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Molecular studies on *klebsiella pneumoniae* as a causative agent of cattle mastitis with special reference to some clinicopathological changes

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Abstract

This study aimed to investigate the prevalence and identification of *Klebsiella pneumoniae* isolates by using PCR reaction for detection of some virulence genes and antibiotic resistance as well as the effect *Klebsiella pneumoniae* on the hematological, biochemical and immunological changes on cows. A total of 150 milk and blood samples was collected from clinically mastitic cows from different farms in Ismailia. Milk samples were subjected to bacteriological examination and molecular identification as well as blood samples subjected to hematological, biochemical and immunological examination. The result of bacteriological examination revealed that the prevalence of *Klebsiella pneumoniae* from the mastitic cows was 13.3% (20/150). All strains of *Klebsiella pneumoniae* were susceptible to Amikacin, Gentamicin, Cephotaxime, Neomycin, Oxytetracycline and Sulphamethoxazole/Trimethoprim. On the other hand, most *Klebsiella pneumoniae isolates* were resistant to Ampicillin (100%), Chloramphenicol, Colistin sulphate, Erythromycin and Streptomycin and Penicillin G (70%-80%). The results of molecular identification of four *Klebsiella pneumoniae* isolates showed that, all the isolate gave a clear band with 16S rRNA at molecular size 130 bp as well as 50% positive for *kfu* gave a band at molecular size 847bp and negative for *rmpA* and *magA* genes, respectively. On the other hand, all four *Klebsiella pneumoniae* isolates were positive with the bla_{tem} gene, and negative with bla_{ctx-m} and bla_{fox} genes.

Hematological studies revealed leucocytosis, neutrophilia and lymphopenia. Significantly lower average values of RBCs, Hb conc. and PCV were observed in the positive *Klebsiella* mastitic cows as compared to healthy cows. Biochemical estimation revealed significantly increased in globulin, AST, Ca, Na, Cl, and K. However, no significant change was observed in Mg and ALT levels. Significant decrease in total protein (TP) level and albumin were observed in the positive *Klebsiella* mastitic cows as compared to healthy animals. The immunological results observed a significant increase in Interleukin-6 (IL-6), tumor necrotic factor-α (TNF-α), alpha globulin and gamma globulin in the blood of positive *Klebsiella* mastitic cows. Finally, it could be concluded that the *Klebsiella pneumoniae* causes mastitis and effects on the immune system of cows by increase level of IL6 and TNF.

Keywords: *Klebsiella pneumoniae, cow, mastitis, Interleukin- 6, TNF, Clinicopathological.*

Introduction

Mastitis adversely affects milk production, and generally makes the infected cattle does not return to full production level, even after its recovery **(Grohn *et al*., 2004)**. The Gram-negative bacteria are responsible for greater reduction in milk production than Gram- positive bacteria and other non-bacterial agents **(Gogoi *et al*., 2014)**. *Klebsiella Pneumoniae* is one of the causes of the primary environment derived *Klebsiella* mastitis and has been the subject of numerous studies **(Dahmen *et al*., 2013 and Ohnishi *et al*., 2013).**

Klebsiella spp., is a common cause of bovine

mastitis, but information regarding it molecular epidemiology is lacking from many parts of the world **(Podder *et al.*, 2014).** *Klebsiella pneumoniae* had the ability to invade the host, by virulent factors such as capsular polysaccharide, lipopolysaccharide, serum resistance, and production of urea and enterotoxin **(Aher *et al.*, 2012). Osman *et al.* (2014)** evaluated *Klebsiella* by both phenotypic and molecular assays through the use of 16S rRNA for identification and virulence genes for *Klebsiella.* Also, The association of the some virulence determinants: *magA* (mucoviscosity-associated gene specific to K1 capsule serotype), *kfu* (iron uptake system gene), and *rmpA* gene (regulator of the mucoid phenotype). The *kfu* chromosomal virulence gene responsible for an iron uptake system (codes for an iron uptake system), is a putative pathogenic gene, significantly associated with the virulent hypermucoviscosity phenotype and purulent tissue infections **(Ma *et al.*, 2005; Yu *et al.*, 2007; Li *et al.*, 2012 and Dora *et al.*, 2013)**.

However, antibiotic resistance properties are the major factor in its pathogenicity that it resists for a wide spectrum of antibiotics and specially β-lactam antibiotics. This is due to the prevalence of infections acquired in hospital, which lead to the orientation of the research on alternative therapies **(Dubey *et al.*, 2013)**. It's noteworthy that β-lactam compounds such as penicillin continues to be one of the most frequently used drugs in veterinary medicine **(Pitkala *et al.*, 2007).** The genes that encode extended spectrum beta lactamase (ESBLs) are usually found in plasmids, and those encoding ESBLs of types CTX-M (gene bla_{ctx-m}), TEM (bla_{tem}), PER (bla_{per}), VER (bla_{ver}) and SHV (bla_{shv}) are the main groups (**Paterson *et al.,* 2003 and Jemima and Verghese, 2008**).

