International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 4s, 2025
https://www.theaspd.com/ijes.php

Molecular Detection Of Klebsiella Pneumonia Isolated From Ruminants Secretion In AL Shatrah City, South Iraq

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Abstract
In this current study showed that detection of Klebsiella species from variation infected cattle discharge. 120 samples and swabs were collected divided in to (40 from nasal secretion, 40 from vaginal discharge and finally 40 from a milk samples). These sample cultured on different culture media and identified by using cultural characteristic and microscopic appearance. klebsiella pneumonia Identified using ViteK II system and confirm the last diagnosed of those organism by using polymerase chain reaction(PCR)assay (16S rRNA). Polymerase chain reaction used to detected some gene K1,K2(which is one of several genes responsible for capsule formation), and also blaSHv gene which is one of the genes related with multy drug resistance. Results explaned that Klebsiella pneumoniae were isolated in rate (10%) from nasal discharge, while isolated in rate (10%) from milk samples, on the other hand, vaginal secretion not appeared positive result for Klebsiella pneumonia. the result involved that K1,K2 genes were not detected in all samples of K. pneumonia. Also, completely isolates of K. pneumoniae harbored blaSHv gene (100%), Also the result included that Klebsiella pneumoniae can be consider as major pathogen responsible for different medical case affected bovine in Al-Shatrah district in Thi-Qar province.
Key words: *Klebsiella pneumoniae , vagial secretion ,nasal discharge.*

INTRODUCTION

Klebsiella species are opportunistic infections that colonize mucosal surfaces without producing disease, yet they have the potential to move from the mucosa to other tissues and endanger life. (Paczosa and Mecsas, 2016). Gram-negative, bacilliform, non-sporulation, non-ciliated bacteria with a thick cell wall, Klebsiella species are the cause of the mucous appearance of colony in a solid-state cultures grown in vitro as well as the invasion of bacteria in vivo. Klebsiella pneumonia is thought to be the most clinically significant of the several Klebsiella species and subspecies in animals as well as humans. Klebsiella species can be found in soil, wastewater, plants, surface water, and other habitats. Klebsiella pneumonia is pathogen capable of causing a different infections. Classically, Klebsiella pneumonia is known the main causes of pneumonia, Urinary tract infections and bacteremia (Choby, et al.,2020). Bacterial pneumonia causes very economic loss to the beef industry and that increase in stress and viral infection, taking antibiotics to at-risk calves is often to prevent the disease. The mass of administration of antibiotic lead to negative result in meat production and that perhaps lead to increase of resistant pathogen (Vulikh et al., 2019).

The most pathogenic bacteria which affected bovine is Gram-negative bacteria which is an significant cause of bovine mastitis all over the world (Schukken, et al., 2013).The Sub clinical mastitis in cattle is a main and silent problem causes greater economic losses to the farmers. One of the primary causes of decreased milk quality and output is mastitis, which is defined as inflammation of the mammary glands' parenchyma and is characterized by pathologic alterations to glandular tissues as well as physical, chemical, and typically bacteriological abnormalities in milk (Kumari et al.,2018).

MATERIALS AND METHODS

Sample collection:
One hundred and twenty swabs and samples was collecting from the cattle, from Thi-Qar province (Shatra dispesary, farms), duing period From the November of the 2024 to January the of 2025, the information including age and location were fixed. milk samples putted in sterile container then in ice box and swabs (nasal and vaginal) putted in transport media then in ice box then the samples and swabs transport to microbiology laboratory in veterinary collage with period less than 24 hours .

Maintenance of bacterial isolates
The following is how the bacterial isolates were maintained:

Short term storage
For several months, the pure isolates of bacteria were kept in screw-capped universal tubes filled with brain-heart agar and cultured for twenty-four hours at 37°C. The slants were then securely wrapped with Parafilm and stored for three months at 4°C (Brown & smith, 2014).

Long term storage
The next day, a loop of pure bacterial culture was used to inoculate a brain heart infusion broth, and after 18 hours of incubation at 37°C , The broth was prepared for incubation by adding glycerol at an ultimate concentration of 20% and keeping it at -20°C for two to eight months (Goldman & Green,2015).

