CONVENTIONAL AND MOLECULAR DETECTION OF *PASTEURELLA MULTOCIDA* IN OUTBREAK OF RESPIRATORY TRACT INFECTION OF SHEEP AND GOATS IN BASRAH PROVINCE

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ABSTRACT

One hundred eight nasal swabs and blood samples from respiratory tract infected animals 66 from sheep and 52 from goats were collected from different sites of Basrah province during a period from December 2012 to April 2013 for isolation and identification of the *Pasteurella multocida* according to PCR assay . Nasal swabs and blood samples were directly cultured on proper media, then five colonies from the agar plate of suspected *P. multocida* cultures were used for extracted DNA and, further used for Polymerase chain reaction PCR . PCR was carried out for amplified the PMOut gene on the previously extracted *P. multocida* DNA . The best amplification of PMOut gene was observed at 45°C annealing temperature . Under these optimal conditions, the expected fragment of 219bp of PMOut gene was successfully amplified. On the other hand , the distinct amplification with a molecular length of 219bp was obtained in 56 positive PCR samples (37 from sheep with distribution rate 56% and 19 from goats with distribution rate 36.5%). The PCR results of the PMOut gene was found to be potentially a useful method for identification of *P. multocida* infections.

INTRODUCTION

Pasteurellosis is a term refers to any of the disease conditions caused by species of the genus *Pasteurella* (1,2) . It is one of the most common disease of

sheep, goats and cattle throughout the world where outbreaks usually lead to high mortality and great economic loss to the ruminant industry (3,4). One of these important genus is *Pasteurella multocida* which is a commensal germ in the upper respiratory tract of many animals (5,6). It is small ,non-motile, Gram-negative coccobacilli of the family pasteurellaceae(7) .Since *Pasteurella*, particularly *Pasteurella multocida* are part of natural flora of the buccal-pharyngeal region, therefore in the animals which are under stress, like the ones that are being transferred, have respiratory infections, have bad nutrition and ventilation, and are being kept in overcrowded places, bacterial growth and proliferation occur in the region and later gets extended to the lower respiratory tract and causes Pneumonic pasteurellosis (8,9,10) .

Within Asia region especially countries like Iraq the pneumonic pasteurellosis is the acute respiratory infectious diseases occur in sheep and goats ,and the outbreaks of the diseases have been noted to occur at the beginning of the winter season, or just before the lush season. The disease characterized by high fever with temperature of 40ºC to 41ºC ,Moist, painful cough, dyspnea (difficulty in breathing). Examination of the lungs may reveal cracklelike sounds, along with nasal and ocular mucopurulent discharge anorexia (loss of appetite) and depression(11). A variety of laboratory diagnostic techniques have been developed over the years for pasteurellosis and used routinely in the laboratory(12) . The organism is identified directly through examination of blood smear from affected animal and can be isolated in suitable culture medium in the laboratory. Various biochemical and serological tests are used for the identification and serotyping of the organism. With development in biotechnological techniques for the detection of nucleic acid, the identification and characterization of etiological agents has become quick, easy and accurate (13) .Recently, polymer chain reaction become a powerful molecular biology technique that was introduced to facilitate the detection of these virulence factors (14) ; for this reason, most studies using this technique worked with isolated colonies and/or extraction and partial DNA purification (15,16).

This study was conducted for isolation and identification of the *Pasteurella multocida* from sheep and goats in different area of Basrah and confirm the identification of these bacteria by polymerase chain reaction PCR assay using a specific primers for amplified the PMOut gene.

MATERIALS AND METHODS

Sample collection : A total of 118 nasal swabs and blood samples from respiratory tract infected animals 66 from sheep and 52 from goats were collected from different area of Basrah province during a period from December 2012 to April 2013.

Bacterial isolates and media: Nasal swabs from all animals were directly cultured on brain heart infusion agar and nutrient agar and incubated at 37ºC for overnight (17,18) .The blood samples were cultured directly . Following incubation, samples from each culture were plated on 7% sheep blood agar and on MacConckey agar. The plates were incubated aerobically at 37ºC for 24-48h (17,18). Following purification through subculturing, the isolated bacterial colonies further identifying using Polymerase chain reaction.

Extraction of DNA from Bacteria and PCR amplification :

Five colonies from the agar plate of suspected as *P. multocida* cultures were transferred into an eppendorf tubes containing 100µL distilled water. The tubes were vortexed and incubated at 100ºC for 15 min.Then 900 µL of distilled water was added and mixed well until the solution is homogeneous. The solution was centrifuged at 12.000 rpm for 10 min. The supernatant which contain the genomic DNA was transferred into an sterile eppendorf tubes for PCR technique .

(PCR) was carried out for amplification of the PMOut gene on the previously extracted DNA sample using Green master mix (Promega, USA) and a specific set of oligonucleotide primer (Bioneer, Korea) which has the following sequences :

Table (1): Oligonucleotide Primers Sequences used for PCR Amplification of *P. multocida* of the PMOut gene.