Neutrophils are the most vital primary mobile phagocytes **(Paape *et al.,* 2003)** in the body of mammals and play a key role in initiating an innate, inflammatory, and specific immune response. With the invasion of pathogens, the neutrophils migrate from the blood into milk and employ cascades of reactions, including both oxidative and non-oxidative mechanisms to destroy the pathogens. Several studies have been carried out on the incidence and treatment of mastitis, but the complex nature of pathogenesis and immune responses in mastitis are still poorly understood **(Bannerman, 2009)**. The mastitis occurrence has significantly increased the parameters of Ca, Na, K, Mg, P, Fe, Zn and Cu and enzymes (LDH, GOT and ALP) in both cattle and buffaloes which is a major challenge for field veterinarians and researchers **(Awale *et al.*, 2012).**

IL-6 is generated by infectious lesion then sent a signal to the entire body. The pathogen-associated molecular patterns, is recognized in the infected lesion by immune cells such as monocytes and macrophages **(Kumar *et al.*, 2011)**. They stimulate a range of signaling pathways, including NF-κB, and enhance the transcription of the mRNA of inflammatory cytokines such as IL-6, tumor necrotic factor (TNF)-α, and IL-1β. TNF-α and IL-1β also activate transcription factors to produce IL-6. IL -6 is crucial for immune function in animals, it induces the final maturation of B cell into immunoglobulin-secreting plasma cell. TNF-α is known to be a key mediator for the induction of apoptosis and development of humeral immune response **(Li *et al.,* 2007).**

Therefore, in the present study, we screened cows affected by clinical mastitis for *Klebsiella pneumoniae* via isolation, biochemical characteristics and molecular virulence and antibiotic resistance genes and confirmation of isolates. In addition, the evaluation of the haemtological, biochemical and immunological alteration in blood of clinical mastitic cows.

Materials and Methods

I-Sample collection

a) Milk sample: The study took place in private dairy farms at Ismailia Governorate. A total of 150 milk samples was aseptically collected from clinical mastitis cows, udders and teats of the randomly selected, lactating ani-

mals were cleaned, dried, and disinfected with 70% ethanol before sample collection. Three streams of foremilk were expressed from each quarter and then discarded. During sample collection,10–15 ml of milk from each quarter was manually expressed into separate sterile 25 ml universal tubes.

After gently suspending each sample, the milk was poured into a separate, unused container and were quickly transported to the laboratory under chilled conditions and stored at 4ºC until bacteriologic analyzed, and had its antimicrobial susceptibility and molecular typing determined.

b) Blood sample: Blood samples were collected from apparently healthy as a control and the positive *Klebsiella pneumoniae* mastitic cows (20 for each) by puncture of the jugular vein, the addition of heparin as an anticoagulant for hematological examination and without heparin for biochemical serum analysis. The serum blood was separated by centrifugation at 1500 g for 10 minutes and stored at -20ºC for a maximum of 60 days until assayed according to the metabolic profile **(Payne *et al*., 1970).**

II- Bacterial isolation and identification:

The milk samples were immediately inoculated on 5% sheep blood agar and MacConkey's agar (HiMedia, Mumbai, India) plates and incubated at 37°C for 18-24 h. Lactose fermented colonies from MacConkey's agar and 5% sheep blood agar plates were picked up and subcultures on brilliant green agar and XLD agar plates to observe the characteristic of *Klebsiella pneumoniae* **(Quinn *et al*., 1994)**. The pure colonies were picked up on nutrient agar slants as pure culture and subjected to biochemical tests as in table (2) **(Quinn *et al*., 1994)**.

III-Antimicrobial susceptibility:

Once they had been isolated and identified, pure isolates of *Klebsiella pneumoniae* were tested for their antibacterial susceptibility against of 12 antibiotics with the disc diffusion assay on Mueller-Hinton agar. Testing was performed according to the recommendation of the Clinical and Laboratory Standards Institute. Antimicrobials were selected for testing based on the licensing for mastitis treatment in the cow, and potential resistant-determinant phenotypes **(FAO/WHO/OIE, 2008 and WHO, 2011)**.

IV-PCR assay:

It was performed in the biotechnology department at animal Health Research Institute.

DNA extraction: DNA extraction from the *Klebsiella pneumoniae* isolates (4) was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations . Briefly, 200 µl of the *Klebsiella pneumoniae* isolates suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate, then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit. The Oligonucleotide Primers were supplied from Metabion (Germany) are listed in table (1)

PCR amplification: Primers were utilized in a 25-µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix **(Takara, Japan)**, 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template**.** The reaction was performed in an Applied biosystem 2720 thermal cycler.

Analysis of the PCR Products: The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the uniplex PCR products were loaded in each gel slot. Gelpilot 100 bp and 100 bp plus DNA ladders (Qiagen, Germany, GmbH) and generally 100 bp DNA ladder (Fermentas, Sigma) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data were analyzed through computer software.

