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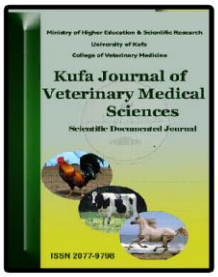

Detection virulence factors of *Klebsiella pneumonia* from cattle by using PCR tec.

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Abstract

Identification of *K. pneumoniae* was avaluated using conventional microbiological characteristics and molecular assays .

Milk specimens were collected from cattle suffered from clinical symptoms of mastitis from different farms of AL-Qadisyiah province.

Out of 45 samples obtained, 20 isolates (44.4 %) were detected as *K. pneumonia* according to morphology of colonies and biochemical features.

Molecular detection of *Klebsiella pneumoniae* based 16S rRNA gene for determination two virulence factor genes(rmpA,magA) by using specific primer.These genes potent the pathogenesis of *Klebisella pneumonia*. The primers were made in this study by using NCBI-GenBank and primer3 plus design online. The primer is made by company in Korea (Bioneer).

Moleculer detection of isolates which give away specific PCR products of of 312bp for *mag*A gene and 835bp for rmpA gene .

The *mag*A and rmpA genes were amplified in six (30%)and five(25%) isolates out of 20 isolates of *Klebsiella pneumonia*.

Keywords: cattle mastitis, Virulence factors, *Klebsiella. pneumoniae*,PCR tech.

الكشف عن عوامل ضراوة الكلبسيلا الرئوية المعزولة من الابقار باستخدام تقنية سلسلة تفاعل البلمرة

جميلة راضي اسماعيل جنان ناظم صادق

كلية الطب البيطري\ جامعة القادسية

الخلاصة

تم تشخيص الكلبسيلا الرئوية الخصائص الميكروبيولوجية التقليدية وطرق القياس الجزيئي .

تم جمع عينات الحليب من ابقار تعاني من علامات سريرية لالتهاب الضرع من حقول مختلفة في محافظة القادسية .

من بين 45 عينة تم الحصول عليها .تم الكشف عن 20 عزلة بنسبة (44,4%).وتم تحديد الكلبسيلا الرئوية على اساس شكل المستعمرات والاختبارات الكيموحيوية .

تم التشخيص الجزيئي للكلبسيلا الرئوية 16srRNAبالاستناد على جين باستخدام بادئات متخصصة(MagA,rmpA)لتحديد عاملين من عوامل الضراوة وهما صممت هذه البادئات في الدراسة باستخدام بنك الجينات وصممت ثلاث بادئات جهزت بواسطة كوريا .تم الكشف الجزيئي للعزلات وحددت نواتج شركة(Bioneer company)

312و 835)للجينين على التوالي . PCR bp)

ظهر تضخيم الجينات في ستة عزلات بنسبة (30%) وخمسة عزلات بنسبة (25%) من اصل 20 عزلة من الكلبسيلا الرئوية .

Introduction

Small number of bacterial species is responsible for most mastitis cases classified into contagious and environmental(1).

Klebsiella pneumonia is present in the normal animals , its an important cause of acquired infection and its one of major organisms among of the gram negative that cause mastitis specially in cattle(2,3) .

Mastitis caused by *K. pneumoniae* is poor response to antibiotic therapy so that case of clinical mastitis caused by it is more sever (4).

Klebsiella pneumonia can arise from cows in environment enter to the udder through milking ,or teats when contamination by feces ,mud and bedding materials(5).

The presence of virulence genes in *Klebsiella pneumonia* promote the pathogenesity to evading the immune of the body (6).

Many sequened virulence genes have been detection in *Klebsiella pneumonia* such as 16 sRNA ,Mag A and rmpA.

analysis and Sequencing of regions within 16S rRNA gene can expand speedy and effective ways to estimate variety of bacteria useful for pathogen and identification(7)

Mag A(mucoviscosity-associated gene A)is causes mucoviscosity,also is encods apsular polymerase and find within gene specific *Klebsiella pneumonia* (8).

While the rmpA gene is related with hypermucoviscous phenotype and encods as plasmid borne regulator of extracellular polysaccharide synthesis, a layer arounding the surface of cell, is hinders the phagocytosis (9)

these two genes associated with invasive infections (10) .

The aim of this study is to determine some virulence genes of *Klebsiella pneumonia* isolated from clinical case of mastitis in cattle in AL-Qadisyiahprovince via microbiological characteristics and molecular methods .

Materials and Methods

This study was carried out in the veterinary medicine collage\ university of AL-Qadisyiah .