Gene	Primers sequences	Length	Reference
PMOut gene	5'- AGGTGAAAGAGGTTATG-3'	17	Hawari *et al.*, 2008.
	5'- TACCTAACTCAACCAAC-3'	17	

PCR was performed in a Thermocycler type (*Esco*, Singapore) in a total reaction volume of 25 µl containing 12.5 µl of green master mix (*Taq* DNA polymerase, dNTPs, MgCl2 and reaction buffers), 1.5 µl of each primer and 9.5µl of

template DNA. Amplification condition was obtained with an initial denaturation step at 94 °C for 2 min, followed by 40 cycles at 94°C for 45sec, and 45°C for 45 min, 72 °C for 1min and final extension 72°C for 5min (19) .

Ten microliters of PCR products were analyzed on 2% agarose gel in 1x Tris-Borate-EDTA (TBE) buffer and run at 75V for 4 hrs. Gels were photographed under UV illumination (E-graph-ATTO-Japan) after staining with 0.5µg/mL ethidium bromide. Fragment size of approximatly 219 bp was verified as positive for *P. multocida* PMOut gene . A 100bp DNA ladder (Bioneer , Korea) was used as a molecular size standard.

RESULTS

The whole bacterial DNA was isolated from suspected *P. multocida* colonies and its concentration was determined to be greater than 100ng/µl using for each samples. On the other hand, the agarose gel electrophoresis was carried out to check the quality and purity of the extracted DNA. The bands of extracted DNA were observed on the gel as shown in figure (1).The agarose gel electrophoresis showed clearly that DNA does not undergo any degradation during extraction.

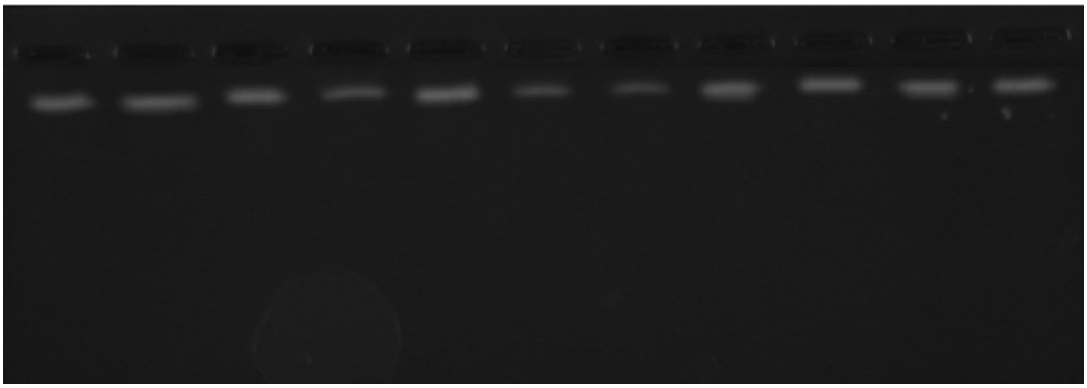

Figure (1): 0.8 % Agarose Gel Electrophoresis of 11 strains of *P.multocida* genomic DNA Bands . All Lanes = Positive Samples (presence of DNA).

The identification of *P. multocida* was confirmed by PCR technique. Amplification of bacterial genomic DNA was conducted by using species-specific primer as mentioned in materials and methods. The best amplification of PMOut gene was observed at 45°C annealing temperature . Under these optimal conditions, the

expected fragment of 219bp of PMOut gene was successfully amplified as shown in figure (2).