Isolation and identification of bacteria
The samples and swabs were collected from cattle from different places from AL Shatrra province .Then, the samples and swab transport and incubation in brain heart infusion on incubator in 37°C for 24 hours then growth on MacConky agar and put on incubation in 37°C for (24 to 48) hours in aerobic environment. Gram stain method done according to (leboffe and Pierce, 2021). Identification by using automated methods [Vitek Ⅱ] system Gram-negative (GN) and Gram-positive (GP) identity cards were produced (Maina & Kagotho, 2014). Molecular studies Extraction of bacterial DNA All Klebsiella pneumoniae isolates had their genomic DNA extracted using the Presto™ Mini gDNA Microbial Kit in accordance with the manufacturer's instructions.

PCR detection of genes
The genes have been amplified using the primers specified in Table (1). A total of 12.5 µl of Mastering Mix, 1 µl of the specific reverse and forward primers for each gene, 3 µl of the DNA template as well as and water that lacks nuclease complete the last reaction in a tube volume of 20 µl.

Primers

Table (3-5) contains a list of all primers used in the present study.

Primer sequences utilized in gene amplification are shown in Table (3-5).

Gene	Primer Sequences (5´- 3´)	Product size	Reference

A:

16SrRNA	F*: AGAGTTTGATCCTGGCTCAG R*: GGTTACCTTGTTACGACTT	1500bp
K1	F: 5'-AGATAGAGGTGTATTGTCGC R:GAGCTCTATATGTTGGATGC	352bp
K2		
	F: TCATACTTGACAGAGGGAGTAG	
balSHV	R: ACGATCGTTACAGTGACAAG	321 bp
	F: GGCCGCGTAGGCATGATAGA	
	R: CCCGGCGATTTGCTGATTTC	714bp

Adanin, T: Thaymin, C: Cytocin, G: Guanin

Five microliters of DNA were combined with dye for loading and put into wells to electrophorese the separated DNA samples. After that, the wells were exposed to an electric field for 45–60 minutes at 70V. Tables listed the thermocycling programs for the K1, K2, blaSHV, and 16SrRNA genes, respectively.

Statistical Analysis

Descriptive statistics were used for the statistical analysis, and percentages (%) were used to represent the nominal variables (source of isolation). Five age groups were created from the age variable, which was regarded as an ordinal variable" To investigate all of the relationships and quality of fit within the variables, the chi-square along with Fisher's accurate tests were employed. The correlation between numerical and interval variables was assessed using the Eta test. Values below 0.05 were deemed statistically significant, taking into account the Alpha significance threshold.

RESULTS AND DISCUSSION

Regarding the distribution of mastitis cases among the age groups, this study found that mastitis was significantly associated ($P<0.05$) with the smaller age groups (1-2 years) table (1). An excellent statistical correlation (Eta value =1) was also found between mastitis and age, Figure(1). Mastitis was recorded at a higher percentage (47.5%) in the age group (1-2 years), followed by the age group 3 years with a percentage of 20%, and the age group 4 years was recorded at 15%. In comparison, the age groups 5 and 6 years were recorded at 12.5% and 5%, respectively.

Table (1): Isolation rate of different cow samples

Age	Positive N0.	Percent	P-value (X^2)
1-2 years	19	47,5%%	
3 years	8	20%	
4 years	6	15%	000
5 years	5	12,5%	(21.250)
6 years	2	5%	
Total	40	100%	