Sample collection: The specimens were milk, collected from cattle undergoing suffers from clinical sings of mastitis from different areas of AL-Qadisyiah province.

All milk samples were cultured on maCconkeys agar and blood agar blates and incubated for 24 hours at 37c according to standerd procedure (11).

Thereafter the isolates were activated by in inoculated on CHROMagar Orintation and incubated at 37C° for overnight.

Identification of isolates based on morphology of colonies ,subculturing of isolates onto maCconkey and incubated for 24 hours at 37c, pink ,mucoid,lactose fermented colonies were considered to be *Klebsiella* spp,On orintation media colonies is metalic blue color,large ,rounded .

The identification of species of *Klebsiella* according to the biochemical reaction such as catalase,oxidase, indole production ,motility and citrate utilization . biochemical reactions carried out according to(12) .

Bacterial genomic DNA extraction:

Bacterial genomic DNA was extracted from *Klebsiella pneumoniae* isolates by

using (PrestoTM Mini gDNA Bacteria Kit. Geneaid. USA). 1ml of overnight bacterial growth on BHI broth was placed in 1.5ml microcentrifuge tubes and than transferred in centrifuge at 10000 rpm for 1 minute. After that, the supernatant was discarded and the bacterial cells pellets were used in genomic DNA extraction and the extraction was done according to company instruction. After that, the extracted gDNA was checked by Nanodrop spectrophotometer, then store in -20C at refrigerator until perform PCR assay.

Polymerase chain reaction (PCR):

PCR assay was performed for confirmative detection of *Klebsiella pneumoniae* based 16S rRNA gene and for determination some virulence factor genes and by using specific primer that designed in this study by using NCBI-GenBank and primer3 plus design online. The primers were provided by (Bioneer company . Korea) as table(1).

Primers	Sequences	Amplicon	GenBank
16S rRNA	**CGCGAAGAACCTTACCTGGT**	**352bp**	**Y17669.1**
	AGTTGCAGACTCCAATCCGG		
MagA	**TAGGTCAGGCAGCTGTTGTG**	**312bp**	**KP973856.1**
	GCTCCGTTGCAATATGACCG		
RmpA	**TGCAAACACGCAAAGGACAA**	**835bp**	**AB289644.1**
	AAGAGTGCTTTCACCCCCTC		

Table (1): **sequences of primers with size of targeted products of the PCR to the *K. pneumonia***

Prepared of the PCR master mix by using (kit of AccuPower® PCR PreMix). from company Bioneer in Korea. Premix tubes of the PCR contain freezer-dried pellet to (dNTPs 250µM ,Tris-HCl (pH 9.0),Taq DNA polymerase 1U, 10mM, KCl 30mM, stabilizer ,MgCl2 , tracking dye , and1.5mM) prepared of the PCR master mix according to the kit of instructions with 20µl total volume ,added 5µl of genomic DNAwas purified and also added 1.5µl of 10p mole of forward primer with same of reverse primer, The PCR premix tube by PCR water (20µl) mixed by Exispin vortex centrifuge. The reaction was carried out in a thermocycler by set up the following thermocycler conditions; initial denaturation temperature of 95 C° for 5 min; followed by 30 cycles at denaturation 95 C° for 30 s, annealing 58 C° for 30 s, and extension 72 °C for 1min and then final extension

at 72 °C for 10 min. The PCR products were examined by electrophoresis in a 1% agarose gel.

Results

growth on Orintation medium where specific color give blue pigment,The growth was also inoculated onto MacConkey agar, where lactose fermenting mucoid colonies producing pink of the medium lactose fermenting. These isolates were positive citrate utilization and negative for H2S production positive for catalase and negative for oxidase reaction according to (11)

In the present study, the prevalence of *Klebsiella pneumonia* was found to be 44.4%.

