Isolation of *Pasteurella multocida* from sheep with molecular identification and typing by using polymerase chain reaction technique

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Summary

Seventy five (75) sheep samples (25 samples from each of nasal cavity ,tonsils ,lungs) were collected from different age , sex of sheep suffering from respiratory problems ,these samples were submitted into classical diagnostic methods to isolate and identificate *pasteuralla multocida.*

Forty two(56%) *Pasteurella multocida isolates were* diagnosed in sheep, higher isolation percentage from lung 17(68%), tonsil 14(56%) and 11(44%) from nasal cavity .

There was no significant effect of sex and age on *pasteurella multocida* isolation which in male 22(83%) in female 20(74%)while isolation percentage in sheep under one years 15(55.5%) and in more than one year 27(56.2%).

Gram negative ,bacilli-coccobacilli have polarity staining with methylene cotton blue and biochemically had positivity into oxidase , catalase , indol but negative to urease and motility .

By using PCR to confirm detection of *P.multocida* with specific finger printing primer gene KMT-1 which appear single bundle on agarose with molecular weight as 460pb.

By using specific primers for each *P.multocida* serotype (A,B,D,E,F) to distinguish the isolated bacteria from sheep by PCR which recorded *P.multocida* type A only .

In conclussion , the result of molecular diagnosis of *P.multocida* , Serotyping by using PCR revealed a high specificity (83.3 %) and sensitivity (97.2%) when compared with routine diagnostic method of this bacteria in sheep.

Introduction:

Pasteurella multocida is common important normal inhabitant bacteria in respiratory and alimentary tracts in healthy animals, and it could be act as primary or secondary pathogenic cause for many infections of upper respiratory tract and mastitis as secondary causes (1, 2) .

Shipping fever , pneumonia and atrophic rhinitis in sheep and goat as well as hemorrhagic septicemia in cattle as the main infectious diseases caused by *Pasteurella multocida*(3).

Bad management , bad nutrition, infectious agent like mycoplasma and virus as well as abnormal hard climate ,all these factor play as stressal factors which suppressed immune system and the bacteria become more active to be pathogenic and signs of severe respiratory problem may occur (4).

To diagnose genus of pasterallaceae family based on classical methods is usually detectable , but to differente between *pasteuralla species* is very difficult because highly similarities of morphology ,biochemical characteristics among them ,therefore, polymerase chain reaction test (PCR) may be used as accurate diagnostic method to identificate these bacteria (5).

Material and methods :

Sample collection : 75 samples were collected from sheep included 25 nasal swabs from nasal cavity and 25 tonsil swabs from tonsil region of Awassi sheep which suffering from respiratory problem (dyspnea , laboured respiratory ,nasal discharging ,coughing as well as rales with auscultation). Also 25 lung specimens were collected from lung of slaughtered sheep which taken from apparent lesion in lungs.

Bacterial isolation and identification:

According to (6) the direct and indirect culturing methods were used on blood agar , MacConkey agar and the bacterial growth subcultured on trypticase soya agar after added cyclohexemide as anti-fungal and erythromycin as antibiotic .

The morphological features of bacterial colonies (shape , size ,colour) and blood hemolysis character ,as well as staining by methylene cotton blue stain and showed biobolarity staining under microscope in order to diagnosis these colonies which could be submitted into biochemical tests (oxidase ,catalase ,urease , indol ,motility test)

Molecular examination using polymerase chain reaction (PCR) :

To confirm identification of *Pasteurella multocida* by using primer KMT-1gene according to (7)

Serotyping the isolates (*Pasteurella multocida*) by using primer of capsule gene CAP which including (A , B , D , E , F) according to (8)

Statistical analysis:

The results were analyzed by using Chi-square test under $p \leq 0.05$ While evaluate sensitivity and specifity for PCR by application the following two equation :