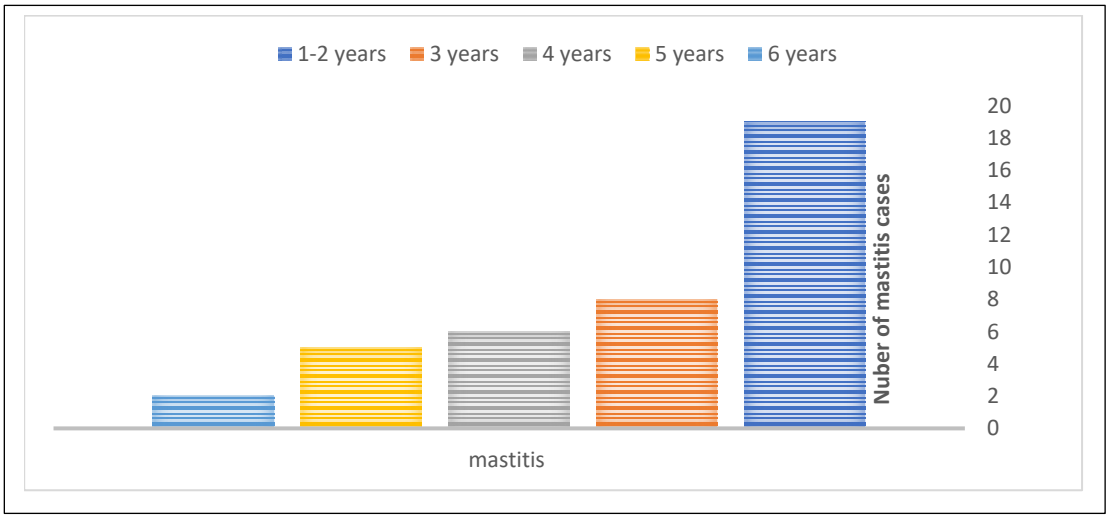


Figure (1): the correlation between the age groups and the prevalence of mastitis (Eta value=1) indicative of excellent correlation

Colony morphology

After 24-48 hours of incubation on agar from MacConkey at 37 °C, the first bacteria from the huge colony were identified, pink, regular edged, spherical colonies with a mucoid texture were produced by Klebsiella on MacConkey agar when the bacteria fermented the lactose.

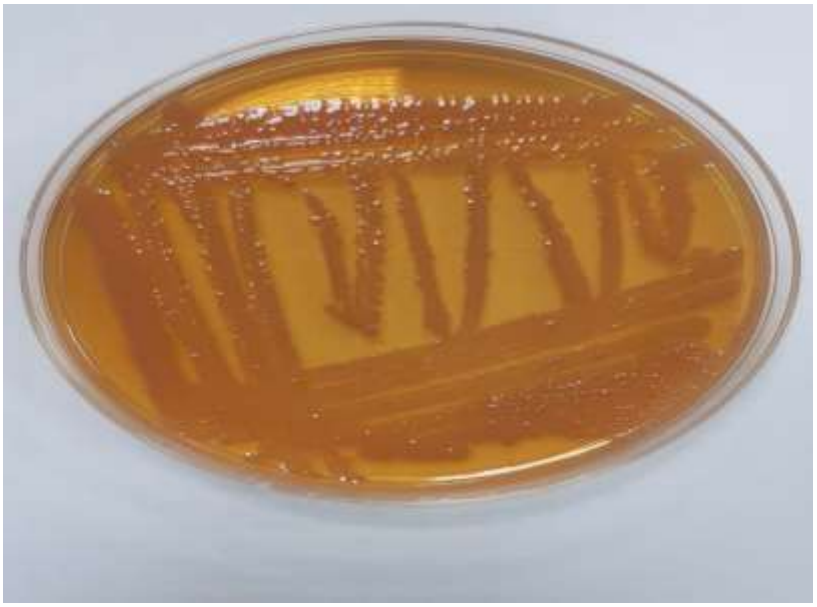


Figure (2) Klebsiella pneumoniae on MacConkey agar

Table (2) represents the results of the distribution of vaginal infection among the age groups. The mean age was 1.95 ±1.2 years, ranging from >1 year to <4 years. This study found significant differences (P<0.05) in the

distribution of vaginal infection regarding the age groups. The highest infection rate was found in the age group >1 year (50%), followed by 25% in the age group 1-2 years old. However, the vaginal infection in the age groups >2-3 and >3-4 was equal (10%) for each. The lowest infection rate was found in the age group >4 years. Similarly, an excellent correlation was found between the age groups and the vaginal infection (Eta value = 98%), figure (3)

Table (2): the distribution of vaginal infection among different cow age groups

Age	Positive No.	Percent	P-value (X2)
<1	20	50%	000 (27.00)
1-2s	10	25%	
>2-3 years	4	10%	
>3-4 years	4	10%	
>4	2	5%	
Total	40	100%	