Figure 1:*Klebsiella pneumonia* on MacConkey agar produced rounded, mucoid (polysaccharide capsule) ,large and pink colonies

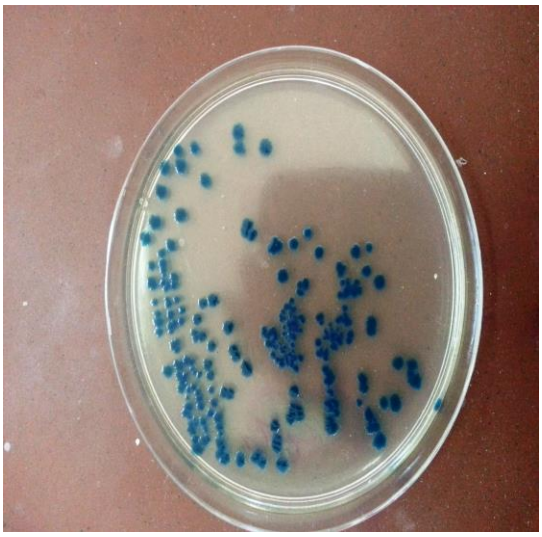

Figure 2:*Klebsiella pneumonia* on chrom agar Orientation ,produce metalic blue ,rounded and large colonies

In this study, *mag*A gene, were tested by PCR by using specific primer sequences with product sizes of 312bp ,Out of total 20 isolates, 6 isolate (30%) was positive for *mag*A gene .
While gene rmpA with product sizes of 835bp, Out of total 20 isolates, clarify that 5 isolate(25%) was positive for rmpA gene while 15 isolates of *Klebsiella* (75 %) were negative to this gene.

Gene	No .of tested isolates	Positive
16S rRNA gene	**20**	**20**
MagA	**20**	**6**
RmpA	**20**	**5**

Table (2):The virulence genes distribution with the numbers of the isolates

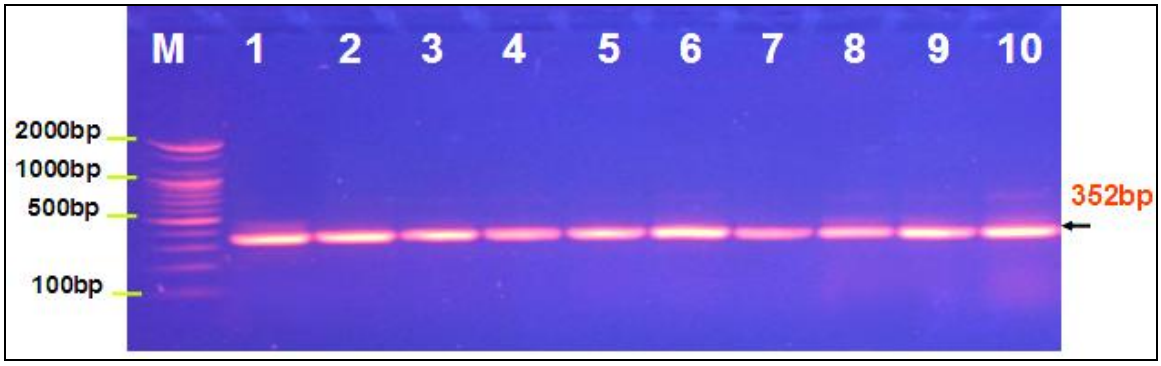

Figure 3: Agarose gel electrophoresis image that show the PCR product analysis of 16S rRNA gene in *Klebsiella pneumoniae* positive isolates. Where M: marker (2000-100bp), lane (1-10) positive Staphylococcus aureus at (352bp) 16S rRNA gene PCR product.

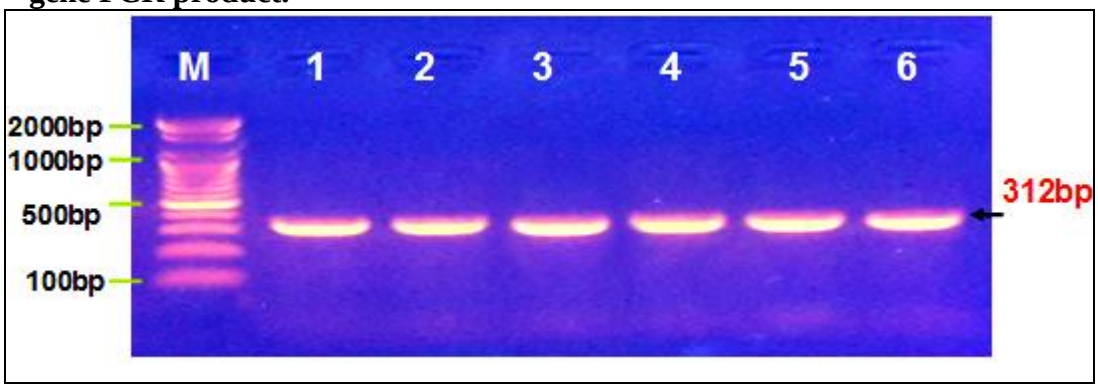

Figure 4: Agarose gel electrophoresis image that show the PCR product analysis of *magA* gene in Klebsiella pneumoniae isolates. Where M: marker (2000-100bp), lane (1-6) positive isolates at (312bp) PCR product.

