutainers without anticoagulants and left at room temperature for 1-2 hours then stored overnight into refrigerator at 4°C. On the following day, sera were separated from blood samples by centrifugation at 5000 rpm for 5 minutes then sera were transferred into new sterile Eppendorf tubes and stored at -20°C till used.

2.2.3. PPR virus identification:

2.2.3.1. Immunocapture Enzyme Linked Immunosorbent Assay (IC-ELISA):

Whole blood, swabs and tissue homogenate antigen samples collected from sheep, goats and gazelles were tested for the presence of PPRV antigen using ID Screen® PPR Antigen Capture Sandwich ELISA Kit for the detection of PPR virus (IDvet Innovative Diagnostics, CIRAD, Montpellier, France) as per the manufacturer's instructions (Libeau *et al.,* 1994).

The microplates were purchased coated with 1X of anti-PPRV-N capture antibody. Reagents were cooled down to room temperature (25°C) and were homogenized by inversion or using vortex. Initially, 25 µl of Dilution Buffer 13 was added to all wells of the plate, then 25 ul of the Negative Control (NC) was added to wells A1 and B1 then 25 µl of the Positive Control (PC) was added to wells C1 and D1 of the plate (Figure 8). Afterwards, 25 µl of each test sample was added to one of the remaining wells of the plate from E1 to H12 (Figure 8). The plates were incubated for 45-50 minutes at 37°C. The wells of the ELISA plate were washed 6 times using an ELISA washer (BioTek (Elx50), USA) with approximately 300 µl of 1X wash solution diluted in D.D.W. Then 100 µl of the 1X conjugate, diluted in Dilution Buffer 19, was added to all wells of the ELISA plate. The plates were incubated at room temperature (21-25°C) for 30 minutes. The wells of the ELISA plate were washed 6 times as before. 100 µl of the Substrate Solution (TMB) was added to each well then the plate was incubated in the dark for 15 minutes at RT. After that, 100 µl of the 0.5 M Stop Solution was added to all wells of the ELISA plate. Finally the optical density (O.D.) of the controls and samples were recorded at 450 nm by reading the plate using an ELISA Reader (BioTek, USA).

2.2.3.2. Calculation and interpretation of the ELISA results:

For each sample, the S/P% expressing the level of PPRV as a percentage of the positive control was calculated as follow:

$$S/P\% = \frac{OD_{sample} - OD_{NC}}{OD_{PC} - OD_{NC}} \times 100$$

Samples yielded less than 20% S/P ratio were considered negative whereas samples yielded S/P ratios equal to or greater than 20% were considered positive.

Controls (Column 1A-1D) **Antigen Samples (Columns 1-12)**

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	NC	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*
B	NC	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*
C	PC	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*
D	PC	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*
E	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*
F	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*
G	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*
H	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*	*TAg*

Figure 8. Layout of the IC-ELISA plate.

Notes:

Controls: NC = Negative control antigen (A1, B1 C1, D1)

PC = Positive control antigen (C1, D1)

Samples: *TAg* = Test Antigen (E1-H12)

Table 6. Steps of the IC-ELISA for detection of PPRV Antigen.

Assay Steps	Assay Conditions			
	Incubation Time	Incubation Temperature	Plate Shaking	Wash Step
Add 25 µl of dilution Buffer 13 Add 25 µl of Positive (+ve) and Negative (-ve) Controls Add 25 µl of the Test Samples	45 min/ 16-20 h	37°C 21-25°C/RT	Yes -	6X
Add 100 µl of the Conjugate diluted in dilution buffer 19	30 min	21-25°C/RT	yes	6X
Add 100 µl of the Substrate Solution	15 min (Dark)	21-25°C/RT	yes	none
Add 100 µl of the Stop Solution	none	none	none	none
Read Reaction (O.D.)	450 nm filter must be in plate reader			

2.2.4. PPR virus isolation:

Vero (Green African Monkey Kidney) cell lines were used for initial PPRV isolation. The prepared antigen samples (buffy coat, swabs and 10% tissue suspension) containing virus were used for PPRV virus isolation in cell culture. Around 500 µl to 1 ml volume of the antigen sample was inoculated into semi confluent monolayers of Vero cells seeded in 25 cm^2 tissue culture flasks, the inoculum was incubated in an incubator at 37°C for 1 hour to allow for virus adsorption to the cells, after that the virus inoculum was emptied and cells were maintained with 5 ml of GMEM medium supplemented with 5-10% newborn calf serum. The cultures were examined microscopically daily for evidence of cytopathic effect (CPE) from the 7th day post inoculation (p.i.). Cells were incubated for 25 days and medium was changed every 4-5 days. When no CPE was detected after 25 days the cells was subcultured for three blind passages. When the CPE reached 80-90% of the infected cell monolayer, the supernatant was harvested and stored at -70°C. The virus suspension was subjected to 2-3 cycles of freezing and thawing and then clarified at 3000 rpm for 30 minutes using refrigerated centrifuge at 4°C. Uninfected cell cultures were treated similarly and maintained as controls.

2.2.4.1. Identification of PPRV isolates:

PPRV in the infected cell culture supernatant was identified using an IC-ELISA and RT-PCR assay using PPRV N-gene specific primers.

2.2.5. Molecular characterization of PPRV:

2.2.5.1. RNA purification:

2.2.5.1.1. RNA purification using RNeasy® Mini kit:

Total Viral RNA was purified from tissue samples using RNeasy® Mini kit (Qiagen, Germany) following the manufacturer's instructions.

Firstly, 1 ml of Trizol was added to 500 µl of whole blood and incubated for 5 min at room temperature then 200 µl of Chloroform was added, tubes were mixed by shacking for 15 seconds then incubated for 3 min at room temperature. Tubes were centrifuged at 12,000 rpm at 4°C for 5 min. The aqueous phase was transferred into a new Eppendorf tube and 600 µl of 70% Alcohol was added and mixed well, then 700 µl of the mixture in the tube was transferred into the RNeasy column and centrifuged at 10,000 rpm at

4°C for 15 sec. 700 µl of buffer RW1 was added to the collection tube and the RNeasy column was centrifuged at 10,000 rpm at 4°C for 15 sec. The RNeasy column was placed into a new 2 ml collection tube and 500 µl of buffer RPE was added to the RNeasy column then the column was centrifuged at 10,000 rpm at 4°C for 15 sec, after that an additional 500 µl of buffer RPE was added to the RNeasy column and centrifuged again at 10,000 rpm at 4°C for 2 min for drying the RNeasy silica-gel membrane. Finally the RNeasy column was placed into a new 1.5 ml collection tube then 30-50 µl of RNase free water was added directly into the silica-gel membrane, after 1 min the column was centrifuged at 10,000 rpm at 4°C for 1 min and the eluted RNA was stored at -70°C till used.

2.2.5.1.2. RNA purification using QIAamp Viral RNA® Mini Kit:

Total Viral RNA was purified from swabs, whole blood samples and viral cell culture supernatants of PPRV isolates using QIAamp Viral RNA® Mini Kit (Qiagen, Germany) following the manufacturer's instructions. RNA purification was performed for the reference virus PPRV Nigeria 75/1 vaccine strain which was used as a positive control in RT-PCR.

560 µl of the prepared Buffer AVL-carrier RNA was added to a 1.5 ml micro-centrifuge tube, then 140 µl of the swabs, whole blood samples and viral cell culture supernatants was added to the mixture in the micro-centrifuge tube. The mixture was mixed and pulse-vortex for 15 sec then incubated at room temperature for 10 min, the tube was centrifuged then 560 µl of 96-100% Ethanol was added to the mixture, mixed and centrifuged as before. 630 µl of the solution was applied carefully to the QIAamp Mini column in a 2 ml collection tube without wetting the rim, the tubes and columns were centrifuge at 8,000 rpm for 1 min. Afterwards, the QIAamp Mini column was placed into a clean 2 ml collection tube, then 500 µl of Buffer AW1 was added, tubes and columns were centrifuged as before, the QIAamp Mini column was placed into a clean 2 ml collection tube. 500 µl of Buffer AW2 was added, the tubes and columns were centrifuged at 14,000 rpm for 3 min. The QIAamp Mini column was placed into a clean 1.5 ml micro-centrifuge tube, then 60 µl of Buffer AVE was added and tubes with the column was incubated at room temperature for 1 min then centrifuged at 8,000 rpm for 1 min. Finally, the eluted RNA was stored at -70°C till used.

2.2.5.2. Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR):

2.2.5.2.1. PCR reaction mixture:

The PCR reaction was performed in a final volume of 20 µl including 18 µl of the master mix (Qiagen®) in addition to 2 µl of the template RNA as follows:

PCR Standard Mixture	1X Volume Reaction
Sterile Water Ultrapure	9.2 µl
1X PCR Buffer 5X	4 µl
0.4 mM dNTPs Mix 10mM	0.8 µl
0.4 pmol/µl Primer NP3	1.6 µl
0.4 pmol/µl Primer NP4	1.6 µl
One step RT-PCR Enzyme mix	0.8 µl
Viral RNA Template	2 µl
Final Volume	20 µl

*A negative control where DNA was replaced by D.D.W. was included.

The partial N-gene sequence (351 bp) was amplified from total viral RNA by a reverse transcription-polymerase chain reaction (RT-PCR) assay designed on a highly conserved sequence of PPRV N-gene. Complementary DNA (cDNA) encoding PPRV partial N-gene sequence (351 bp) was synthesized from RNA by a reverse transcription (RT) reaction followed by PCR for amplification of the respective gene using Qiagen One-Step RT-PCR Kit (Qiagen, Germany) and N-gene specific primers set: [NP3 forward Primer: NP gene 1232-1255 (5'-TCT CGG AAA TCG CCT CAC AGA CTG-3') and NP4 reverse Primer: NP gene 1583-1560 (5'-CCT CCT CCT GGT CCT CCA GAA TCT-3')] following the procedure described previously by Couacy-Hyamann *et al.* (2002).

The PCR cycling conditions were as follow: cDNA synthesis in 1 cycle of reverse transcription performed at 50°C for 30 min, RT inactivation and initial polymerase activation at 95°C for 15 min followed by 40 cycles of cDNA amplification corresponding to denaturation at 95°C for 10 sec, annealing at 55°C for 30 sec, extension at 72°C for 30 sec, and finally 1 cycle for termination of the PCR reaction by heating at 72°C for 5 min. The PCR amplification was performed using a thermal cycler (Peqlab Biotechnology GmbH, Germany).

Table 7. PCR cycling conditions.

Step	Temperature	Time	No. of cycle
Reverse-Transcription (RT)	50°C	30 min	One cycle
RT inactivation and initial polymerase activation	95°C	15 min	One cycle
Denaturation	95°C	10 sec	40 cycle
Annealing	55°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	5 min	One cycle
Preservation	12°C	∞	-

To analyze the size of PCR amplicon, 5 µl of DNA was mixed with 2 µl of DNA loading buffer, stained with ethidium bromide and size separated by 1.5% agarose gel electrophoresis.