Table (1). Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primer sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
16S-23S *RNA*	F:ATTTGAAGAG GTTGCAAACGAT R:TTCACTCTGA AGTTTTCTTGTG TTC	130	94°C 5 min.	94°C 30 sec.	55°C 30 sec.	72°C 30 sec.	72°C 7 min.	**Turton *et al.* (2010)**
***Kfu* (iron)**	F:ATC CTC TGG TCG CTA ACT G R:CTG CAC TGG AAGAACTGTTCT	847	94°C 5min.	94°C 30sec	50°C 40sec	72°C 50sec	72°C 10min	**Ewers *et al.* (2007)**
MagA	F: GGTGCTCTTTA-CATCATTGC R: GCAATGGCCATT TGCGTTAG	1282	94°C 5min.	94°C 30sec.	50°C 40sec	72°C 1.2 min.	72°C 12 min	**Yeh *et al.* (2007)**
RmpA	F:ACTGGGCTAC CTCTGCTTCA R:CTTGCATGAG CCATCTTTCA	535	94°C 5min.	94°C 30sec.	50°C 40sec.	72°C 40sec.	72°C 10 min	
Bla_{FOX}	F:AACATGGGGT ATCAGGGAGAT G R:CAAAGCGCGT AACCGGATTGG	190	94°C 5min	94°C 30 sec.	55°C 30sec.	72°C 30 sec.	72°C 7 min.	**Pérez-Pérez and Hanson (2002)**
Bla_{CTX}	F:ATGTGCAGYA CCAGTAARGTK ATGGC R:TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593	94°C 5 min.	94°C 30 sec.	54°C 40 sec.	72°C 40 sec.	72°C 10 min.	**Archambault *et al.* (2006)**
Bla_{TEM}	F: ATCAG-CAATAAACCAG C R:CCCCGAAGAA CGTTTTC	516	94°C 5min.	94°C 30 sec.	54°C 40 sec.	72°C 40 sec.	72°C 10min.	**Colom *et al.* (2003)**

V-Hematological studies: Red blood corpuscles (RBCs10^6/µl), hemoglobin concentration (Hb, conc.), packed cell volume (PCV%), Total leucocytic count (WBCs 10^3 /µl) and differential leucocytic count were estimated according to **Jain (2000).**

VI-Biochemical serum analysis: ALT, AST, Ca, Cl, K, Mg, Na, P, TP, and albumin were estimated by using fully automated biochemical analyzer (Hitachi 912) in hospital Suez Canal University.

VI-Immunological studies:

a) Protein electrophoresis were done by using SDS- Polyacrylamide gel electrophoresis according to **Laemmli (1970)** in the Animal Health Research Institute in the biochemistry department.

b) Determination of interleukin-6: The level of IL-6 was detected using ELISA kits according to **Nishimoto *et al.* (2008).**

c) Determination of Tumor Necrotic Factor: The test was drawn according to **Dowlati *et al.* (2010)**.

VII-Statistical analysis: The data were statistically analyzed of clinicopathological results according to **Snedecor & Cochran (1982).**

Results

Out of the (150) mastitic cows milk, twenty isolates were typically identified as *Klebsiella pneumoniae* with prevalence ratio (13.3%) according to cultural, morphological and biochemical characters as in (Table, 2).

Table (2). Specific Identification of *Klebsiella pneumoniae* Isolates

Test	Characteristics
MacConkey Agar	(lactose fermeter) Pink colonies
Brillant green Agar	Yellow green acid colonies
XLD Agar	Yellow(acid)colonies
Indole test	-ve
Methyl Red test	-ve
Voges Proskeur test	+ve
Citrate utilization test	+ve
Urease test	+ve
Triple Sugar Iron (TSI) agar	Yellow/Yellow/H_2S
String test for mucoviscosity	+ve
Gelatine liquefaction test	-ve

+ ve: positive reaction -ve: negative reaction

The results of antimicrobial susceptibility were recorded in Table (3), revealed that all strains of *Klebsiella pneumoniae* were susceptible to Amikacin, Gentamicin, and Cephotaxime (100%). Most strains were susceptible to Neomycin (80 %); Oxytetracycline (70%) and Sulphamethoxazole/Trimethoprim (60%). On the other hand, most *Klebsiella pneumoniae* were resistant to Ampicillin (100%), Chloramphenicol, Colistin sulphate, Erythromycin and Streptomycin and Penicillin G (70%-80%).