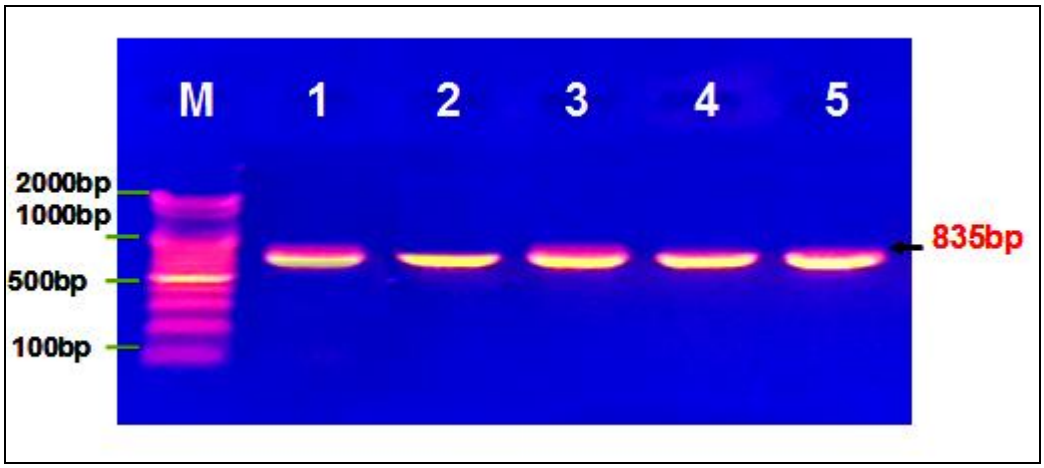

Figure 5: Agarose gel electrophoresis image that show the PCR product analysis of *rmpA* gene in Klebsiella pneumoniae isolates. Where M: marker (2000-100bp), lane (1-5) positive isolates at (835bp) PCR product.

Disccusion

The most frequent problem in the farms of dairy cattle in the world is mastitis **(**13)The occurance of mastitis in cattle differ according to the regions due to religious law ,also varies according to management accounting,and hygiene measures.

In clinical mastitis the most common gram negative bacteria is *Klebsiella pneumonia* (14).

sheeding of Klebsiella spp in feces promote the distrpution of pathogens in the environment ,material of bedding and then suggested recycled transmission of *Klebsiella* pneumonia in dairy farms.(15).

(16)found that large number of mastitis outbreaks by *Klebsiella pneumonia*.

The contamination of milk may result from contact with cow which infected or may be

contact with contamination of environment (17).

The occurance of mastitis by *Klebsiella* increases under humid and hot conditions(18)

outbreaks by *Klebsiella* are reported in many countries in highly percent exemplify USA

and Eroupe(19).

sample collection methods may play a role in heterogeneity of *Klebsiella* in milk samples

Our high rates are proximate to the results in south America and Asia which have

registrated in rates between 33.5% and 45%(20)(21),While the findings of other study in

france a high values which have registrated 92%.(22).The pathogenic strains of

Klebsiella pneumonia existent in the environment in broud variety(23).

(24)were observed that both of serotypes of capsular related to *Klebsiella pneumonia*

which cause mastitis and human consider as source of infection.

Species of *klebsiella* can be detected by 16SrRNA sequencing of gene, but due to the nucleotide variation is limited , 16SrRNA sequences not be used to differentiation within the phylogenetic groups (22),So 16S rDNA analysis of

phylogenetic used in classifying and identifying K. pneumonia.

MagA and rmpA causing the bacteria s the most invasive and resistant to immune defence (25)

A prior studies has shown that the phenotype of mucoid colony may be to a gene

specified rmpA (26).

in our study gene rmpA was detected in only 5 isolates,this less from previous study (27)

The public health importance of this study is to know unique regulator of hypermucoviscous capsule composition and formation (28).

Regarding analysis of molecular of *Klebsiella pneumonia* confirmed in highly specifity of primers from region of 16SrRNA.

Conclusion

((1)) molecular methods maesurment instrument helpful in diagnostics of mastitis ((2)) control of *Klebsiella* in farms of dairy cattle by hygiene ((3)) Detection of the virulence factors in *K. pneumoniae* that cause mastitis in cattle will be aid in detection of this disease .

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