X^2 = Chi-square test, degree of freedom=4

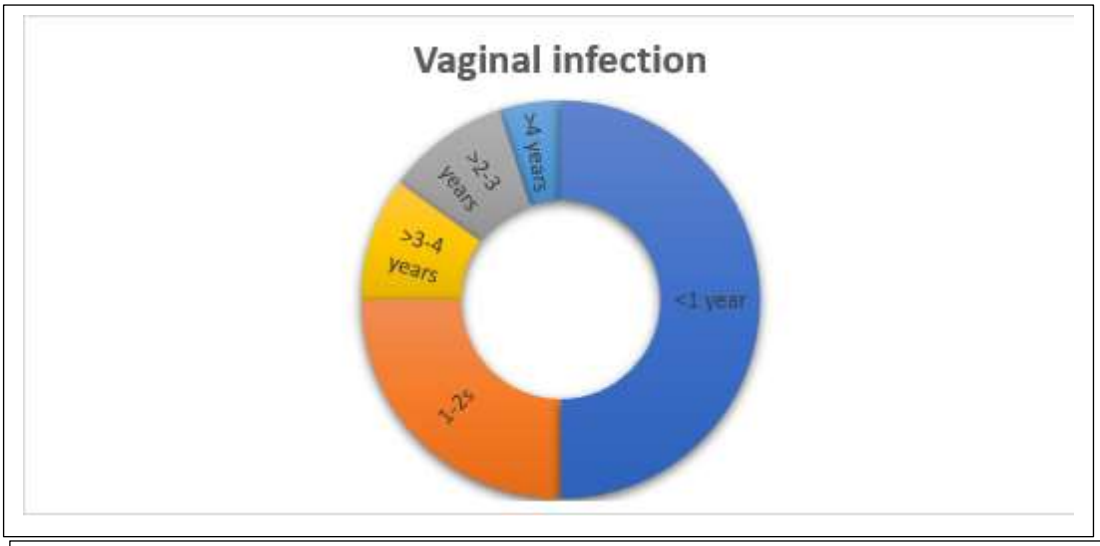
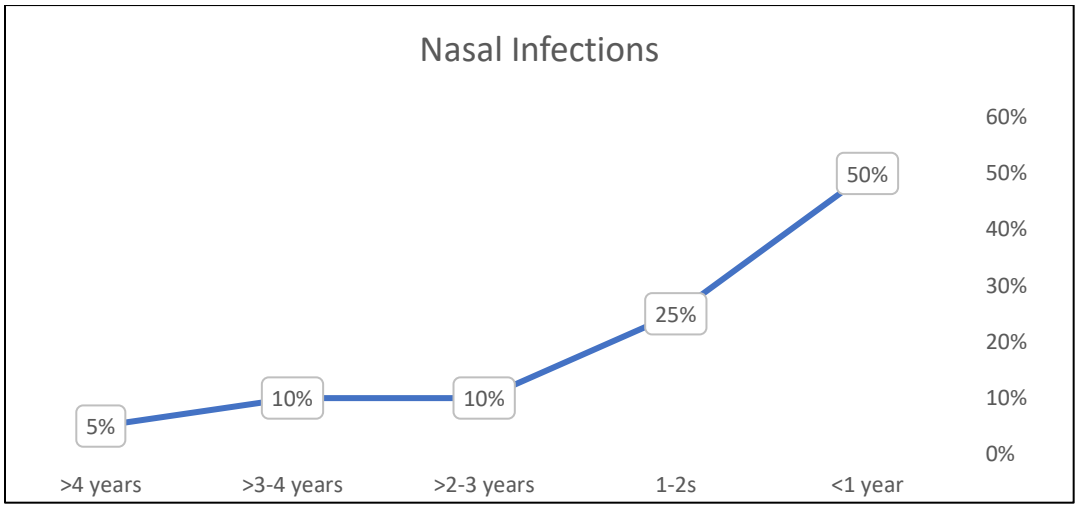


Figure (3): the correlation based on Eta test between age groups and the vaginal infection

Regarding to the nasal infections, this study found that the distribution of the nasal infection among the different age groups was statistically significant (P<0.05). The highest nasal infection rate (50%) was found in the smaller age group (<1 year old), followed by the 25%, which accounted for the age group (1-2 years old). On the other hand, the age groups >2-3 years and >3-4 years recorded 10% for each, and finally the lowest infection rate was found in the older age group (>4 years). Statistically, there is an excellent correlation (Eta value = 96%)between the age groups and the nasal infection rate. Figure (4)

Table (3): the distribution of nasal infection among different cow age groups

Age	Positive No.	Percent	P-value (X2)
<1	20	50%	000 (27.00)
1-2year	10	25%	
>2-3 years	4	10%	
>3-4 years	4	10%	
>4	2	5%	
Total	40	100%	



Figure(4): the distribution of nasal infection among the different age groups, an excellent correlation (eta value = 96%)

This study found no significant differences (p>0.05) between the positivity of VitekII and PCR technique in identifying K. pneumonia, as both techniques showed only 4/54(7.40%) were K. pneumonia. Table (4).