2.2.5.3. Agarose gel electrophoresis:

1.5% agarose gel was prepared by dissolving 1.5 g of ultrapure agarose in 100 ml of 1X Tris Acetate EDTA (TAE) puffer, melted by boiling in a microwave oven and cooled in a water bath at 56°C. After that 3 µl of ethidium bromide was added to the gel, mixed well, poured into a horizontal gel electrophoresis apparatus and left to solidify at RT for 30 min. The gel was covered by 1X TA gel running buffer containing ethidium bromide.

To analyze the size of PCR amplicon, 5 µl of the resulting PCR product was mixed with 2 µl DNA loading buffer then pipetted into pockets in the gel. Samples were analyzed along with 1 Kbp DNA marker. Separation of DNA occurred at 90 V for 30 min. DNA fragments were visualized by an UV transilluminator and the expected size of the PCR product was determined.

2.2.6. Genetic characterization of PPRV:

2.2.6.1. DNA sequencing, sequence analysis and alignment of sequence data:

The 351 nt partial sequences of the 3’ end (1232-1583 nt) of PPRV N-gene, from samples of sheep, goats and gazelles, were determined by sequencing the yielded PCR amplicons in an automated DNA Sequencer using DNA Sequencing kit. DNA

sequencing using Sanger Sequencing method was carried out at BGI Company, China. The yielded nucleotide sequences of PPRV partial N-gene (351 nt = 1232-1583 nt) of sheep, goats and gazelles samples were analyzed and compared with the respective N-gene sequences of PPRV strains available in the GenBank database using BLASTn (http://blast.ncbi.nlm.nih.gov/).

2.2.6.2. Phylogenetic analysis for lineage identification of PPRV:

The partial N-gene sequences of sheep, goats and gazelles samples were aligned with the respective N-gene sequences of other PPRV isolates available in the GenBank database using ClustalW multiple alignment program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). For PPRV lineage identification, a phylogenetic analysis for the aligned PPRV N-gene partial sequences was performed using the MEGA7 (Molecular Evolutionary Genetics Analysis) program (Kumar *et al.,* 2016). Subsequently, a phylogenetic tree was generated using the Neighbor-Joining method, the distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004). Bootstrap confidence intervals were calculated on 1,000 replicates (clusters were supported by bootstrap values > 70 shown at node).

2.2.7. Detection of PPRV antibodies:

2.2.7.1. Competitive-Enzyme Linked Immunosorbent Assay (C-ELISA):

For detection of PPRV antibodies, a competitive ELISA (C-ELISA) assay directed against PPRV nucleoprotein using a competitive screening ELISA kit "ID Screen® PPR Competition kit for the detection of antibodies against PPR in sheep and goats serum and plasma" developed by CIRAD-EMVT, Montpellier, France (IDVet Innovative Diagnostics, France). The procedure for the C-ELISA was performed following the manufacturer's instructions. Reagents were cooled down to room temperature (25°C) and were homogenized by inversion or using vortex. Initially 25 µl of Dilution Buffer 13 was added to all wells of the plate, then 25 ul of the Positive Control (PC) was added to wells A1 and B1 of the plate and 25 µl of the Negative Control (NC) was added to wells C1 and D1 (Figure 9). Afterwards, 25 µl of each test sample was added to one of the remaining wells of the plate from E1 to H12 (Figure 9). The plates were incubated for 45-50 minutes at 37°C. The wells of the ELISA plate were washed 3 times each with approximately 300 µl of 1X wash solution diluted in D.D.W. using an ELISA washer

(BioTek (Elx50), USA). Then 100 µl of the 1X conjugate, diluted in Dilution Buffer 4, was added to all wells of the ELISA plate. The plates were incubated at room temperature (21-25°C) for 30 minutes. The wells of the ELISA plate were washed 3 times as before. 100 µl of the Substrate Solution (TMB) was added to each well then plate was incubated in the dark for 15 minutes at 21-25°C. 100 µl of the 0.5 M Stop Solution was added to all wells of the ELISA plate. Finally the plate was read using an ELISA Reader (BioTek, USA) and the optical density (O.D.) of the controls and samples were recorded at 450 nm. The resulting coloration depends on the quantity of specific antibodies present in the sample to be tested that means firstly in the absence of antibodies, a blue solution appears which becomes yellow after addition of the stop solution, secondly in the presence of antibodies no coloration appears.

Controls
(Column 1A-1D)

Serum Samples
(Columns 1-12)

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	PC	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*
B	PC	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*
C	NC	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*
D	NC	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*
E	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*
F	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*
G	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*
H	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*	*TS*

Figure 9. Layout of the C-ELISA plate.

Notes:

Controls: PC = Positive control serum (A1, B1

NC = Negative control serum (C1, D1)

Samples: *TS* = Test serum (E1-H12)

Chapter III
Results

3.1. PPR in sheep and goats:

3.1.1. Locations of areas of PPR outbreaks:

During 2015 to 2018, many suspected PPR outbreaks occurred among sheep and goats were recorded in many localities within different States of the Sudan. These outbreaks were recorded during 2015-2018 in Khartoum State (Central Sudan), during 2016-2017 in Gezira State (Central Sudan) and in River Nile State (Northern Sudan), during 2015-2017 in Elgedarif State (Eastern Sudan) and finally in 2017 in Western Kurdufan State (Western Sudan).

3.1.2. Description of PPR clinical disease in sheep and goats:

Sheep and goats from PPR suspected outbreaks were recognized mostly by the appearance of the typical clinical signs of the disease including pyrexia, anorexia, profuse serous nasal and ocular discharges while some animals showed mucopurulent discharges, dry muzzle, respiratory signs and diarrhea (Figure 10). The cases were associated with a higher morbidity and mortality rates particularly among young animals.

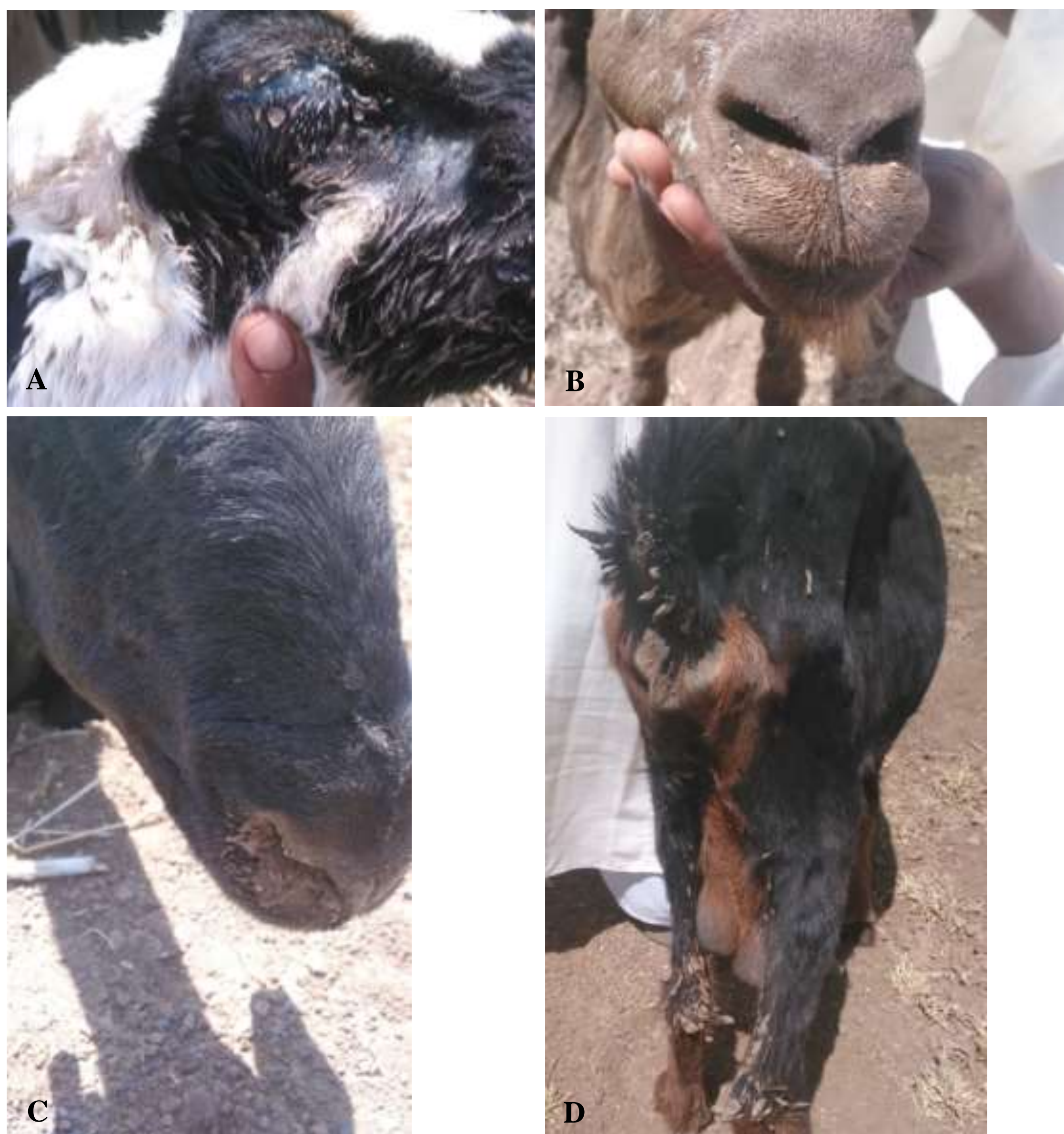

Figure 10. Clinical signs of PPR in sheep and goats infected by PPRV in Gezira State during 2016. A) Ocular discharges. B) and C) Nasal discharges. D) Diarrhoea in goat.

3.1.3. Immunocapture ELISA (IC-ELISA) for detection of PPRV antigen:

3.1.3.1. IC-ELISA for detection of PPRV antigen in samples collected from sheep and goats from PPR suspected outbreaks and from slaughter houses:

A total of 359 whole blood and tissue samples of sheep and goats collected from ten different States of the Sudan were screened for the presence of PPRV antigen by an IC-ELISA assay. The results showed that PPRV antigen could be detected in 180/359 samples (50.1%) while 179/359 samples (49.9%) were found negative (Table 8).

On the species basis, of the 288 sheep samples analyzed, 135/288 (46.9%) were positive whereas the majority of 153/288 (53.1%) samples were found negative. Additionally, of the 71 goat samples tested by the IC-ELISA, 45/71 (63.4%) samples were positive while 26/71 (36.6%) samples were found negative (Table 8).

Table 8. IC-ELISA for detection of PPRV antigen in samples collected from sheep and goats from PPR suspected outbreaks and from slaughter houses.

Animal Species	Whole blood and tissue samples		
	Total No. tested (%)	No. +ve (%)	No. -ve (%)
Sheep	288 (100%)	135 (46.9%)	153 (53.1%)
Goats	71 (100%)	45 (63.4%)	26 (36.6%)
Total	359 (100%)	180 (50.1%)	179 (49.9%)

3.1.3.2. IC-ELISA for detection of PPRV antigen in whole blood and lung samples collected from sheep and goats from PPR suspected outbreaks:

Of the total 276 antigen samples from sheep and goats collected from suspected PPR outbreaks and analyzed by IC-ELISA, 160/276 samples (58%) were found positive for PPRV antigen while 116/276 samples (42%) were found negative (Table 9). The overall antigenic prevalence of PPRV antigen was 58% (Table 9).