Sensitivity = b + d / d

Specifity = c + a / a

a: really positive
b :false positive
c :false negative
d :really negative

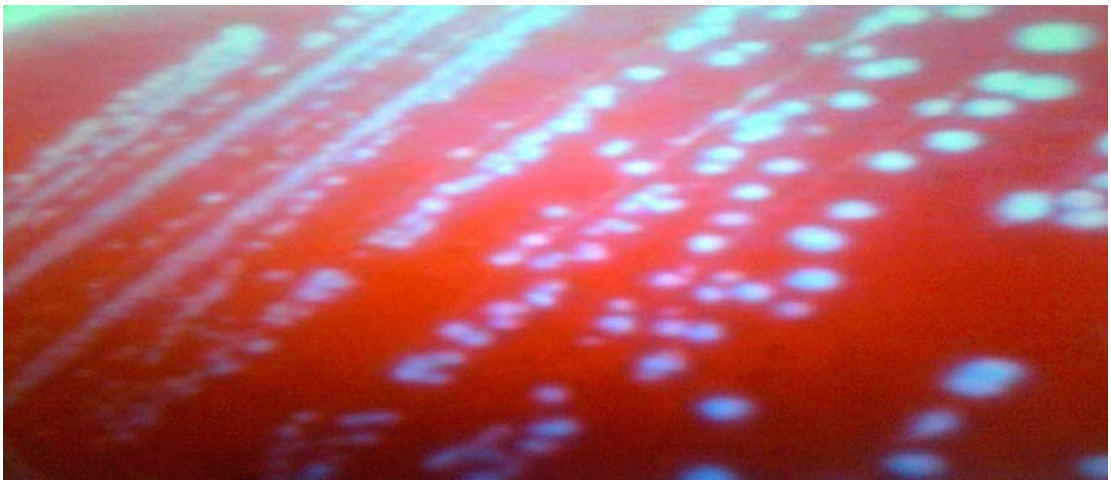
Results :

Forty two isolates of *Pasteurella multocida* were diagnosed from examined sheep at percentage 56% (table 1).

Table (1) :Percentage of *Pasteurella multocida* isolation from Awasssi sheep

Animal	Number of examined sample	Sample positive	%	Sample negative	%
Sheep	75	42	56%	33	44%

Semi-transparent gray-white small colony –not hemolyse blood on blood agar (figure -1) , non growing on MacConkey agar and microscopically appeared short bacilli-coccobacilli ,Gram negative and the polar staining appeared under microscope by staining with methylene cotton blue stain . (figure -2)



Figure(1): Colony of *Pasteurella multocida* which grow on blood agar (non hemolyse to blood)