Table (4): the positivity of VitekII and PCR in identification of K. pneumonia from different cow samples

Screening Techniques	Positive	Negative
VitekII	4	54
PCR	4	54
P-value	0.642	

Fisher exact test

Vitek Ⅱ system for identification of Klebsiella pneumonia:

Klebsiella pneumonia identification by vitek Ⅱ system rapidly in this approach with environment free of kind of pollution that may have prevented the presence of the pathogen from being identified in any other way. The result from this system's testing corroborated those from the morphological and PCR test.

Table(5):This table showed the vitek result of present of klebsiella

samples	Total number	Positive+	Percent	Negative-p
Milk	20	2	20%	18
Nasal secretion	20	2	20%	18
Vagina secretion	18	0	0%	18

RESULTS OF PCR TECHNIQUE

The results of PCR technique showed the genomic DNA of all Klebsiella pneumoniae samples showed in Fig (5).

The present results recorded that (4/4;100%) of K. pneumoniae isolates giving positive results for 16SrRNA gene. The bands of this gene which determined the size of 16SrRNa gene, nearly 1500bp, as shown in Fig. (6). While, K1,K2 genes were not detected in all samples of K. pneumonia. Also, completely isolates of K. pneumonia harbored blaSHv gene (100%), as in fig (7)

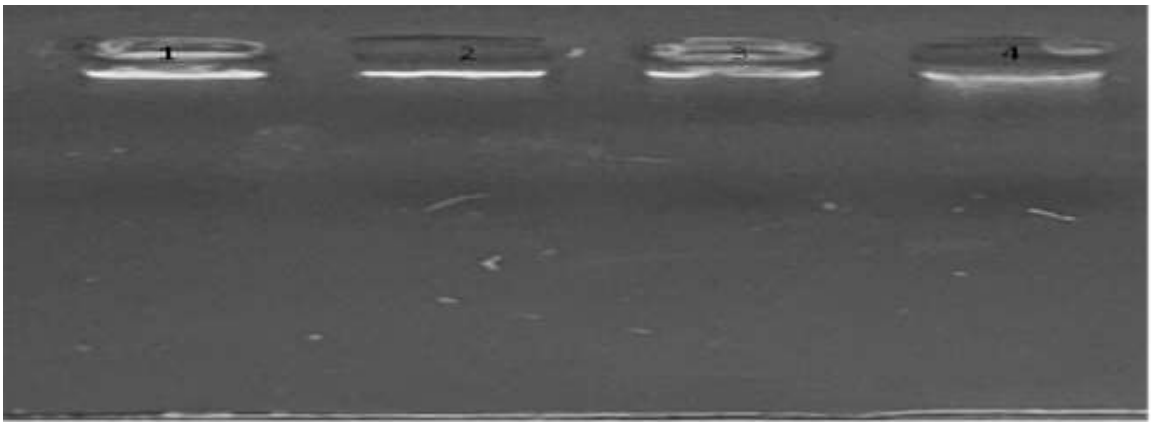


Fig (5): genomic DNA of K. pneumoniae

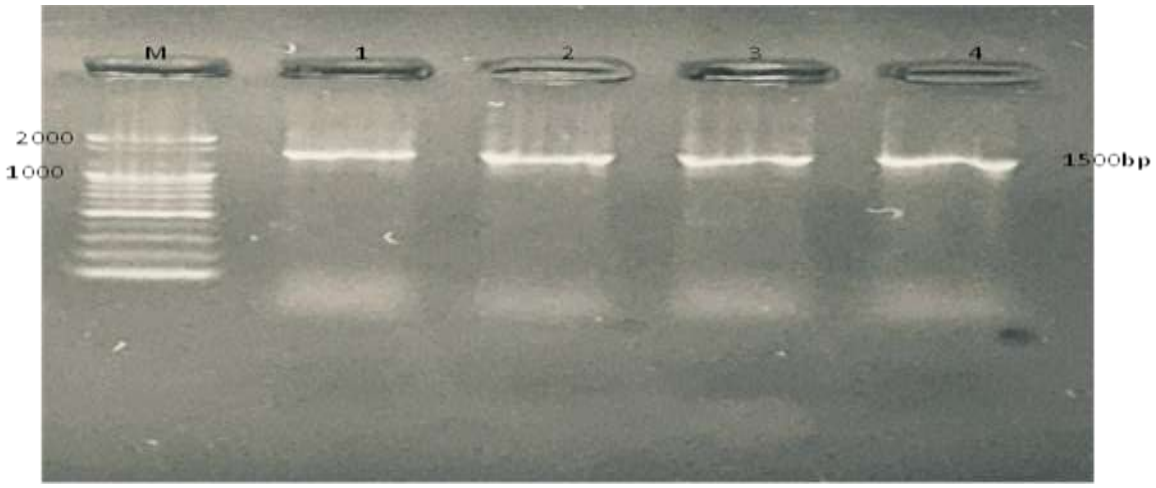