Table (3). Antimicrobial susceptibility patterns of *Klebsiella pneumoniae* isolates (n=20)

Antimicrobial agents	Susceptibility					
	Susceptible		Intermediate		Resistance	
	No.	%*	No.	%*	No.	%*
Neomycin (30 µg)	16	80	4	20	0	0
Amikacin (10mg)	20	100	0	0	0	0
Gentamycin (10 µg)	20	100	0	0	0	0
Ampicillin (10 µg)	0	0	0	0	20	100
Cephotaxime (30 µg)	20	100	0	0	0	0
Chloramphenicol (30 µg)	0	0	4	20	16	80
Erythromycin (15 µg)	0	0	4	20	16	80
Streptomycin (10 µg)	0	0	4	20	16	80
Oxytetracycline (30 µg)	14	70	6	30	0	0
Colistin Sulphate (10 µg)	0	0	4	20	16	80
Sulphamethoxazole / Trimethoprim (1.25 µg+23.75 µg)	12	60	4	20	4	20
Penicillin G (10 IU)	0	0	6	30	14	70

*Percentage were calculated according to the number of *Klebsiella pneumoniae* isolated tested

The results of PCR assay observed in Fig. (1); Showed that, the positive amplification of the 130 bp fragment of primer specific for the 16S rRNA gene in all of the examined *Klebsiella pneumoniae* (4/4; 100%). The isolated *Klebsiella pneumoniae* strains revealed that *rmpA* (0%) (Fig. 2), *magA* (0%) genes (Fig.3) and *kfu* (2/4; 50%) (Fig. 4), and. Bla_{TEM} gene was positive in all four isolates (fig.5) whereas bla_{CTX} (fig.7) and bla_{FOX} (fig.6) were negative in all four isolates.

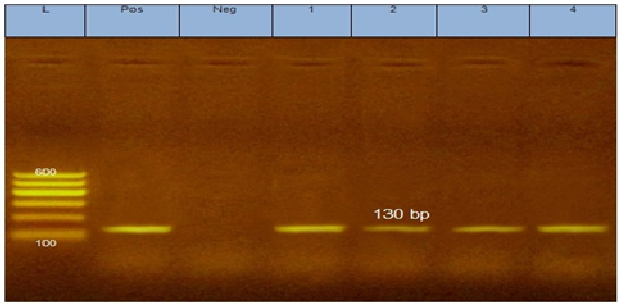

Figure (1): Agarose gel electrophoresis showing positive amplification of the product 130 bp fragment of 16SrRNA gene of *Klebsiella pneumoniae* performed with specific primer. L: 100–600 bp DNA ladder; Pos: positive control of *K. pneumoniae*; lanes1-4: *K. pneumoniae* from cow milk; Neg: negative control of *E. coli.*

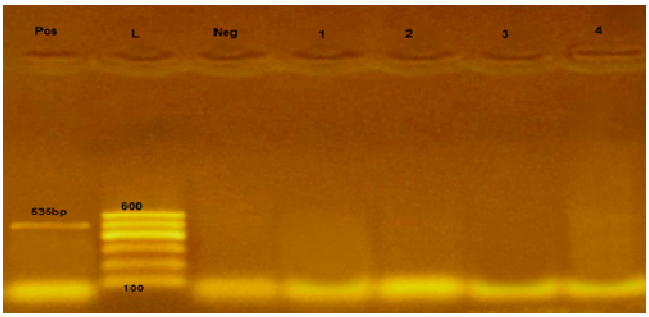

Fig. (2): Results of the m-PCR amplification of products *rmpA*, 535 bp .Lane L: 100–1500 bp DNA ladder; Pos: positive control of *K. pneumoniae*; lanes 1-4: –ve; Neg: negative control of *E. coli*.

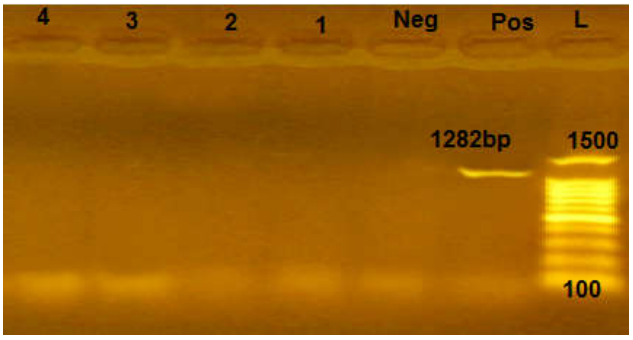

Fig. (3): Results of the m-PCR amplification of products *magA*, 1282 bp .Lane L: 100–1500 bp DNA ladder; Pos: positive control of *K. pneumoniae*; lanes 1-4–ve; Neg: negative control of *E. coli*

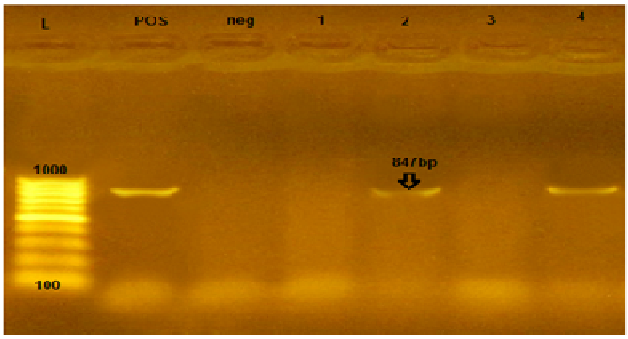

Fig. (4): Results of the m-PCR amplification of products *kfu*, 847 bp .Lane L: 100–1000 bp DNA ladder; Pos: positive control of *K. pneumoniae*; lanes 1-4: 2 and 4 +ve result of *K. pneumoniae* from cow milk; lane1, and 3 –ve; Neg: negative control of *E. coli.*