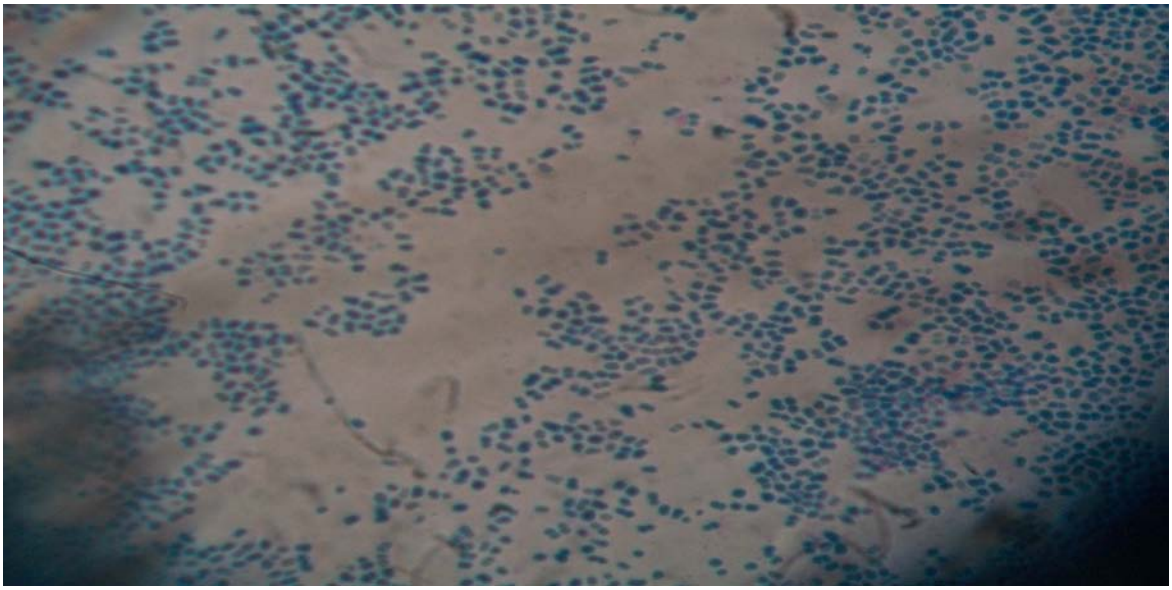


Figure (2): Polar staining for *Pasteurella multocida*(methylene cotton blue stain ,100X).

These isolation showed biochemically positive to oxidase , catalase , indol and H_2S producing test but , it is negative for urase and motility test .

The highest percentage isolation of *Pasteurella multocida* from lung 17 (68%) than 14(56%)from tonsil and 11(44%) from nasal cavity .Table (2)

Table (2) :Isolation percentage of *Pasteurella multocida* from different samples of examined sheep

Sample type	Number examined sample	Number of positive sample	%
lung	25	17	68
tonsil	25	14	56
Nasal cavity	25	11	44

Chi-square_{cal} = 5.143 Chi-square_{tab} = 5.99 Df =2

There was no significant effect of sex and age of animal on isolation percentage of *P.multocida* which 22(8.3%) in male and 20(74%) in female (table 3).

Table (3): The effect of animal sex *P.multocida* isolation percentage

Animal sex	Number examined sample	Number of positivity sample to *P.multocida*	%
Male	22	38	8.3
female	20	37	7.4

In table (4) showed percentage isolation of *P.multocida* from sheep under and more than one years as 15(55.5%) , 27 (56.5%) respectively Table (4).

Table (4) :The effect of age of animal on *P.multocida* isolation percentage

Animal sex	Number examined sample	Number of positivity sample to *P.multocida*	%
Under one years	27	15	55.5
More than one years	48	27	56.5

Molecular examination :

Confirm diagnosis of *P.multocida* by PCR ,the result of amplification of specific primer KMT-1 gene which represent of DNA finger printing of *P.multocida* which molecular weight 460 pb (figure-3)

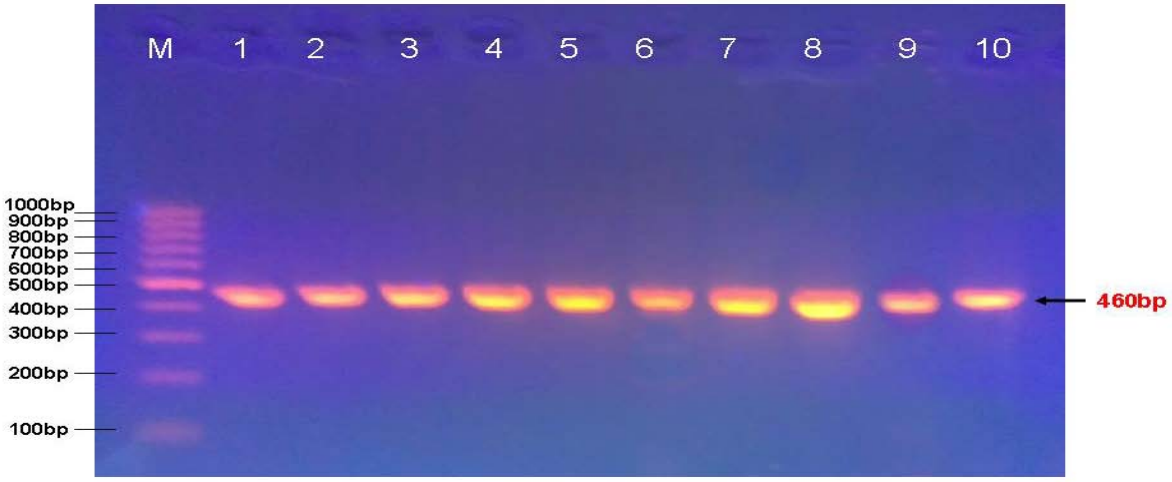


Figure (3): Results of amplification specific KMT-1 gene for *Pasteurella multocida* isolated from sheep columns M represent DNA ladder while the numbers from(1-10) represent the positive isolation to examination of PCR (460 pb)