Fig. (6): Agarose gel electrophoresis of 16SrRNA gene amplification, where M: ladder, 1-4: positive results.

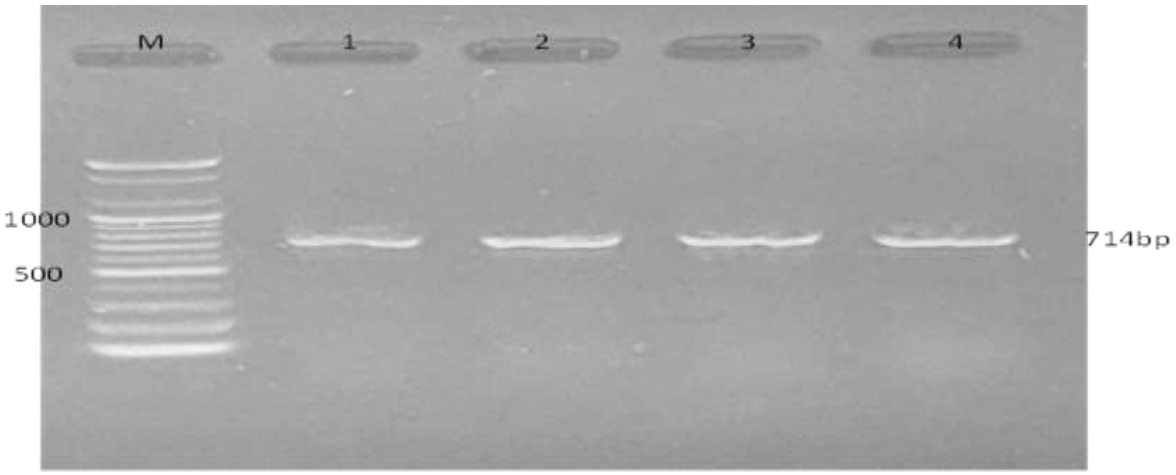


Fig. (7): Agarose gel electrophoresis of blaSHV gene amplification, where M: ladder, 1-4: positive results.

Mutation result show most genetic variation in 16s rRNA

Sample Variant Position in the PCR fragment

Sample	Variant	Position in the PCR fragment
S2,S3	C7T	7
S1,S4	C87T	87
S2,S3	T103A	103
S2,S3	A122T	122
S2,S3	A130G	130
S2,S3	A154T	154
S2,S3	C338T	338
S2,S3	C359G	359
S1,S4	Gins360	360
S2,S3	Ains360	360
S1,S4	G361A	361
S2,S3	G365A	365
S2,S3	A373C	373
S2,S3	C375T	375
S1,S2,S3,S4	C377T	377
S1,S2,S3,S4	Gins378	378
S2,S3	T379C	379
S2,S3	T380C	380
S2,S3	G381C	381
S2,S3	C494T	494
S2,S3	C498T	498
S2,S3	G504A	504
S2,S3	T541C	541

S2,S3	C544T	544
S2,S3	G553A	553
S2,S3	A557T	557
S2,S3	T564C	564
S2,S3	A649G	649
S2,S3	A719C	719
S2,S3	T730C	730
S2,S3	A778G	778
S2,S3	G791A	791
S1,S2,S3,S4	T1078C	1078
S1,S2,S3,S4	G1079T	1079

The phylogenetic tree as shown in figure (3) described the most relative sequences to studied samples were found in very closed related with K. pneumoniae isolates. The samples (S1 and S4) were closely related in this phylogenetic tree with (KT588650.1, CP166017.1, PQ432922.1 K. pneumoniae), with adjacent branch; those samples were binding with other branch with ref sequence.