Considering the animal species, of the total 223 antigen samples (216 whole blood + 7 lung) collected from sheep from PPR suspected outbreaks, 123/216 (56.9%) whole blood samples and 4/7 (57.1%) lung were found positive for PPRV antigen (Table 10). Of the

total 53 antigen samples (50 whole blood + 3 lung) collected from goats from PPR suspected outbreaks, 31/50 (62%) whole blood samples and 2/3 (66.7%) lung were found positive for PPRV antigen (Table 10).

Considering the type of samples analyzed by IC-ELISA for detection of PPRV antigen, of the 266 whole blood samples from both sheep and goats, 154/266 (57.9%) samples were positive while 112/266 (42.1%) samples were negative. PPRV could be detected in 123/216 (56.9%) of the whole blood samples of sheep and in 31/50 (62%) of the whole blood samples of goats (Table 10). When lung samples from both sheep and goats were analyzed by IC-ELISA, of the 10 lung samples 6/10 (60%) samples were positive while 4/10 (40%) samples were negative. PPRV could be detected in 4/7 (57.1%) lung tissues collected from sheep and in 2/3 (66.7%) lung tissues collected from goats (Table 10).

Table 9. IC-ELISA for detection of PPRV antigen in samples collected from sheep and goats from PPR suspected outbreaks.

Animal Species	Samples from PPR suspected outbreaks		
	Total No. tested (%)	No. +ve (%)	No. –ve (%)
Sheep	223 (80.8%)	127 (56.9%)	96 (43.1)
Goat	53 (19.2%)	33 (62.3%)	20 (37.7%)
Total	276 (100%)	160 (58%)	116 (42%)

Table 10. IC-ELISA for detection of PPRV antigen in whole blood and lung samples collected from sheep and goats from PPR suspected outbreaks.

Animal Species	Total No. of samples	Type of samples					
		Whole Blood			Lung		
		Total No. tested (%)	No. +ve (%)	No. -ve (%)	Total No. tested (%)	No. +ve (%)	No. -ve (%)
Sheep	223 (80.8%)	216 (100%)	123 (56.9%)	93 (43.1%)	7 (100%)	4 (57.1%)	3 (42.9%)
Goats	53 (19.2%)	50 (100%)	31 (62%)	19 (38%)	3 (100%)	2 (66.7%)	1 (33.3%)
Total	276 (100%)	266 (100%)	154 (57.9%)	112 (42.1%)	10 (100%)	6 (60%)	4 (40%)

3.1.3.2.1. IC-ELISA for detection of PPRV antigen in samples collected from PPR suspected outbreaks occurred in five different states of the Sudan:

To determine the antigenic-prevalence of PPRV in five different States of the Sudan, samples from sheep and goats from suspected PPR outbreaks were analyzed by IC-ELISA. 160/276 (58%) were positive with 58% overall antigenic prevalence (Table 11). Within States under study, results indicated that the highest antigenic-prevalence was demonstrated in Western Kurdufan State (81.2%), followed by River Nile State (65.6%), then Gezira State (58.5%), then Khartoum State (53.5%) and finally the lowest incidence was present in Elgedarif State (50%) (Table 11).

To determine the antigenic-prevalence of PPRV considering the animal species in five different States of the Sudan, samples were collected from sheep (223) and goats (53) from suspected PPR outbreaks and analyzed by IC-ELISA. Among sheep, results revealed that 127/223 (57%) samples were positive while 96/223 (43%) samples were negative. Within states under study, results indicated that the highest antigenic-prevalence among sheep was demonstrated in Western Kurdufan State (81.2%), followed by River Nile State (64.3%), then Gezira State (62.1%), then Gedarif State (50%) and finally the lowest incidence was present in Khartoum State (48.6%) (Table 12).

Among goats, results revealed that 33/53 (62.3%) samples were positive while 20/53 (37.7%) samples were negative. Within states under study, results indicated that the highest antigenic-prevalence among goats was demonstrated in River Nile State (75%), followed by Khartoum State (69.7%) and finally the lowest incidence was present in Gezira State (43.8%) (Table 12).

Table 11. IC-ELISA for detection of PPRV antigen in samples collected from PPR suspected outbreaks occurred in five different states of the Sudan.

Place of collection	Sheep and goats		
	Total No. tested (%)	No. +ve (%)	No. -ve (%)
Khartoum State	142 (100%)	76 (53.5%)	66 (46.5%)
Gezira State	82 (100%)	48 (58.5%)	34 (41.5%)
River Nile State	32 (100%)	21 (65.6%)	11 (34.4%)
Gedarif State	4 (100%)	2 (50%)	2 (50%)
Western Kurdufan State	16 (100%)	13 (81.2%)	3 (18.8%)
Total	276 (100%)	160 (58%)	116 (42%)

Table 12. IC-ELISA for detection of PPRV antigen in sheep and goats samples collected from PPR suspected outbreaks occurred in five different states of the Sudan.

Place of collection	Sheep			Goats		
	Total No. tested (%)	No. +ve (%)	No. –ve (%)	Total No. tested (%)	No. +ve (%)	No. –ve (%)
Khartoum State	109 (100%)	53 (48.6%)	56 (51.4%)	33 (100%)	23 (69.7%)	10(30.3%)
Gezira State	66 (100%)	41 (62.1%)	25 (37.9%)	16 (100%)	7 (43.8%)	9 (56.2%)
River Nile State	28 (100%)	18 (64.3%)	10 (35.7%)	4 (100%)	3 (75%)	1 (25%)
Elgedarif State	4 (100%)	2 (50%)	2 (50%)	-	-	-
Western Kurdufan State	16 (100%)	13 (81.2%)	3 (18.8%)	-	-	-
Total	223 (100%)	127 (57%)	96 (43%)	53 (100%)	33 (62.3%)	20 (37.7%)

3.1.3.3. IC-ELISA for detection of PPRV antigen in sheep and goats lung samples collected from slaughter houses:

Screening of lung samples (83) from sheep (65) and goats (18) collected from slaughter houses by IC-ELISA for detection of PPRV antigen, indicated that 20/83 (24.1%) were positive while 63/83 (75.9%) were negative. Results indicated an overall antigenic prevalence of 24.1% (Table 13).

Table 13. IC-ELISA for detection of PPRV antigen in sheep and goats lung samples collected from slaughter houses.

Animal Species	Lung samples		
	Total No. tested (%)	No. +ve (%)	No. -ve (%)
Sheep	65 (78.3%)	8 (12.3%)	57 (87.7%)
Goats	18 (21.7%)	12 (66.7%)	6 (33.3%)
Total	83 (100%)	20 (24.1%)	63 (75.9%)

3.1.3.3.1. IC-ELISA for detection of PPRV antigen in sheep and goats lung samples collected from slaughter houses in five different States of the Sudan:

To determine the antigenic-prevalence of PPRV in five different States of the Sudan, sheep and goats lung samples from slaughter houses were analyzed by IC-ELISA. Within States under study, results indicated that the highest antigenic-prevalence was demonstrated in Northern State (80%), followed by Northern Kurdufan State (9.5%), then Red Sea State (8.3%), then White Nile State (4%) and finally no incidence was present in Kassala State (0%) (Table 14).

To determine the antigenic-prevalence of PPRV, considering the animal species, in lung samples from slaughter houses in five different States of the Sudan, results revealed that 8/65 (12.3%) sheep samples were positive while 57/65 (87.7%) sheep samples were negative. Within States under study, results indicated that the highest antigenic-prevalence among sheep was demonstrated in Northern State (80%), followed by Northern Kurdufan

State (9.5%), then Red Sea State (8.3%), then White Nile State (4%) and finally no incidence was present in Kassala State (0%) (Table 15).

Among goats, results revealed that 12/18 (66.7%) samples were positive while 6/18 (33.3%) samples were negative. Within states under study, results indicated that the highest antigenic-prevalence among goats was demonstrated in Northern State (80%) while no incidence was present in Kassala State (0%) (Table 15).

Table 14. IC-ELISA for detection of PPRV antigen in lung samples collected from slaughter houses in five different States of the Sudan.

Place of collection	Sheep and goats		
	Total No. tested (%)	No. +ve (%)	No. -ve (%)
White Nile State	25 (100%)	1 (4%)	24 (96%)
Northern State	20 (100%)	16 (80%)	4 (20%)
Kassala State	5 (100%)	0 (0%)	5 (100%)
Red Sea State	12 (100%)	1 (8.3%)	11 (91.7%)
Northern Kurdufan State	21 (100%)	2 (9.5%)	19 (90.5%)
Total	83 (100%)	20 (24.1%)	63 (75.9%)

Table 15. IC-ELISA for detection of PPRV antigen from sheep and goats lung samples collected from slaughter houses in five different states of the Sudan.

Place of collection	Sheep			Goats		
	Total No. tested (%)	No. +ve (%)	No. -ve (%)	Total No. tested (%)	No. +ve (%)	No. -ve (%)
White Nile State	25 (100%)	1 (4%)	24 (96%)	-	-	-
Northern State	5 (100%)	4 (80%)	1 (20%)	15 (100%)	12 (80%)	3 (20%)
Kassala State	2 (100%)	0 (0%)	2 (100%)	3 (100%)	0 (0%)	3 (100%)
Red Sea State	12 (100%)	1 (8.3%)	11 (91.7%)	-	-	-
Northern Kurdufan State	21 (100%)	2 (9.5%)	19 (90.5%)	-	-	-
Total	65 (100%)	8 (12.3%)	57 (87.7%)	18 (100%)	12 (66.7%)	6 (33.3%)

3.1.4. PPRV isolation and identification:

Isolation of PPRV from filed samples was attempted in Vero cells which were maintained in Glasgow minimum essential medium (GMEM) supplemented with 5% fetal bovine serum. PPRV isolation was successful only in Vero cells inoculated with 10% lung homogenate. Two PPRV isolates were successfully isolated in Vero cells, the first one was isolated from goat lung from Bahri (Elfaki hashim), Khartoum State and designated as "PPRV/tc/Sudan/Khartoum/Bahri/2015 isolate" while the second one was isolated from sheep lung from Elgedarif State and designated as "PPRV/tc/Sudan/Gedarif/2015 isolate". Typical PPRV cytopathic effect (cpe) consisting of cell rounding, detachment of the cells from the monolayer sheet, destruction of the monolayer sheet and syncytia formation appeared starting from day 15 post infection (p.i.) and completed at 27 d.p.i. (data not shown). Cells were sub-cultured in 9 days intervals till the 27 d.p.i. Uninfected Vero cells, kept as control, were treated in a similar way with the cell monolayer remained intact up to day 27 (data not shown). At 27 d.p.i., viral supernatant and the infected associated cells were harvested by three cycles of freezing and thawing and stored at -70°C until used.

For Identification of PPRV isolates, PPRV and viral RNA in the harvested viral cell culture supernatant were identified using IC-ELISA and RT-PCR assays, respectively.