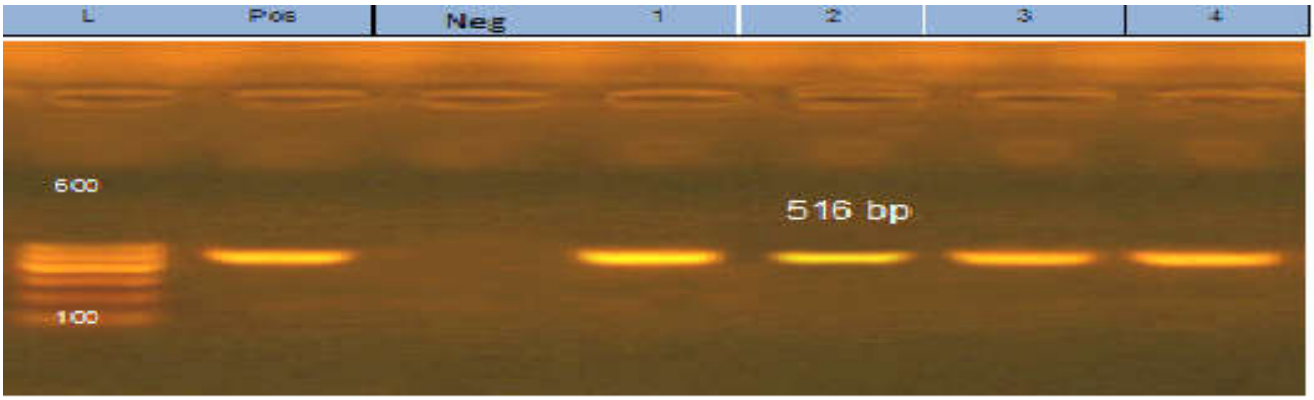

Fig. (5): Agarose gel electrophoresis showing positive amplification of the product 516 bp fragment of bla_{TEM} gene of *Klebsiella pneumoniae* performed with specific primer. L: 100–600 bp DNA ladder; Pos: positive control of *K. pneumoniae*; lanes1-4: *K. pneumoniae* from cow milk; Neg: negative control of *E. coli*

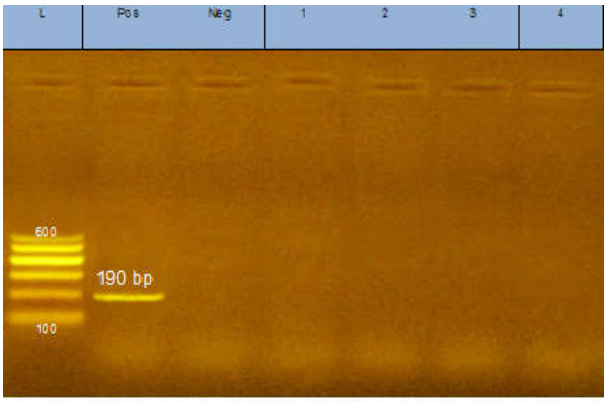

Fig. (6): Results of the m-PCR amplification of products bla$_{FOX}$, 190 bp .Lane L: 100–600 bp DNA ladder; Pos: positive control of *K. pneumoniae*; lanes 1-4:–ve; Neg: negative control of *E. coli*.

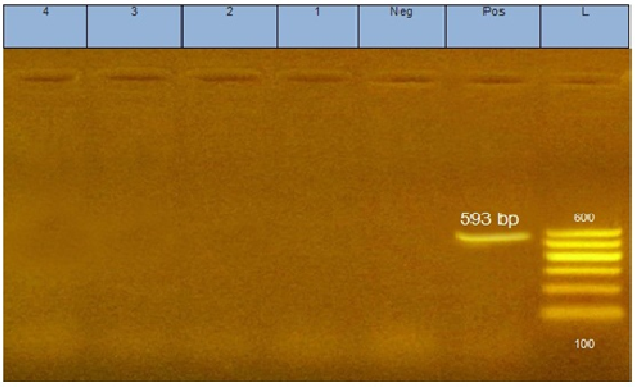

Fig. (7): Results of the m-PCR amplification of products bla$_{CTX}$, 593bp .Lane L: 100–600 bp DNA ladder; Pos: positive control of *K. pneumoniae*; lanes 1-4:–ve; Neg: negative control of *E. coli*

Hematological studies revealed lower average values of RBCs, Hb conc. and PCV. Also, leucocytosis, neutrophilia and lymphopenia significantly (P< 0.05) were observed in a *klebsiella pneumoniae* mastitic cows as compared with healthy animals (Table, 4).