While, samples (S2,S3) were closely related in the current phylogenetic tree, and two samples separated from compared samples with separated branch. The results of phylogenetic tree and genetic variation detected by DNA sequencing improved the results of similarity and relation between (S2, and S3) sample.

DISCUSSION

Isolation of Klebsiella isolates:

One of the most significant opportunistic pathogens in animals, Klebsiella pneumoniae mostly causes mastitis and respiratory infections. In a recent research. The samples were examined for presence of Klebsiella isolates. A total of 120 clinical samples and swabs were included (40from milk, 40from nasal discharge and40 from vagina discharge) about 62 of these samples and swabs are no growth and 58(48%) of bacteria culture belong to different Gram negative bacteria .

From the milk samples were examined and the results, referred that20 (50%) which contained bacterial isolates, on the other hand, 20 (50%) of samples were negative for bacterial isolates. From positive cultures of milk samples several Gram negative bacterial isolates were identified such as E.coli 5 (25%),Enterobacter cloacae complex 3(15%), Leclerciaadecarboxylata2(10%), Klebsiella pneumoniae2(10%) Acinetobacterspp. 2(10%)Stenotrophomonasmaltophillia 2 (10%),while other bacterial isolates were Pseudomonas alcaligenes, , Providenciarettgeri, Pseudomonasstutzeri, and Pseudomonas aerugiosa 1(5%) respectively .

From nasal swabs the total numbers were 40 samples. no growth was 20 the other negative bacteria 20(50%) include E.coli 13(65%), Burkholderiacepacia 1(5%), Coronobactersakazakii 1(5%), Achromobacterdenitrificans 1(5%), Pseudomonasalcaligenes 1 (5%), Burkholderiagaldioli 1(5%) and Klebsiellapneumonia 2(10%).

From 40 vaginal swabs isolated the present study involved no growth were in 22 swabs,while the positive numbers were 18(45%) included E.coli 13(72,5%), Escherichiahermannii 1(5,5%), Achromobacterlowffii 1(5,5%), Aeromonashydrophila1(5,5%), Pseudomonasaerugiosa 1(5,5%) and Enterobaxtercloacaecomplex 1(5,5%).

Klebsiella pneumonia were isolated from millk samples in rate (2\20 (10%) . On the other hand, its isolated from nasal discharge in rate 2\20(10%) Nevertheless, K. pneumonia could not be isolated from all vaginal secretion 0/18(0%).

This result conceder nearly similar to Ramadan, M. M. (2023) which was examined samples and swaps from cattle and showed that Klebsiellapneumonia number was in nasal swaps 24/233(10.3%) and 5/60(8.33%) milk and no recorded of Klebsiell apneumoniae from vagina secretion . The current study which agreement to other study done by Chiaverini and his group (2022), who examined samples from wild animals and the Klebsiella pneumonia isolate recorded13/130 in rate 13%. In addition to that, he detected the same cultural characters of klebsiella species and recorded that the isolated bacteria appeared as pink, large , mucoid colonies on McConkey agar for 24 h at 37 C.

Higher isolation rate of klebsiella species from different sources most of them were from pig, cattle, milk and the rest of isolates were isolated from vegetables, pets, livestock and farm animals (Klaperetal., 2021)

Identification of Klebsiellapneumoniae by culturing and colony morphology and staining reaction
The bacteria were originally identified from the colony, which is a big, spherical, regular-edged colony with a mucous membrane texture and pink due to the acids generated by Klebsiella on the agar from MacConkey when the microbes fermented the lactose, after being incubated on the agar at 37 degrees Celsius for 24 to Identification of Klebsiellaspp. by using selective enriched media involved cultivating materials on MacConkey agar with crystal violet and bile salts which is favor to growth of Enterobacteriaceae and similar enteric Gram-negative rod while discouraging the development of Gram-positive bacteria and other very fussy Gram-negative bacteria. in this media the present of lactose can be differentiation between lactose-fermenting bacteria from non- fermenting bacteria. Fermenting bacteria when the Ph. is low enough, turns colonies pink from the breakdown of neutral red indicator dye.
Klebsiellapneumonia under a microscope were confirmed to be non-motile, Gram-negative, tiny straight rods that were either single or in pairs, as previously described by (Garrityetal., 2005) which similar to Dubey et al (2013).