3.1.5. Molecular characterization of PPRV:

3.1.5.1. Detection of PPRV RNA in suspected samples by reverse-transcription polymerase chain reaction (RT-PCR):

3.1.5.1.1. RT-PCR for detection of PPRV RNA in samples collected from sheep and goats from PPR suspected outbreaks and from slaughter houses:

To confirm the positive results obtained by the IC-ELISA assay, selected sheep and goat samples which were positive by IC-ELISA were further analyzed using PPRV N-gene based RT-PCR assay. All of the analyzed samples 32/32 (100%) yielded positive RT-PCR results (Table 16) showing bands of around 351 bp corresponding to the partial N-gene (Figure 11).

Table 16. RT-PCR for detection of PPRV partial N-gene in samples collected from sheep and goats from PPR suspected outbreaks and from slaughter houses.

Place of collection	Whole blood and tissue samples		
	Total No. tested (%)	No. +ve (%)	No. -ve
Khartoum State	10 (100%)	10 (100%)	0
Gezira State	8 (100%)	8 (100%)	0
Northern State	2 (100%)	2 (100%)	0
River Nile State	3 (100%)	3 (100%)	0
Red Sea State	1 (100%)	1 (100%)	0
Elgedarif State	2 (100%)	2 (100%)	0
White Nile State	1 (100%)	1 (100%)	0
Northern Kurdufan State	1 (100%)	1 (100%)	0
Western Kurdufan State	4 (100%)	4 (100%)	0
Total	32 (100%)	32 (100%)	0

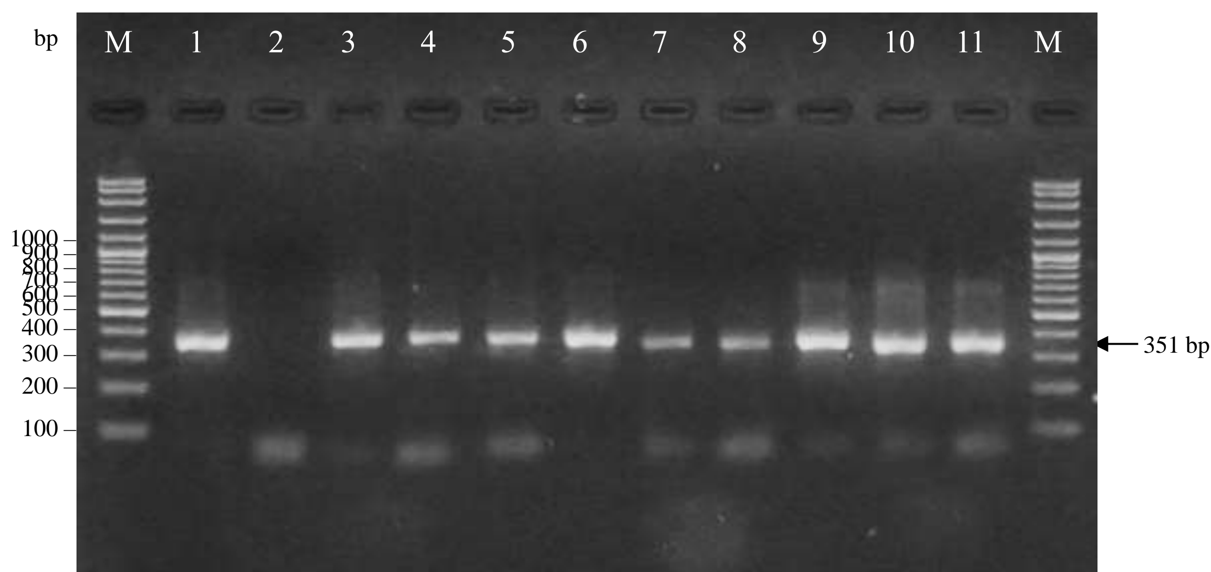

Figure 11. Agarose gel electrophoresis for identification of PPRV N-gene sequence (351 bp) in samples from sheep and goats. Reverse-transcription PCR (RT-PCR) was performed for amplification of PPRV N-gene partial sequence (351 bp) using NP3 and NP4 primer sets, the amplified products were analyzed by agarose gel electrophoresis on 1.5% agarose gel stained with ethidium bromide. Lane M: Marker (DNA MW marker), lane 1: PPRV Nigeria 75/1 vaccine strain (Positive Control), lane 2: Negative Control, lane 3: PPRV/Sudan/Khartoum/2015, lane 4: PPRV/Sudan/Gedarif/2015, lane 5: PPRV/Sudan/Gezira/Kab-Elgidad/2016, lane 6: PPRV/Sudan/Northern/Dongola/2017, lane 7: PPRV/Sudan/River-Nile/Garie/2016, lane 8: PPRV/Sudan/Red-Sea/Port-Sudan/2017, lane 9: PPRV/Sudan/White Nile/Kosti/2017, lane 10: PPRV/Sudan/Northern-Kurdufan/El-Obeid/2017, lane 11: PPRV/Sudan/Western-Kurdufan/Abuzabad/2017, lane M: Marker (DNA MW marker).

3.1.5.1.2. RT-PCR for detection of PPRV RNA in whole blood and lung samples collected from sheep and goats from PPR suspected outbreaks:

Twenty-seven (27) sheep and goat samples from PPR suspected outbreaks which were positive by IC-ELISA were selected and further analyzed using PPRV N-gene based RT-PCR assay. All of the analyzed samples 27/27 (100%) were found positive (Table 17).

On the species basis, all sheep samples 18/27 (66.7%) and all goats samples 9/27 (33.3%) analyzed by RT-PCR were positive (Table 17). Considering the type of samples analyzed by RT-PCR for detection of PPRV RNA, 18 (14 whole blood and 4 lungs) samples from sheep while 9 (6 whole blood and 3 lungs) samples from goats were found positive (Table 18). These sheep samples were from Khartoum, Gezira, River Nile, Elgedarif and Western Kurdufan States whereas goat samples were from Khartoum, Gezira and River Nile States (Table 17, 18).

Table 17. RT-PCR for detection of PPRV RNA in whole blood and lung samples collected from sheep and goats from PPR suspected outbreaks.

Place of collection	Total No. tested (%)	Whole blood and lung samples			
		Sheep		Goats	
		No. tested	No. +ve	No. tested	No. +ve
Khartoum State	10	6	6	4	4
Gezira State	8	5	5	3	3
River Nile State	3	1	1	2	2
Elgedarif State	2	2	2	-	-
Western Kurdufan State	4	4	4	-	-
Total	27	18	18	9	9

Table 18. RT-PCR for detection of PPRV RNA in whole blood and lung samples collected from sheep and goats from PPR suspected outbreaks.

Place of collection	Sheep				Goats			
	Whole Blood		Lung		Whole Blood		Lung	
	No. tested	No. +ve	No. tested	No. +ve	No. tested	No. +ve	No. tested	No. +ve
Khartoum State	4	4	2	2	1	1	3	3
Gezira State	5	5	-	-	3	3	-	-
River Nile State	1	1	-	-	2	2	-	-
Elgedarif State	-	-	2	2	-	-	-	-
Western Kurdufan State	4	4	-	-	-	-	-	-
Total	14	14	4	4	6	6	3	3

3.1.5.1.3. RT-PCR for detection of PPRV RNA from lung samples collected from sheep and goats from slaughter houses:

Five lung samples were selected from samples which were collected from slaughter houses and tested positive by IC-ELISA. These samples were further tested by RT-PCR. RT-PCR results confirmed that all 5/5 (100%) samples analyzed were positive (Table 19).

On the species basis, 4/5 of the analyzed lung samples were from sheep while only one lung sample was from goat (Table 19). These 4 sheep samples were from White Nile, Northern, Red Sea and Northern Kurdufan States whereas the only goat sample was from Northern State (Table 19).

Table 19. RT-PCR for detection of PPRV RNA in lung samples collected from sheep and goats from slaughter houses.

Place of collection	Total No. tested (%)	Sheep		Goats	
		No. tested	No. +ve (%)	No. tested	No. +ve (%)
White Nile State	1	1	1	-	-
Northern State	2	1	1	1	1
Kassala State	0	0	0	0	0
Red Sea State	1	1	1	-	-
Northern Kurdufan State	1	1	1	-	-
Total	5	4	4	1	1

3.1.6. Genetic characterization of PPRV:

3.1.6.1. DNA sequencing:

The identity of the specific PPRV strains was determined by sequencing of RT-PCR amplicons in an automated DNA Sequencer using DNA Sequencing kit (BGI Company, China). For determining the partial N-gene sequencing of PPRV, nine samples were selected from 9 States except of Kassala State. These nine samples were selected from sheep and goat samples which were positive when analyzed by IC-ELISA and PPRV N-gene based RT-PCR. PPR viruses sequenced in this study were designated as "PPRV/Sudan/Khartoum/2015; PPRV/Sudan/Gedarif/2015; PPRV/Sudan/Gezira/Kab-

Elgidad/2016; PPRV/Sudan/River-Nile/Garie/2016; PPRV/Sudan/Northern/Dongola/2017; PPRV/Sudan/Red-Sea/Port-Sudan/2017; PPRV/Sudan/White-Nile/Kosti/2017; PPRV/Sudan/Western-Kurdufan/Abuzabad/2017; PPRV/Sudan/Northern-Kurdufan/El-Obeid/2017".

PPRV N-gene partial nucleotide sequences were then assembled using CodonCode Aligner, the portions overlapping and the primer sequences were eliminated appropriately. The 351 nt partial PPRV N-gene sequences of the nine Sudanese PPRV were compiled and deposited in the NCBI GenBank database under accession numbers MK371448.1 (PPRV/Sudan/Khartoum/2015); MK371449.1 (PPRV/Sudan/Gedarif/2015); MK371450.1 (PPRV/Sudan/Gezira/Kab-Elgidad/2016); MK371451.1 (PPRV/Sudan/River-Nile/Garie/2016); MK371452.1 (PPRV/Sudan/Northern/Dongola/2017); MK371453.1 (PPRV/Sudan/Red-Sea/Port-Sudan/2017); MK371454.1 (PPRV/Sudan/White-Nile/Kosti/2017); MK371455.1 (PPRV/Sudan/Western-Kurdufan/Abuzabad/2017); and MK371456.1 (PPRV/Sudan/Northern-Kurdufan/El-Obeid/2017), respectively.

3.1.6.2. Sequence analysis and comparison:

Subsequently, sequences of PPRV partial N-gene of the sheep and goat samples were analyzed and compared with the respective N-gene sequences of PPRV strains available in the NCBI GenBank database using BLASTn (http://blast.ncbi.nlm.nih.gov/) to find the percentages of nucleotide sequence identities with other PPR viruses. Comparison of the resulting PPRV sequences obtained from sheep and goat samples using BLASTn revealed a close identity to PPRV lineage IV strains. All nine PPRV from sheep and goat from nine different states of the Sudan shared the highest nucleotide sequence identity of 100% with PPRV strain Georgia/G1, 99% with Egyptian PPRV strains [Ismailia 3/Egypt/2010, Ismailia 1/Egypt/2010, Ismailia 2/EGY/2012], PPRV Georgia/Tbilisi/2016 and PPRV Ethiopia 2010 and 2014, 98% with Egyptian PPRV strains [Ismailia 1/2014 and El-Kalubeya], 97% with PPRV strains [Morocco 2008, Shiraz 101, Kurdistan 2011, Algeria/S15] with a lower identity of 95-96% with other PPRV lineage IV isolates.