Serotyping of *P.multocida* :

By using specific primer of different serotype of *P.multocida* (CAPA ,CAPB , CAPD , CAPE , CAPF) only the serotype CAPA give apparent single bundle which had

1044 pb (figure - 4)

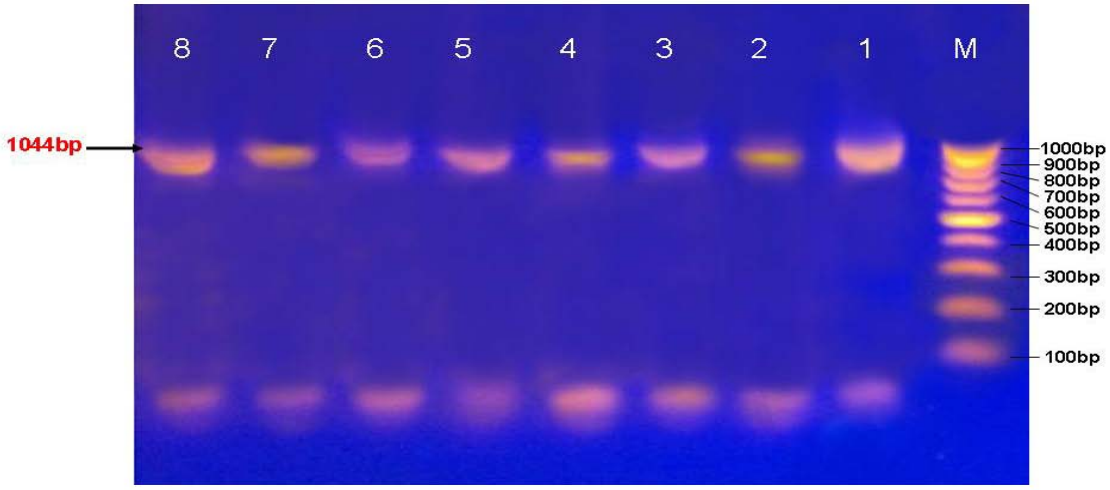


Figure (4) : Results of amplification specific CAPA gene for *Pasteurella multocida* isolated from sheep columns M represent DNA ladder while the numbers from (1-8) represent the positive isolation to examination of PCR (1044pb)

The PCR was highly specifity (97.2%) and high sensitivity(83.3%)when compared with classical method to diagnose *P.multocida* in sheep. (table 5)

Table (5): number of positive isolates to classical methods and PCR technique

Animal type	Number of positivity isolates to *P.multocida* by using classical methods	Number of positivity isolates to *P.multocida* in PCR technique
sheep	42	35

Discussion:

The result of isolation percentage of *P.multocida* from sheep higher than many studies like (9) 14% ,(10) and (11) as 42%,these variance in resultant due to either many animal could be take high doses of antibiotic as treatment , as well as the study season of study effect on isolation percentage because *P.multocida is* opportunistic bacteria which become active and pathogenic at hard climate and in nourished animal than in normal climate and in healthy animal.

Also , *P.multocida is regarding* the common infective bacteria of respiratory system in livestock animal(12) and (13).

P.multocida isolation from lung higher than tonsil and nasal swabs , that agreement with results by (14) and (15) , the examined lungs could be had apparent isolation and that increased the probability to be infective with *P.multocida* which that lead to high isolate

The results of bacterial identification of growing colonies on blood agar is agreement with the descriptive feature by (16) while ,on MacConkey agar was non-growing due to inability to grow on media contain bile salts which that also recorded by (17) , (18) and (19).

Staining the bacterial swab by methylene cotton blue was appear microscopical features as bacilli to cocci-bacilli ,have polarity staining because chromatin bodies located in two polar of bacteria which have concentrate stain that give polarity shape ,these insimilarity of results by (20) , the present results about the positivity reaction of *P.multocida* with catalase ,oxides and indol tests were agreement with (21) and (22) , as well as the negative reaction of their isolate against motility , urease which in similar by (23).while , (22) is disagreement with the present result about the positivity reaction of *P.multocida* against oxidase test because he recorded many *P.multocida* isolates gave negative reaction with oxidase test.