Table (4). Hematological parameters of apparently healthy and *Klebsiella pneumoniae* mastitic cows.

Groups / Parameter	Apparently healthy (n=20)	Cows mastitis (n=20)
RBCs x10^6/µl	8.3±0.3	6.51±0.2**
Hb g/dl	11.41±0.34	8.76 ±0.27**
PCV %	33.2±0.2	29.2±0.3**
WBCs x10^3/µL	8.94 ±0.11	11.66±0.26**
Neutrophile (%)	38.79±0.5	52.62±0.4**
Lymphocyte (%)	54.26 ± 0.32	41.06±0.24**
Monocyte (%)	5.65±0.4	5.12±0.3
Eosinophile (%)	1.30± 0.11	1.20±0.2

*Significant increase (p≤0.05) ** highly significant (p≤0.05)

Table (5), Biochemical estimation revealed a significant increase in AST, Ca, Cl, Na, and K in positive *klebsiella pneumoniae* mastitic cows as compared with healthy cases. However, significant decreased in P in positive *klebsiella pneumoniae* mastitic cows as well as no significant ($P \leq 0.05$) changes were observed in ALT and Mg levels.

Table (5). Biochemical parameters of apparently healthy and *klebsiella pneumoniae* mastitic cows**.**

Parameter \ Groups	Apparently healthy (n=20)	Cows mastitis (n=20)
ALT U/L	11.23±0.16	10.95±0.15
AST U/L	80.3±1.14	95.3±1.4*
Ca mg/dl	8.4±0.5	11.35±0.6**
P mg/dl	7.33±0.2	5.65±0.25*
Mg mg/dl	3.42±0.22	3.25±0.13
Cl mEq/l	85.20±1.4	95.04± 1**
Na mEq/l	145.68±0.6	162.20±0.7**
K mEq/l	3.30±0.05	4.11±0.06*

*Significant increase (p≤0.05) ** highly significant (p≤0.05)

In table (6), showed that, a significant decrease in total protein (TP), and albumin level as well as a significant increase in globulin, alpha globulin and gamma globulin in the positive *klebsiella pneumoniae* mastitic cows as compared to apparently healthy cows.

Table (6). Protienogram of apparently healthy and *klebsiella pneumoniae* mastitic cows.

Parameter \ Groups	Apparently healthy (n=20)	Cows mastitis (n=20)
TP g/dl	8.39±0.1	6.20±0.07*
Albumin (gm/dl)	4.62±0.20	1.30 ± 0.8**
Globulin (gm/dl)	3.77±0.55	4.90 ± 0.7**
α– globulin (gm /dl)	1.32 ± 0.58	1.89± 0.3*
β– globulin (gm /dl)	0.75 ± 1.04	0.81± 0.5
ϒ – globulin (gm /dl)	1.70 ± 0.95	2.20± 0.6*

*Significant increase (p≤0.05) ** highly significant (p≤0.05)

In Table (7), revealed a significant increase in interleukin-6 (IL-6) and tumor necrotic factor (TNF-α) in mastitic cows as compared to control group.

Table (7). The levels of interleukin-6 (IL-6) and tumor necrotic factor-α (TNF-α) production in serum of apparently healthy and *klebsiella pneumoniae* mastitic cows.

Parameter \ Groups	Apparently healthy (n=20)	Cows mastitis (n=20)
IL-6 (pg/ml)	130.68 ± 1.30	148.2 ± 1.20*
TNF-α (pg/ml)	53.32 ± 0.40	70.3 ± 0.78**

*significant increase (p≤0.05) ** highly significant (p≤0.05)

Discussion

Bovine mastitis is one of a major economic disease on the dairy industry throughout the world **(Bachaya *et al*., 2011).** Clinical mastitis during early lactation, markedly and negatively influences the reproductive performance of dairy cows **(Ahmadzadeh *et al*., 2009 and Yang *et al*., 2012)**. It is characterized by the changes in the content of milk, the bacteriological status of milk and pathological changes in glandular tissues **(Ranjan *et al*., 2010).**

The existence of an oro-fecal transmission cycle has been suggested for *Klebsiella pneumoniae* in dairy herds, with fecal shedding resulting in the contamination of feed and water and the subsequent re-ingestion of the organism, resulting in renewed fecal shedding **(Munoz *et al.,* 2006 and Zadoks *et al.,* 2011).** Fecal shedding of *Klebsiella spp.*, contributes to pathogen loads in the environment, including in the milking machines, recycled manure, bedding material, alleyways, and holding pens **(Sampimon *et al.,* 2006; Verbist *et al.,* 2011 and Zadoks *et al.,* 2011)**. Studies have illustrated a correlation between bovine teat colonization by *Klebsiella* and the use of saw dust bedding **(Zdanowicz *et al.,* 2004).**

In the present study, the result of bacteriological examination revealed that the prevalence of *Klebsiella pneumoniae* from the mastitic cows was 13.3% (20/150). This result similarly to **(Osman *et al.,* 2014**). A low prevalence of *Klebsiella spp*., had also been reported by **(Yimer and Asseged, 2007)** at the rate of 1.3% and **Aher *et al.* (2012)** revealed prevalence of *klebsiella pneumoniae* at a rate of 5.4%.