3.1.6.3. Phylogenetic analysis for PPRV lineage identification:

This analysis was extended by comparison of the assembled sheep and goats PPRV sequences with the respective N-gene sequences of other PPRV isolates available in the GenBank database using ClustalW multiple alignment program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic analysis to determine the specific virus lineage was performed for the aligned PPRV N-gene partial sequences using the MEGA7.0.26 (Molecular Evolutionary Genetics Analysis) program (www.megasoftware.net/mega.html). The resulting phylogenetic tree confirmed that the nine PPRV strains from sheep and goats in the Sudan belonged to the PPRV lineage IV genotype and shared the closest sequence identity with PPRV strains originating from North African countries (Figure 12). This lineage contains a number of sub-lineages, one of which includes a number of previously published PPRV strains from the Sudan (2000-2009) (Kwiatek *et al.,* 2011), Egypt/Ismailia strains (2010 and 2012) and PPRV strains from Eritrea, Ethiopia, Tunisia, Algeria, Morocco, Saudi-Arabia, Niger, Gabon and Cameroon (Figure 12). Interestingly, the nine PPRV sequenced in this study are located in this particular sub-lineage in which a close relationship is evident with other African strains mentioned indicating possible transnational virus spread (Figure 12).

Of note, the other three PPRV strains identified in this study, PPRV/Sudan/Gedarif/2015, PPRV/Sudan/River-Nile/Garie/2016 and PPRV/Sudan/Northern-Kurdufan/El-Obeid/2017, shared an identical sequence in the specific region of the N gene under investigation and were clustered together into one sub-lineage (Figure 12). The remaining six sequences namely PPRV/Sudan/Khartoum/2015, PPRV/Sudan/Gezira/Kab-Elgidad/2016; PPRV/Sudan/Northern/Dongola/2017, PPRV/Sudan/Red-Sea/Port-Sudan/2017, PPRV/Sudan/White-Nile/Kosti/2017 and PPRV/Sudan/Western-Kurdufan/Abuzabad/2017 were clustered into other sub-lineage with other Sudanese and African strains (Figure 12).

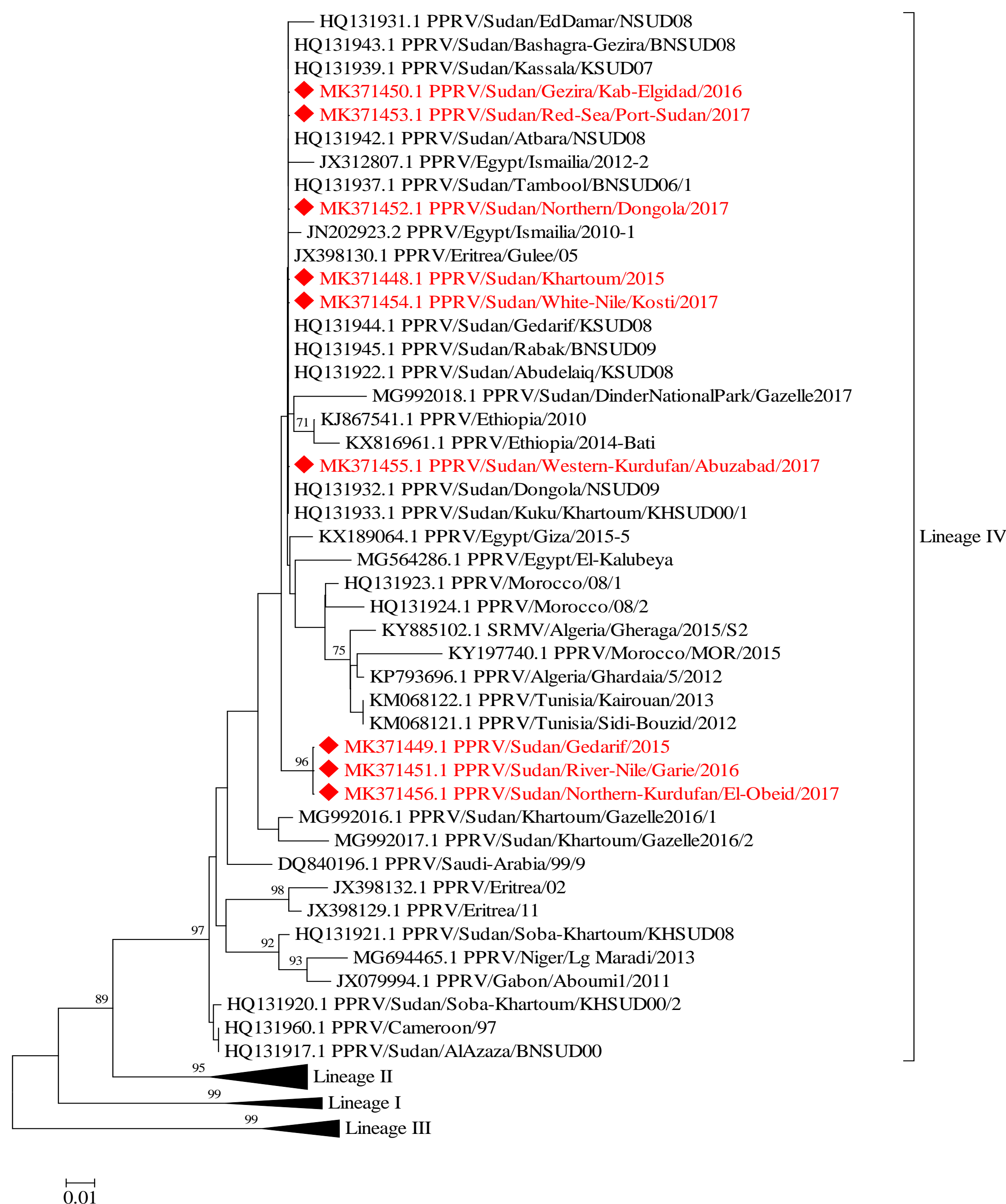

Figure 12. Neighbor-joining tree for PPRV strains constructed on the basis of the 255 nt partial N-gene sequences of PPRV strains. The analysis was conducted in MEGA7.0.26 (Molecular Evolutionary Genetics Analysis) (www.megasoftware.net/mega.html) by analyzing 1,000 bootstrap replicates, clusters were supported by bootstrap values >70 (PPRV Sudanese Strains from sheep and goats are represented by red font and red diamond circle). The Neighbor-joining tree represents PPRV lineage III and IV strains.

3.2. PPR in Dorcas gazelles *"Gazella dorcas"*:

3.2.1. Clinical investigations and description of PPR outbreak:

Concerning PPR clinical disease in gazelles, the six apparently healthy gazelles *"Gazella dorcas"* from the different three areas of Khartoum "Elazhari, Soba East and Kuku Animal Zoo" did not show any apparent clinical signs of the disease. However, gazelles *"Gazella dorcas"* from the Dinder National Park suspected PPR outbreak exhibited typical clinical signs of PPR including pyrexia, ocular and nasal discharges, pneumonia, diarrhea and sudden death of young gazelles.

3.2.2. Detection of PPRV antigen by an immunocapture ELISA (IC-ELISA) assay:

For detection of PPRV antigen, from the 11 gazelle samples analyzed by an immunocapture ELISA (IC-ELISA) assay, PPRV antigen could be detected in the four whole blood samples taken from gazelles from Elazhari and Soba East, Khartoum State (Table 20). In contrast, PPRV antigen could not be detected in the two whole blood and nasal swab gazelle samples from Kuku Animal Zoo, Khartoum State (Table 20). This assay also provided that the suspected PPR outbreak in gazelles in Dinder National Park was due to PPRV as antigen could be detected in lung tissue and two whole blood samples (Table 20).

3.2.3. PPRV isolation and identification:

Virus isolation was attempted in Vero cells which were maintained in Glasgow minimum essential medium (GMEM) (Sigma-Aldrich) supplemented with 5% fetal bovine serum (Sigma-Aldrich). PPRV isolation on Vero cells was attempted several times using Buffy coat, swab supernatants or a 10% lung tissue homogenate. However, PPRV isolation was successful only in Vero cells inoculated with 10% lung homogenate of a gazelle from the Dinder National Park outbreak (Table 20). PPRV isolate was designated as "PPRV/Gazelle/tc/Sudan/DNP2017". Infected Vero cells, started to show characteristic cytopathic effects (CPE) of PPRV such as cell rounding at 7 days post-infection (d.p.i.), with progressive destruction of the cell monolayer between 11-15 d.p.i. (Figure 13). Uninfected Vero cells, kept as control, were treated in a similar way with the cell monolayer remained intact up to day 15 (Figure 13). At 15 d.p.i., combined cell associated,

and supernatant virus components were harvested and stored at -70°C for the purpose of identification of the virus isolate.

For Identification of PPRV isolates, PPRV and viral RNA in the harvested viral cell culture supernatant were identified using IC-ELISA and RT-PCR assays, respectively.

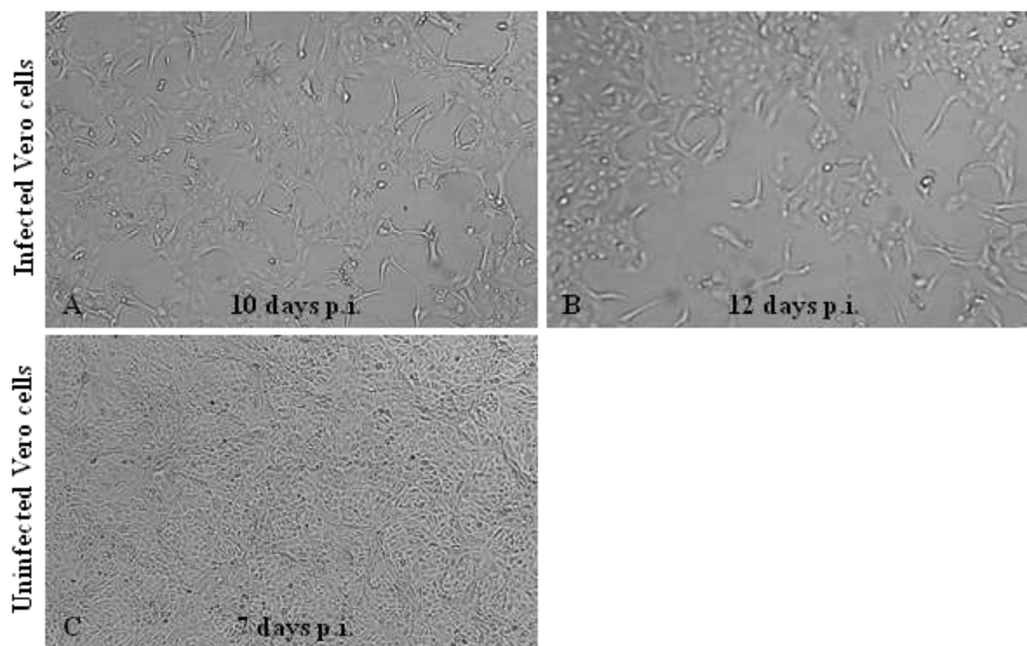

Figure 13. PPRV isolation from gazelle lung from Dinder National Park outbreak in Vero cell lines. A) and B) Vero cells were infected with 10% lung homogenate of a gazelle from the Dinder National Park outbreak, PPRV isolate was designated as PPRV/Gazelle/tc/Sudan/DNP2017. C) Normal Vero cells (control). Photos were acquired using inverted microscope Olympus CKX41 and camera (Olympus Lifescience).