Non-significant effect of sheep sex on *P.multocida* isolation is agreement with (9) and (25) because animal is rearing under the same managemental and nutrition regimen in Iraq .

The age was not effective in isolation percentage , which in small age (under one year) and aged animals is closely percentage , the same result was reported by (26) , but (27) whom recorded that age of sheep not effective in non-epidemic area and in open – system rearing while they showed the isolation percentage higher in small aged animal in epidemic area .

The small aged sheep could be less resistance against stressal factor like bad management (bad ventilation , crowding , grazing at distanced area as well as abnormal hard weather) all and another factors decrease the immune response of animal which lead to opportunistic bacteria to be growth and effective .

Molecular identification :

The highly similarity between genus of pasteurallaceae family in phenotypic and serological characteristic as well as in antigenic determinant , therefore the difficulties in distinguished between the genus was reported (5)

The PCR as highly specific primer , that test appeared 97.9% , 83.3% as sensitivity and specifity in diagnosis these isolate , which is agreement with results by (28) that appear on gel molecular weight as 460 pb when compeer with leader , which like in resultant by (29) and(30).

The results of genotyping of isolates which appear only (serotype A) which in similarity to results (31) and(32) , while there was not showed any bundle on gel concern with another type (B , D, E, F) which closely to information by (5)which detect type D infect pig , type F was infect Turkey fowl type , type E infective in cattle and type B ,not isolate from sheep , there for only type A infect sheep .

عزل جرثومة الباستوريلا ملتوسيدا من الأغنام وتشخيصها وتنميطها جزيئيا باستخدام تقنية تفاعل سلسلة البلمرة

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الخلاصة :

استخدمت 75 عينة (25 عينة من كل من المسحات الأنفية ، مسحات من اللوزتين ،عينات رئة) جمعت العينات من اعمار واجناس مختلفة من أغنام تعاني من مشاكل تنفسية ، تم فحص العينات باستخدام الطرق التقليدية لغرض عزل وتشخيص جرثومة *Pasteurella multocida* تم تشخيص 42 عزلة (56%) في الأغنام ، اعلى نسبة عزل كانت من الرئات 17 (68%) ، اللوز 14(56%) و 11(44%)من المسحات الأنفية.

لم يظهر العمر والجنس أي تأثير على نسبة العزل لجرثومة *Pasteurella multocida* في الذكور 22 (8.3%) ، في الإناث 20 (7.4%) بينما كانت نسبة العزل في الأغنام تحت عمر السنة 15 (55.5%) ، في الأعمار أكثر من سنة 27 (56.2%).

اظهر الفحص ألمجهري للعزلات بانها سالبة لصبغ ة كرام ،عصوية ، عصوية- كروية تمتلك خاصية التصبيغ القطبي عند تصبيغها بصبغة ازرق المثيل ،موجبة للأختبارات الكيموحيوية الأوكسيديز ،الكاتيلي ز والأندول ،لكن سالبة لكل من انتاج اليوريز واختبار الحركة .

استخدمت تقنية ال PCR لتاكيد عزلات *Pasteurella multocida* باستخدام بادئات نوعية (KMT-1) ، أظهرت حزمة مفردة واحدة عند ترحيلها على الاكاروز وبوزن جزيئي (460) زوج قاعدي .

استخدمت عدة بادئات نوعية لكل الأنواع المصلية لجرثومة ال *Pasteurella multocida* والتي تشمل كل من (A , B , D , E , F) لتمييز العزلات البكتيرية باستخدام تقنية تفاعل سلسلة البلمرة حيث سجلت النوع المصلي من (A) لجرثومة ال *Pasteurella multocida* المعزولة من الأغنا م .

خلصت الدراسة الى إن نتائج التشخيص الجزيئي للجرثومة باستخدام التقنية تفاعل سلسلة البلمرة وأنماطها المصلية كانت ذات خصوصية (97.2%) وحساسية(83.3%) في الأغنام .

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