Antibiotic resistance of Klebsiellapneumoniae isolates:

In this study the isolated strain of Klebsiellapneumonia tested against 14 anti-biotic with various mechanisms of action such as (Ampicilin/ sulbactam, Piperacillin/Tazobactam, Cefotaxime, Ceftazidime, Ceftazidine/Avibactam, Ceftazidine/Tazobactam, Cefepime, Impenem, Meropenem, Amekacin, Gentamicin , Ciprofloxacin, Tigecycline and Trimethroprim/sulfamethoxazole). The result of this study revelant that all isolated of Klebsiellapneumonia exhibited sensitive to all this antibiotic in rate 100%.

The other studes showed that Klebsiella pneumonia's resistance rate to some antibiotic as the following (ampicillin98,1%, ciprofloxacin35,9%,

trimethoprim/sulfamethoxazole35%, cefotaxime34%, gentamicin31,1%,

tetracycline31,1%) according to Haradet et al (2016).

Moreover, Gomaa (2021) verified that all Klebsiella pneumonia isolates were susceptible to the drugs a antibiotic such as trimethoprime/sulphamethaxole and trimethoprime (54.54% each), but resistant to the antibiotic ampicillin and amoxacillin-clavulinicacid (100%) following cefepime (72.72%), imipenem (82%) followed by aztreonam (55%), and amikacin and azithromycin (45%).

Montsoetal. (2019) showed that The highest multi drug resistance rate were found among klebsiellaisoltes from cattle in rate reached to (85–100%)

Brisse and Duijkeren (2005) found that from 78 isolates, resistance was common against ampicillin (99%) and cephalexin (43%), but not against tetracycline,ceftazidime, , enrofloxacin,ceftiofu, gentamicin and trimethoprimsulfamethoxazole.

Ramadan, M. M. (2023) study showed that the biofim formation module indicated that all strains were biofilm formed strains.

It was shown that 81.81% of the pneumonia (K. pneumonia) isolates were high (33.33%), moderate (22.22%), or weakly (44.44%) polymeric producers, compared to 18.18% of the total number of isolates that did not form biofilms (Gomaa, 2021). This result supports the findings of Massé et al. (2020).

The objective of the current study was to identify the evolutionary relationships by phylogenetic tree of all samples of Klebsiella pneumoniae isolated from cattle's milk and nasal secretions, as well as to identify genetic variants of the 16SrRNA genes by DNA sequencing.

Life-threatening illnesses are caused by Klebsiella pneumoniae. Nonetheless, the organisms have been linked to the onset of sickness (Villegas et al., 2006). Reports that bacteria that cause Klebsiella pneumoniae can spread swiftly across wide geographic areas are especially worrisome (Navon-Venezia et al., 2009).

The 16SrRNA gene sequencing used to identified the K. pneumoniae that isolated from various sources as samples obtained from animal, or from clinical isolates such as urine, blood, feces, sputum, which were aligned and compared to the sequence of strains that deposited in GenBank database (Budiarsoetal., 2021).

Despite their potential health risks, street meals are popular in nations that are developing due to their low prices and preference among the lower and middle classes. According to Rowbotham and Ruegg (2016) and Fuenzalida and Ruegg (2019), Klebsiella pneumoniae and Klebsiella oxytoca are commonly found in farm surroundings, cow skins and the milk, teet-end cotton swabs and clinical mastitis. Additionally, the Klebsiella pneumoniae species was a significant pathogen linked to the health of both humans and animals. Urinary tract infections, liver abscesses, and pneumonia have all been linked to Klebsiella pneumoniae (Bengoechea and Pessoa 2019; Fuenzalida and Ruegg 2019).

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CONCLUSION

-Klepsiella pneumoniae isolated from nasal secretion, milk secretion, and not found in vagina discharge. Klepsiella pneumoniae can be identified directly by vitek II system and conformed by PCR to detect 16s rRNA. All Klepsiella pneumoniae isolated optened to be sensitive to most antibiotic tested. Klepsiella pneumoniae isolated does not have K1,K2 and contain blaSHv gene.

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