Table 20. Detection of PPRV antigen by IC-ELISA and PPRV isolation from specimens of Dorcas gazelles *"Gazella dorcas"*.

Collection Date	Location	Type of Samples	Total No. of Samples	IC-ELISA		Virus Isolation	
				No. tested	No. +ve (%)	No. tested	No. +ve (%)
Nov. 2016	Soba East, Khartoum State	WB	2	2	2 (100%)	2	0 (0%)
Dec. 2016	Elazhari, Khartoum State	WB	2	2	2 (100%)	2	0 (0%)
April 2017	Kuku Animal Zoo, Khartoum State	WB	2	2	0 (0%)	2	0 (0%)
		NS	2	2	0 (0%)	2	0 (0%)
May 2017	Dinder National Park, Sinnar and Elgedarif States	WB	2	2	2 (77.7%)	2	0 (0%)
		L	1	1	1 (33.3%)	1	1 (100%)

Notes: WB = Whole blood; NS = Nasal Swab; L = Lung

3.2.4. Molecular characterization of PPRV:

3.2.4.1. Detection of PPRV RNA in suspected samples by RT-PCR:

For detection of PPRV RNA by RT-PCR, three samples were selected from gazelle samples which were positive by IC-ELISA and were further analyzed using PPRV N-gene based RT-PCR. These included two whole blood samples from Soba East and Elazhari, Khartoum State, designated as "PPRV/Gazelle/Sudan/Khartoum2016-1" and "PPRV/Gazelle/Sudan/Khartoum2016-2" respectively, and one lung sample from a gazelle with suspected PPR from Dinder National Park which was designated as "PPRV/Gazelle/Sudan/DNP2017". The reference PPRV Nigeria 75/1 vaccine strain, a PPRV lineage II, was used as a control. The partial PPRV N-gene sequence (351 bp, 1232-1583 nt) was amplified using Qiagen One-Step RT-PCR Kit and N-gene specific primers. This resulted in PCR amplicons in all samples with an expected size of 351 bp upon visualization by electrophoresis on an ethidium bromide-stained 1.5% agarose gel (Table 21, Figure 14).

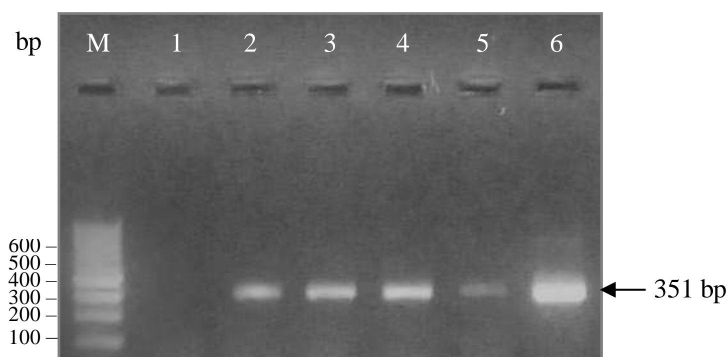

Figure 14. Agarose gel electrophoresis for identification of PPRV N-gene sequence (351 bp) in samples from Dorcas gazelles "*Gazella dorcas*" and PPRV isolate. Reverse-transcription PCR (RT-PCR) was performed for amplification of PPRV N-gene partial sequence (351 bp) using NP3 and NP4 primer sets, the amplified products were analyzed by agarose gel electrophoresis on 1.5% agarose gel stained with ethidium bromide. Lane M: Marker (DNA MW marker), lane 1: Negative Control, lane 2: PPRV/Gazelle/Sudan/Khartoum2016-1, lane 3: PPRV/Gazelle/Sudan/Khartoum2016-2, lane 4: PPRV/Gazelle/Sudan/DNP2017, lane 5: PPRV Isolate "PPRV/Gazelle/tc/Sudan/DNP2017", lane 6: PPRV Nigeria 75/1 vaccine strain (Positive Control).

Table 21. Molecular characterization of PPRV in specimens from Dorcas gazelles *"Gazella dorcas"* by RT-PCR and DNA sequencing.

Collection Date	Location	Type of Samples	Total No. of Samples	RT-PCR		DNA Sequencing	
				No. tested	No. +ve (%)	No. tested	No. +ve (%)
Nov. 2016	Soba East, Khartoum State	WB	2	1	1 (100%)	1	1 (100%)
Dec. 2016	Elazhari, Khartoum State	WB	2	1	1 (100%)	1	1 (100%)
April 2017	Kuku Animal Zoo, Khartoum State	WB	2	-	-	-	-
		NS	2	-	-	-	-
May 2017	Dinder National Park, Sinnar and Elgedarif States	WB	2	-	-	-	-
		L	1	1	1 (100%)	1	1 (100%)

Notes: WB = Whole blood; NS = Nasal Swab; L = Lung

3.2.5. Genetic characterization of PPRV:

3.2.5.1. DNA sequencing:

The identity of the specific PPRV strains was determined by sequencing of RT-PCR amplicons in an automated DNA Sequencer using DNA Sequencing kit. PPRV N-gene (351 nt) partial nucleotide sequences were then assembled using CodonCode Aligner, with overlapping sequences and primer sequences removed prior to analysis. These sequences were compiled and deposited in the NCBI GenBank database under accession numbers MG992016.1 (PPRV/Gazelle/Sudan/Khartoum2016-1), MG992017.1 (PPRV/Gazelle/Sudan/Khartoum2016-2) and MG992018.1 (PPRV/Gazelle/Sudan/DNP2017), respectively.

3.2.5.2. Sequence analysis:

Subsequently, sequences of PPRV partial N-gene of the gazelle samples were analyzed and compared with the respective N-gene sequences of PPRV strains available in the NCBI GenBank database using BLAST Nucleotide to find the percentages of nucleotide sequence identities with other PPR viruses. Comparison of the resulting PPRV sequences obtained from gazelle samples using BLAST nucleotide revealed a close identity to PPRV lineage IV strains. All three PPRV from gazelles shared the highest nucleotide sequence identity of 96-98% with Egyptian PPRV strains [Ismailia 3/Egypt/2010, Ismailia 1/Egypt/2010, Ismailia 2/EGY/2012, Ismailia 1/2014, El-Kalubeya], PPRV Georgia/Tbilisi/2016 and PPRV Ethiopia 2010 and 2014 with a lower identity of 93-95% with other PPRV lineage IV isolates.

3.2.5.3. Phylogenetic analysis for PPRV lineage identification:

This analysis was extended by comparison of the assembled gazelle PPRV sequences with the respective N-gene sequences of other PPRV isolates available in the GenBank database using ClustalW multiple alignment program. Phylogenetic analysis to determine the specific virus lineage was performed for the aligned PPRV N-gene partial sequences using the MEGA7.0.26 (Molecular Evolutionary Genetics Analysis) program. The resulting phylogenetic tree confirmed that the three PPRV strains from gazelles in the Sudan belonged to the PPRV lineage IV genotype and shared the closest sequence identity with PPRV strains originating from North African countries (**Figure 15**). This lineage contains a number of sub-lineages, one of which includes a number of previously published PPRV strains from the Sudan (2000-2009) [14], Egypt/Ismailia

strains (2010 and 2012) and PPRV Eritrean strains from 2005. Interestingly, PPRV/Gazelle/Sudan/DNP2017 is located in this particular sub-lineage in which a close relationship is evident with lineage IV Ethiopian strains (2010 and 2014) (Figure 15) indicating possible transnational virus spread. Of note, the other two PPRV strains identified in this study, Khartoum "PPRV/Gazelle/Sudan/Khartoum2016-1" and "PPRV/Gazelle/Sudan/Khartoum2016-2", shared an identical sequence in the specific region of the N gene under investigation and were clustered together into one sub-lineage (Figure 15).

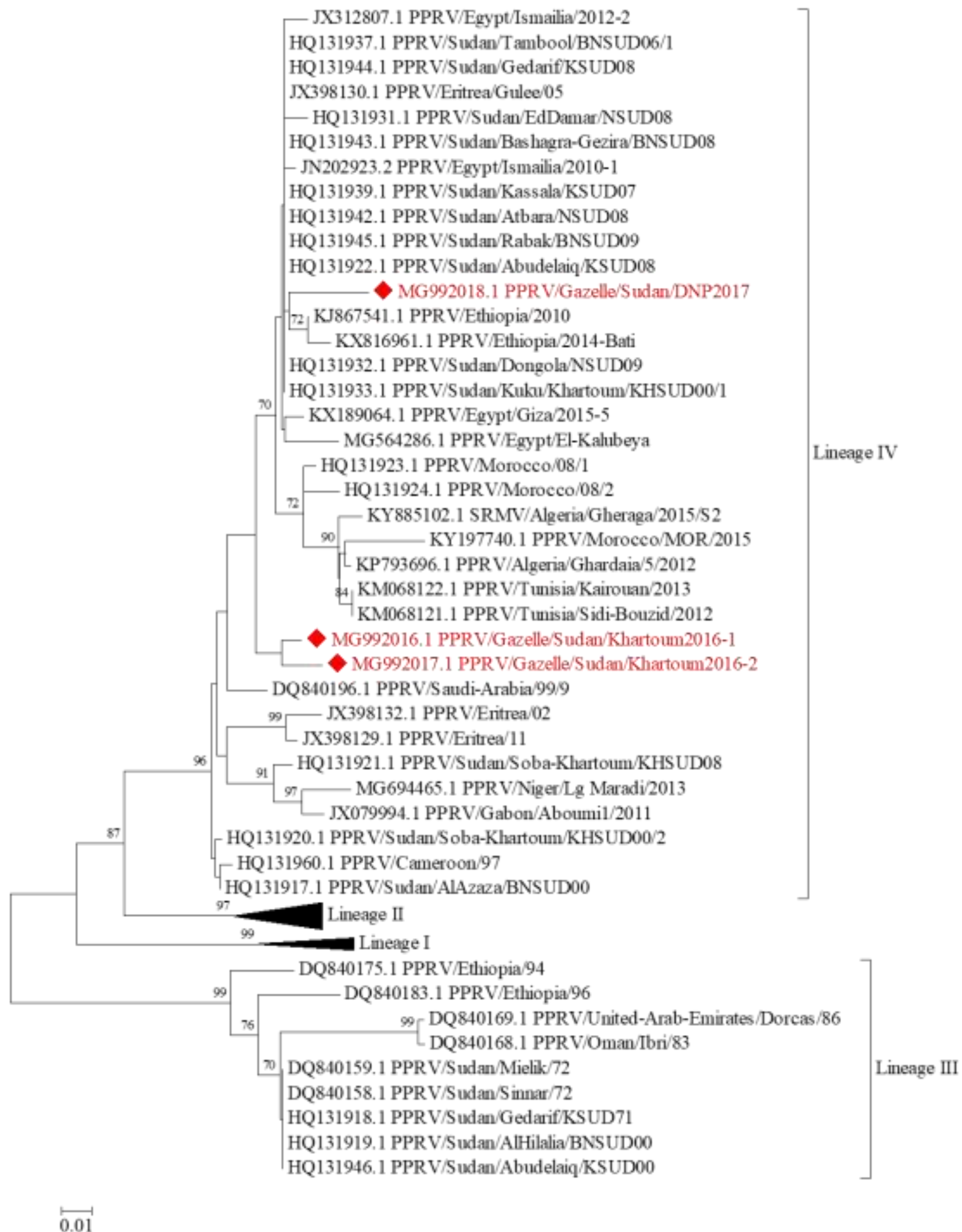

Figure 15. Neighbor-joining tree for PPRV strains constructed on the basis of the 255 nt partial N-gene sequences of PPRV strains. The analysis was conducted in MEGA7.0.26 (Molecular Evolutionary Genetics Analysis) (www.megasoftware.net/mega.html) by analyzing 1,000 bootstrap replicates, clusters were supported by bootstrap values >70 (PPRV Sudanese Strains from gazelles are represented by red font and red diamond circle). The Neighbor-joining tree represents PPRV lineage III and IV strains.