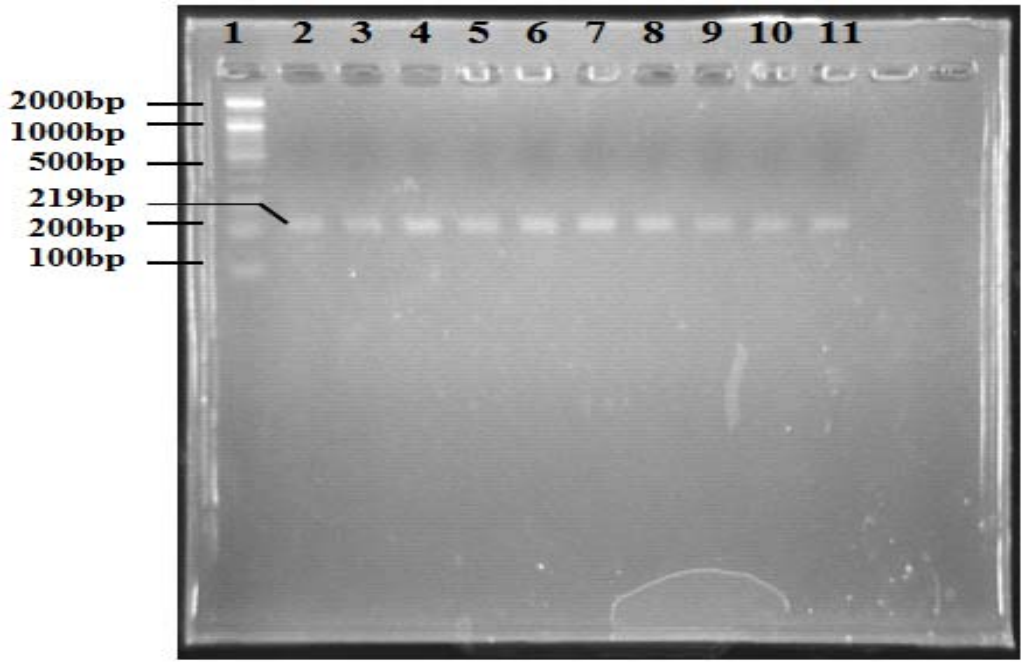

Figure (2) : PCR Products of *P. multocida* PMOut gene .Lane 1: 100 bp DNA Ladder. Lanes 2 to 5 : PCR Products of *P. multocida* PMOut gene Isolated from Sheep . Lanes 6 to 11 PCR Products of *P. multocida* PMOut genes gene Isolated from Goats.

On the other hand , the correct amplification with a molecular length of approximately 219bp was obtained in 56 positive pcr samples (37 from sheep with distribution rate 56% and 19 from goats with distribution rate 36.5%) as shown in table (1).

Table (2): The distribution rate of *P. multocida* PMOut gene in sheep and goats for 118 specimens.

Animal	No. positive	%	No. negative	%	Total	%
Sheep	37	56	29	44	66	56
Goat	19	36.5	33	63.5	52	44
Total	56	47.5	62	52.5	118	100

DISCUSSION

Recently, the isolation of *P. multocida* from domestic animals has been the issue of studies, consequently, the present paper describes as a first report for identification of *P.multocida* in Iraqi sheep and goats based on molecular technique. Although *P.multocida* is present as a normal microflora of the upper respiratory tract and environment of sheep and goats, but certain strains designated as pathogenic pasteurellosis possess specific virulence factors and are able to cause disease (20,21,22) . This disease is a serious problem for the animal industry, since it causes great economic loss. Even though there are a wide range of different virulence factors that may play a role in the pathogenesis of *p. multocida* we have investigated the presence of only one of these virulence genes factors (PMOut). In this study, the presence of the PMOut genes that derived from Omp gene (23)and encodes for outer *P.multocida* bacterial proteins was confirmed by PCR analysis(24).

PCR- based system have been designed for the detection of many virulence genes and are often the most sensitive , rapid and specific methods for detecting them; however, using these techniques for the screening of more than one gene is labor intensive and costly. Many studies and researcher (19,24) were reported the use of PCR technique to identification the *P. multocida* in domestic animals .One of these studies (19) using PMOut primer sequences for screening *P.multocida* from sheep and goats nasal and throat swabs samples, further more (24) they also used the PMOut primer for successfully identified the *P.multocida* from Turkey sheep and goats. To explain the possibility of same strain of *P.multocida* to transmitted among domestic animals especially between sheep and goats it may be that *P. multocida* is shared between domestic sheep and goat population through nose to nose contact in small ruminant herds (25,26,27) .

In conclusion : we have devised and validated a *P. multocida*-specific PCR assay ,based on a PMOut gene , that allow the rapid and specific detection of the recently described *P.multocida* . This assay also facillate the studies needed to define the prevalence ,distribution and epidemiology of pathogenic bacteria.

الكشف التقليدي والجزيئي لجرثومة *Pasteurella multocida* في الثورة التنفسية الخمجيه لأغنام ومعزَ محافظه البصرة

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الخلاصة

في هذه الدراسه تم جمع ١١٨، مسحه انفيه وعينه دم من الاصابات التنفسيه (٦٦ من الاغنام و ٥٢ من المعز) من مناطق مختلفه من محافظه البصره خلال الفتره من كانون الاول ولغايه نيسان ٢٠١٣وذلك لغرض عزل وتشخيص *Pasteurella multocida* من الاغنام والمعز وبالاعتماد على تفاعل البلمره التسلسلي PCR. زرعت المسحات الانفيه وعينات الدم مباشره على الاوساط الزرعيه الملائمه ثم جمعت خمس مستعمرات من الاطباق الزرعيه المشتبهه بنمو بكتريا *Pasteurella multocida* لغرض عزل الحامض النووي DNA ليستخدم لاحق ا في تفاعل PCR . حيث استخدم تفاعل PCR في تضخيم جين PMOut باستخدام عينات DNA المستخلصه سابقا وكان افضل تضخيم لجين PMOut قد لوحظ في درجه حراره ارتباط ٤٥ م° وتحت هذه الظروف المثلى فان حجم القطعه المتوقعه 219bp لجين PMOut قد ضخمت بنجاح . ومن جهه اخرى فان التضخيم الصحيح والممثل بالوزن الجزئي 219bp قد لوحظ في ٥٦ عينه موجبه PCR (٣٧ في الاغنام وبمعدل انتشار ٥٦% ، ١٩ في المعز وبمعدل انتشار ٣٦.٥%) . ان نتائج PCRلتضخيم جين PMOutقد بينت و بقوه كفائه هذه التقنيه في تشخيص اصابات *Pasteurella multocida* .

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