The prolonged use of antibiotics in the treatment of mastitis lead to the problem of antibiotic resistant strains. *Klebsiella's* economic impact may be more as many cows die or end up being culled **(Schukken *et al.,* 2012).** *Klebsiella* is usually prone to cause severe clinical mastitis, and respond poorly to treatment and as a result of infections tend to be severe and long lasting with a fatal outcome **(Erskine *et al.,* 2002; Munoz and Zadoks 2007a; Paulin-Curlee *et al*., 2008 and Lucheis, 2012).**

The antimicrobial susceptibility of udder pathogens differs between studies. The disc diffusion technique is the most methods for determining the susceptibility of animal pathogens. Therefore, any comparison with studies that use other methods of susceptibility testing is not acceptable **(Schwarz *et al.,* 2010)**. Antibiotic-resistant *Klebsiella* on fresh vegetables and in sawdust pose a potential health problem with respect to bovine mastitis (**Talbot *et al.,*. 1980).** The documented over use of antibiotics in both agriculture and medical practice lead to antibiotic resistance *Klebsiella* **(WHO, 2011 and FDA, 2012).** A possible explanation for the resistance phenomenon could be from the most widely used for treatment of Ampicillin, Chloramphennicol, Colistin sulphate, Erythromycin, Streptomycin, and Penicillin G for many years. The environment can contain a large variety of *Klebsiella pneumoniae* strains with pathogenic potential **(Struve and Krogfelt, 2004; Munoz *et al*., 2006; Munoz and Zadoks 2007a; Munoz *et al*., 2007b and Paulin-Curlee *et al*., 2008)** and mastitis of dairy animals are resulted from many different strains.

The 16S rRNA gene represents a highly accurate method for the identification of bacteria to the species level, even when the species are difficult to identify by biochemical methods **(Song *et al.*, 2003)**. Four isolates were subjected to the molecular identification through PCR amplification of 16S rRNA using K 16S-F and K 16S-R primers which represents specific primers for the PCR amplification of *Klebsiella pneumoniae* 16S rRNA. Results showed that the PCR amplified fragments were about 130 bp in size as shown in figure (1), which the same size is obtained by **Turton *et al.* (2010)** when they used the same primer.

In the present study, *kfu* genes were detected by PCR using specific primer sequences which yielded product sizes of 847 bp, respectively. Out of total 4 isolates, 2 isolates (50%) were positive for *kfu* gene (Figure 3). The results of the present study are partially in agreement with reports of **(Ma *et al.,* 2005 and Yu *et al.,* 2006)** who showed that the prevalence of viru-

lence associated genes viz., *kfu* genes were found at the rate of 25%. **Yu *et al.* (2006)** who detected prevalence of virulence associated genes viz., *kfu* genes at the rate of 35%. **Liu *et al.* (2008)** refered to the importance of *fur* gene (ferric uptake regulator) because of it is role in regulation of cps synthesis genes (*rcsA*, *rmpA*, and *rmpA2*) by iron a *7T*cquisition, *7T*[*Fe(II)*] this gene effect on regulator *cps* synthesis genes*7T*, *7T* since these genes affected with presence of iron. In addition, *Klebsiella pneumoniae* produces several other extracellular virulence factors which affect the host cell metabolism **(Aher *et al*., 2012)**.

Among ESBL-producing (extended-spectrum beta-lactamase) microorganisms, *Klebsiella* is the genus that produces the greatest variety of such enzymes **(Paterson *et al.*, 2003).** Treatments with β-lactams in cows with intra mammary infection caused by ESBL-producing bacteria are not likely to succeed, leading to economic losses **(Erskine *et al.,* 2003)**. There is very little evidence supporting an increase in antimicrobial resistance due to mastitis treatments **(Erskine *et al.,* 2002)**.

Bla_{TEM} gene was positive in all four isolates where as bla_{CTX} and bla_{fox} were negative in all isolates. TEM-type ESBLs (mainly derived from TEM-1) are capable of hydrolyzing cephalosporins **(Hammad *et al.,* 2008; Locatelli *et al.,* 2009)**. The importance of TEM and SHV enzymes has been diminished due to the rapid growth of the third group of ESBLs, and CTX-M enzymes **(Woodford 2010). Gonzalez *et al. (*1994)** reported that the *klebsiella* strain had a comparatively lower level of resistance to extended spectrum cephalosporins and aztreonam. The FOX family of class C cephalosporinases is distinctly different in amino acid sequence when compared with other class C β-lactamases. All eight variants of the FOX family of β-lactamases that have been described thus far show substrate specificity for cephalosporins, including cephamycins **(Jacoby, 2009 and Miró *et al.,* 2013)**. Significantly, the presence of FOX β-lactamases can complicate the laboratory detection of extended-spectrum β-lactamases by yielding false -positive or false-negative results **(Robberts *et al.,* 2009).**