3.2.6. Serological detection of PPRV antibodies in gazelle sera using C-ELISA:

C-ELISA was used for assessing the serological profile of PPRV antibodies in gazelle sera. From the 6 gazelles sera analyzed, PPRV antibodies could not be detected in the two captive gazelle's sera from the two apparently healthy gazelles from Kuku Animal Zoo, Khartoum State (Table 22). In contrast, PPRV antibodies could be detected in all four gazelle sera which were collected from surviving free-ranging gazelles from Dinder National Park outbreak (Table 22).

Table 22. Serological detection of PPRV in sera of Dorcas gazelles *"Gazella dorcas"* by C-ELISA.

Collection Date	Location	Serum samples		
		Total No. of Sera	No. tested	No. +ve (%)
Nov. 2016	Soba East, Khartoum State	0	0	0 (0%)
Dec. 2016	Elazhari, Khartoum State	0	0	0 (0%)
April 2017	Kuku Animal Zoo, Khartoum State	2	2	0 (0%)
May 2017	Dinder National Park, Sinnar & Gedarif States	4	4	4 (100%)

Chapter IV
Discussion

Peste des Petits Ruminants (PPR) is an acute and highly contagious viral disease that affects both domestic and wild small ruminants (Parida *et al.,* 2015; OIE, 2019). PPR is one of the economically important diseases of small ruminants with variable prevalence in several parts of Asia and Africa. During 1971-1972, the first outbreaks of a rinderpest-like disease infecting sheep and goats were reported in Gedarif, Eastern Sudan and Sinnar and Mieliq, Central Sudan (Elhag Ali, 1973). The presented clinical signs is typical to PPR, however, PPR viruses were isolated firstly in 1984 (Elhag Ali and Taylor, 1984). Since then outbreaks of the disease were continually reported from different parts of the country (El Hassan *et al.,* 1994; Saeed, 2004; Osman, 2005; Saeed *et al.,* 2010; 2017; Osman *et al.,* 2018; Ahmed, 2019; Ahmed Ali, 2019; Abdalla, 2019, Al Hussein, 2019).

The present study was designed to identify, isolate and characterize PPRV circulating during 2015-2018 in small ruminants "sheep and goats" and gazelles in the Sudan. Additionally, molecular and genetic characterization were performed to identify the lineage of PPRV which is currently circulating in these animal hosts in the country and to update the disease current situation in the country.

4.1. PPR in sheep and goats:

Screening of whole blood and tissue samples from sheep and goats, collected from ten different States of the Sudan, for the presence of PPRV antigen by an IC-ELISA assay revealed an overall antigenic prevalence of 50.1%. On the species basis, higher antigenic-prevalence was obtained from goats samples (63.4%) compared to sheep samples (46.9%). A slightly lower PPRV overall antigenic prevalence of 42.6% was demonstrated among sheep in the Sudan during 2008 (Saeed *et al.,* 2010) whereas a much lower overall antigenic prevalence (18.3%) was demonstrated in recent years (Saeed *et al.,* 2017).

During 2015 to 2018, many suspected PPR outbreaks were occurred among sheep and goats in many localities within different States of the Sudan. These PPR suspected outbreaks were recognized mostly by the appearance of the typical clinical signs of the disease in small ruminants and were associated with a higher morbidity and mortality rates. An overall antigenic prevalence of 58% was demonstrated among sheep and

goats, from PPR suspected outbreaks, by an IC-ELISA. Compared with previous study, a relatively lower PPRV antigenic prevalence (42.6%) was demonstrated among sheep in the Sudan during 2008 (Saeed *et al.,* 2010). In another study, very lower PPRV antigenic prevalence was obtained in samples collected from sheep (15.4%) and goats (21.1%) from slaughter houses and from outbreaks of the disease (Saeed *et al.,* 2017).

Considering the type of samples, 57.9% antigenic prevalence was demonstrated when whole blood samples from both sheep and goats were analyzed by an IC-ELISA for detection of PPRV antigen. Moreover, PPRV could be detected in 62% and 56.9% of the whole blood samples of goats and sheep, respectively. Screening of lung tissues from both sheep and goats revealed 60% antigenic prevalence and PPRV could be detected in 66.7% and 57.1% of the lung tissue samples of goats and sheep, respectively. It is evident that the antigenic prevalence is higher in lung tissue samples than in whole blood samples. In a previous study, PPRV antigen was detected in 11%, 35% and 30% of swab samples, tissue samples and whole-blood samples using an IC-ELISA assay (Ishag *et al.,* 2015) indicating the higher viral detection rate in tissue samples which is in agreement with our findings.

In the five States where outbreaks occurred, the highest antigenic-incidence was demonstrated in Western Kurdufan State (81.2%), followed by River Nile State (65.6%), then Gezira State (58.5%), then Khartoum State (53.5%) and finally the lowest incidence was present in Elgedarif State (50%). These finding is typical to Saeed *et al.* (2010) who reported the highest incidence among the Sudan was in Kurdufan State, Western Sudan (62.5%) then Gezira State, Central Sudan (27.3%).

In this study, investigation for the presence of PPRV in sheep and goats lung tissues collected from slaughter houses revealed very lower antigenic prevalence of 24.1%. The presence of PPRV antigen in lungs of apparently healthy small ruminants indicated its association with respiratory infection and circulation of PPRV among animal populations. In five different States of the Sudan where slaughter houses are available, the highest antigenic-prevalence was demonstrated in Northern State (80%) while no incidence was present in Kassala State, Eastern Sudan (0%). Our findings indicated the higher presence of PPRV in Northern Sudan, although it is considered as a disease-free zone, compared to other parts of the country. Considering the animal species, the antigenic-prevalence of PPRV was much higher in sheep (87.7%) than in goats (66.7%). These results are in contrary to our findings among infected animals form PPR suspected outbreaks and to previous study which demonstrated higher prevalence

among goats (Saeed *et al.,* 2017). To our knowledge, PPRV infection is more severe in goats than in sheep in the same environmental conditions (Khan *et al.*, 2008; Munir *et al.,* 2009; Merck, 2010).

Isolation of PPRV from two lung samples was performed in Vero cells, with appearance of the characteristic cytopathic effects of PPRV consisting of rounding of cells and syncytia formation, as described previously in other studies (Saeed *et al.,* 2004; Osman, 2005; Balamurugan *et al.,* 2010).

Detection of PPRV nucleic acid using RT-PCR was achieved from 27 whole blood and lung samples, which were positive by IC-ELISA, collected from sheep and goats from PPR suspected outbreaks and slaughter houses from all States of the Sudan. This finding is typical with previous reports that described the high sensitivity of RT-PCR using NP3/NP4 primers for detection of PPR nucleic acids (Couacy-Hymann *et al.,* 2009; Muthuchelvan *et al.,* 2014).

Genetic characterization studies based on the partial PPRV N-gene sequence clustered old PPRV Sudanese isolates from 1970th (PPRV SUD 71 Gedarif, PPRV SUD 72/1 Sinnar and PPRV SUD 72/2 Mieliq strains), and few new isolates after 2000, into PPRV lineage III genotype along with East-African and Arabian Peninsula's isolates (Banyard *et al.,* 2010; Kwiatek *et al.,* 2011). Interestingly, most new PPRV strains isolated from the Sudan after the year 2000 identified as a PPRV lineage IV, indicating the co-existence of a lineage IV beside lineage III (Kwiatek *et al.,* 2011). In this study, molecular typing of 9 PPR viruses from sheep and goats samples from nine different States of the Sudan provided strong evidence of the presence of PPRV lineage IV which is circulating in the country since 2000 (Kwiatek *et al.,* 2011). The sequence similarity between Sudanese PPRV strains from sheep and goats and PPRV strains from African countries including Egypt, Ethiopia, Morocco and Algeria can better explain this lineage circulation between countries bordering Sudan suggesting a common origin for these outbreaks.

The successful isolation of the virus and molecular findings of this study confirmed the wide distribution of PPRV in wide belt of the country in herds of sheep and goats and even in slaughter houses, active and progression of lineage IV among populations of sheep and goats in the Sudan, suggesting risks for potential spread of the disease to currently free areas.

4.2. PPR in Dorcas gazelles "Gazella Dorcas":

The present study describes for the first time occurrence of PPRV infection in gazelles as a potential wild small ruminant host for the disease in the Sudan. In this study, PPR viral antigen and RNA could be demonstrated in samples of four apparently semi-captive healthy gazelles from two areas in Khartoum State "Soba East and Elazhari". The presence of PPRV associated with the absence of a clinical disease suggests that those gazelles might be exposed to, and largely recovering from, PPRV infection. In fact, PPRV is endemic in Khartoum where sheep and goats normally share grazing areas with other animal species. Thus, those semi-captive gazelles might be exposed to PPRV while mingled together with infected small ruminants. Exposure of gazelles to PPRV suggests that the vast majority of the animals around Khartoum have been infected with PPRV.

PPRV subclinical infection of gazelles has been well documented previously in Goitered Gazelle (*Gazella subgutturosa subgutturosa*) from Turkey (Gür and Albayrak, 2010) and in Grant's gazelle from Tanzania (Mahapatra *et al.,* 2015). The presence of PPRV associated with the absence of a clinical disease suggests gazelles as subclinical hosts and carriers of the virus thus represent potential virus source for other domestic species as has been well documented previously (Gür and Albayrak, 2010; Aziz-ul-Rahman *et al.,* 2018).

The present study represents the first evidence for PPR infection and the presence of a typical clinical disease in wildlife Dorcas gazelles (*Gazella dorcas*) in Dinder National Park, Sudan. PPR infection or serologic detection of antibodies in gazelles in Saudi Arabia (Abu Elzein *et al.,* 2004; Sharawi *et al.,* 2010), United Arab Emirates (Furley *et al.,* 1987; Kinne *et al.,* 2010; cited in Parida *et al.,* 2015), and Turkey (Gür and Albayrak, 2010; cited in Parida *et al.,* 2015) were reported previously. Laboratory investigations demonstrated the presence of PPRV antigen and RNA in samples of diseased gazelles using IC-ELISA and RT-PCR, respectively. Recently, PPRV antigen or RNA has been identified in wildlife animal species in Tanzania (Mahapatra *et al.,* 2015), Mongolia (Shatar *et al.,* 2017) and China (Li *et al.,* 2017). Also seroconversion of gazelles against PPRV in Dinder National Park outbreak was demonstrated using C-ELISA. Moreover, the seropositivity of PPRV antibodies was reported in gazelle's sera in Turkey (Gür and Albayrak, 2010), Tanzania (Mahapatra *et al.,* 2015), Nigeria (Bello *et al.,* 2016) and Sudan (Saeed *et al.,* 2017). Isolation of a unique PPRV from the lung tissue from an infected gazelle further supports PPRV as the causative agent responsible

for the outbreak and the clinical disease occurred in gazelles. In many reports, PPRV was isolated from gazelles in United Arab Emirates (Furley *et al.,* 1987) and Saudi Arabia (Abu Elzein *et al.,* 2004; Sharawi *et al.,* 2010) although Kinne *et al.* (2010) failed to isolate a virus.

Concerning Dinder National Park suspected PPR outbreak of gazelle's, the presence of a clinical disease in infected Dorcas gazelles *"Gazella dorcas"* which is typical to that seen in sheep and goats indicating PPRV infection of all gazelles examined so far and suggests gazelles as potential wild small ruminants host for the disease in the country as has been described previously in Saudi Arabia and United Arab Emirates (Abu Elzein *et al.,* 2004; Kinne *et al.,* 2010; cited in Aziz-ul-Rahman *et al.,* 2018).

In the present study, partial N-gene sequences based molecular characterization of PPRV from the whole blood and lung specimens of the subclinically and clinically infected gazelles, respectively, identified PPRV lineage IV strains resembling new PPRV Sudanese isolates from 2000 to 2009. The presence of the Asian lineage IV in the country indicates the introduction of this lineage into the Sudan from neighboring African countries where lineage IV is present. This can be a result of transboundary animal movement most possibly from the nearby neighboring countries such as Ethiopia or Eritrea in the East and Egypt in the North. The sequence similarity between Sudanese PPRV gazelle strains and PPRV strains from Egypt, Ethiopia, Eritrea, Morocco, Algeria and Tunisia can better explain this lineage migration from countries bordering Sudan suggesting a common origin for these outbreaks.

The present study represents the first evidence describing PPRV infection in gazelles in the Sudan as well as in Africa and suggests a significant role of gazelles in the epidemiology of PPR in the Sudan. The results concluded that gazelles are considered as potential wild small ruminant host of PPR in the country. Since Sudan is one of PPR endemic countries and thus a part of the global campaign to eradicate the disease, the current situation of the disease and the possible role of other domestic and wildlife animal hosts should be investigated to aid in the currently ongoing global eradication program of the disease.

Conclusions

Based on the results of this study, it can be concluded that:
Detection of PPRV antigen and nucleic acid in sheep and goats samples from PPR suspected outbreaks indicated infection of these animals. Similarly, detection of PPRV from lung tissues of sheep and goats from slaughter houses indicated exposure of these animals to PPRV through contact with infected animals. Demonstration of the presence of PPRV in ten different States of the Sudan indicated the wide distribution of the disease among sheep and goats populations in different parts of the country. Molecular and genetic characterization performed for PPR viruses from sheep and goats from outbreaks and slaughter houses clustered them in PPRV lineage IV genotype which is circulating in the Sudan since 2000. Sequence comparison and phylogenetic analysis revealed that the characterized PPRV strains from sheep and goats in the Sudan shared the closest sequence identity with other PPRV strains originating from North African countries.

PPRV antigen and nucleic acid were detected in samples from semi-captive and free-range Dorcas gazelles *"Gazelle Dorcas",* from Khartoum and Dinder National Park outbreak, using IC-ELISA and RT-PCR. Further, genetic characterization clustered all PPRV from gazelles into lineage IV genotype. One PPRV was isolated in Vero cell from lung tissue of gazelles from Dinder National Park outbreak. PPRV antibodies were detectable in sera from surviving gazelles from Dinder National Park outbreak using a C-ELISA assay.

Infection of PPRV were reported in free-range Dorcas gazelles *"Gazelle Dorcas"* in Dinder National Park that is considered the first study describe PPRV infection among gazelles in the Sudan and also in Africa. The result of this study demonstrates that gazelles are a potential wild small ruminant host for PPRV and may influence the epidemiology of PPR in the Sudan.

Recommendations

1- PPR was wide spread in the Sudan with high antigenic incidence, thus, we recommend systematic vaccination to contain outbreaks in affected areas and geographically linked surrounding areas to which the disease could potentially spread due to different epidemiological linkages.

2- Further studies should be done in other species (cattle, buffalo, camel, wild animals…..etc) to investigate the possible role that other species might play (or not) in PPRV transmission and epidemiology of PPR.

3- Since it is very difficult to isolate PPRV in Vero cell lines, using another sheep or goats cells or derivatives of Vero cell lines such as Vero-dogSLAM, which enable rapid isolation of PPRV in less than one week, is recommended.

4- Our finding showed that PPRV Lineage IV is currently the dominant lineage circulating among small ruminant in the Sudan, this information is crucial for further research aimed at defining strategies for the efficient prevention and control of PPRV.

5- More genetic analyses are required to understand the epidemiology of the disease in order to aid in eradication of PPR.

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Appendix

1. Buffers and Solutions

1.1. Deionized Distilled Water (D.D.W):

Deionized distilled water (D.D.W) was used in preparation of solutions besides washing of equipment. Water was distilled to get rid of salts and passed through deionizer to get free of ions. D.D.W. was then sterilized by autoclaving at 121°C for 30 minutes.

1.2. Phosphate Buffered Saline (PBS): 1 Litre

One pouche of Phosphate Buffered Saline was dissolved in 1000 ml/1 L of D.D.W., the solution was then sterilized by autoclaving at 121°C for 30 minutes and stored at 4°C till used.

2. Antibiotics

2.1. Penicillin-Streptomycin:

One gram of Streptomycin powder and contents of two vials of Penicillin (10^6 IU/ vial) were dissolved in 10 ml of sterile D.D.W., 1 ml of Penicillin-Streptomycin prepared solution contained 100 mg of Streptomycin and 20,000 IU of Penicillin. The solution was kept at -20°C.

2.2. Gentamycin:

10 mg of Gentamycin was dissolved in 1 ml of sterile D.D.W. The prepared solution contains 10,000 µg Gentamycin per 1 ml. The solution was kept at -20°C.

2.3. Nystatin:

One vial of Nystatin (50,000 µg) was dissolved in 10 ml of sterile D.D.W.

The stock solution for tissue culture medium was used to give a final concentration of 200 units per ml Penicillin, 100 µg Streptomycin, 50 units Nystatin and 10 µg Gentamycin.

3. Cell culture medium and buffers

3.1. 5X Growth Minimum Essential Medium (GMEM)

Growth Minimum Essential Medium (GMEM)	125.7 g
D.D.W. completed to	2000 ml

Medium was sterilized by filtration, then aliquoted in amount of 0.5 litres and frozen at -20°C.

3.2. 1X Growth Minimum Essential Medium (GMEM)

GMEM 5X conc.	200 ml
Lactalbumin Hydrolysate	25 ml
Yeast Extract	25 ml
$NaHCO_3$	8 ml
Penicillin and Streptomycin	2 ml
Gentamycin	1 ml
Mycostatin	0.5 ml
D.D.W. completed to	1000 ml

3.3. Outgrowth Medium:

GMEM 5X conc.	85 ml
Tryptose Phosphate Broth	5 ml
Calf serum	10 ml

3.4. Maintenance Medium:

GMEM 5X conc.	90 ml
Tryptose Phosphate Broth	5 ml
Calf serum	5 ml

3.5. Phosphate Diluent (P.D):

NaCl	16.0 g
KCl	0.4 g
Na_2HPO4 (anhydrous)	2.3 g
KH_2PO_4 (hydrous)	0.4 g
D.D.W. completed to	2000 ml

The solution was sterilized by autoclaving at 121°C for 30 minutes, left to cool and antibiotics were added.

P.D. is used for the preparation of cell dispersing solutions such as 0.25% Trypsin and 0.02% Versene.

4. Tissue Culture Additives:

4.1. Tryptose Phosphate Broth (TPB) 5%:

Tryptose Phosphate Broth powder	7.5 g
D.D.W.	100 ml

The solution was sterilized by autoclaving at 121°C for 30 minutes.

4.2. Yeast Extract 1%:

Yeast Extract powder	1 g
D.D.W.	100 ml

The solution was sterilized by autoclaving at 121°C for 30 minutes.

4.3. Lactalbumin Hydrolysate 5%:

Lactalbumin powder	5 g
D.D.W.	100 ml

The solution was sterilized by autoclaving at 121°C for 30 minutes.

4.4. Sodium Bicarbonate ($NaHCO_3$) 7.5%:

$NaHCO_3$	7.5 g
D.D.W.	100 ml

The solution was sterilized by autoclaving at 121°C for 30 minutes.

5. Cell Dispersing Solutions:

5.1. Trypsin 7.5%:

Trypsin powder	37.5 g
P.D. completed to	500 ml

The solution was sterilized by filtration.

5.2. Versene 5%:

Versene	25 g
P.D. completed to	500 ml

The solution was sterilized by filtration.

5.3. Trypsin-Versene Solution:

Trypsin	6 ml
Versene	4 ml

P.D. completed to	90 ml

6. Hank's Balanced Salt Solution (HBSS):

Solution (A):

NaCl	8 g
KCl	0.4 g
$MgSO_4.2H_2O$	0.2 g
$Na_2HPO_4.2H_2O$	0.6 g
KH_2PO_4	0.6 g
Glucose	1 g
Phenol Red	0.04 g
D.D.W.	800 ml

Solution (B):

$CaCl_2$	0.14 g
D.D.W.	100 ml

Solution (C):

$NaHCO_3$	0.35 g
D.D.W.	100 ml

Each solution was sterilized by autoclaving separately at 121°C for 30 minutes, then solution (A) and (B) were added to solution (C).

7. Agarose gel electrophoresis buffers:

7.1. 1X Tris Acetate EDTA (TAE) buffer for agarose gel (100 ml):

TAE 10X conc.	10 ml
D.D.W.	90 ml

7.2. 1X Running buffer (500ml):

TAE 10X conc.	50 ml
D.D.W.	450 ml

7.3. Agarose (1.5%):

Agarose	1.5 gm
TAE 1X	100 ml
Ethidium Bromide	3 µl