The hematological results revealed a significant ($P< 0.05$) decrease in red blood cell, hemoglobin concentration, and packed cell volume level in positive *Klebsiella pneumoniae* mastitic cows in comparison to healthy animals. These findings are in accordance with (**Zaki *et al.*, 2008; Zaki *et al.,* 2010; Krishnappa *et al.,* 2016 and Sarvesha *et al.,* 2017)** who reported that anemia in mastitic cows or buffalo, were due to decrease in RBCs, Hb conc. , and PCV levels. The results in contrast with the observation of (**Sischo *et al.,* 1997)** who reported that PCV and Hb conc. did not exhibit any specific trend in the animals suffering from mastitis.

Higher leukocyte and neutrophils count were recorded in the present study in *Klebsiella pneumoniae* mastitis affected cows. However, lymphocyte count was observed to be significantly lower in *Klebsiella pneumoniae* mastitic cows. TLC increased in *Klebsiella pneumoniae* cows of mastitis due to invasion by a pathogen inside the mammary gland. An increase in erythrocyte sedimentation rate and white blood cells in the blood of mastitic cows had been observed by (**Padhy *et al.,* 2014** and **Sarvesha *et al*., 2017)**. Increased TLC with an increase in absolute number of eosinophils and neutrophils in mastitis buffalo and cow were reported by (**Khan *et al.,* 1997; Krishnappa *et al.,* 2016 and Sarvesha *et al*., 2017).** These results similarly with (**Zaki *et al.,* 2008; Zaki *et al.,* 2010 and Alhussien *et al.,* 2015)** reported an increase in total leucocytic count in affected animals with mastitis along with a higher neutrophils and eosinophils count. However, these results were different to that obtained in Murrah buffaloes in their earlier studies **(Dang *et al.,* 2007).**

The highly significant increases detected in AST values of *Klebsiella pneumoniae* mastitic cow are in line with the reports of **Bayumi *et al.* (2005); Chandrasekaran *et al.* (2015); Krishnappa *et al.* (2016) and Sarvesha *et al.* (2017)** which could be due to stressful conditions.

Serum calcium level of the *Klebsiella pneumoniae* mastitic cow was significantly (P< 0.05) higher than the healthy animals which are attributed to the reduced milk production in affected animals which causes decreased Ca excretion in milk **(Wegner & Stull, 1978). Singh *et al.*, (2014) ; Krishnappa *et al.* (2016) and Sarvesha *et al.* (2017)** reported higher plasma levels of Ca in mastitis affected buffaloes and cows which is similar to our findings. But in contrast to the present study **Zaki *et al.* (2008) and Zaki *et al.* (2010)** observed a reduction in the Ca values in the infected animals.

There is significant decreased in phosphorous in *Klebsiella pneumoniae* mastitic cows that may be resulted from the injury in the udder wall of cows lead to high secretion of phosphorus in milk, resulting in decreasing phosphorus in milk which is in accordance with the observation of **Dwivedi *et al.*, (2004)**; **Siddiqe *et al.* (2015); Krishnappa *et al.* (2016) and Sarvesha *et al.* (2017)**.

On the other hand, there is no significant change in magnesium level was recorded in a *Klebsiella pneumoniae* mastitic cow. This result in agreement with the findings of **Dwivedi *et al.*, (2004); Krishnappa *et al.* (2016) and Sarvesha *et al.* (2017)**. In contrast, the significant increase in serum Mg of buffaloes suffering from acute mastitis was reported by **Singh (1999)** and decreased serum Mg levels were recorded by **Siddiqe *et al.* (2015).**

Sodium, chloride and Potassium levels of the *Klebsiella pneumoniae* mastitic cows were significantly (P< 0.05) higher than the healthy animals. Which was in line with the findings of **Atroshi *et al.* (1996); Krishnappa *et al.* (2016) and Sarvesha *et al.* (2017)**. This was attributed to the reduced milk yield during mastitis which elevates the level of sodium, potassium, and chloride in the blood due to minimal loss of the mastitis affected the animal. Increased levels of plasma K in buffaloes suffering from mastitis were also reported by **Singh (1999).**

Blood serum proteins play roles in the maintenance of colloid osmotic pressure, as a rapid substitute for indispensable amino acids, assuring glucose through gluconeogenesis, in the transport of minerals and hormones, in building of enzymes and in the immune system of the organism. Therefore, blood serum proteins have an exceptional significance in the homeostasis maintenance.

In the present study, there is a significant decrease in total protein (TP) level and albumin, which observed in *Klebsiella pneumoniae* mastitic cows as compared to healthy animals. This may be attributed to the decreased albumin levels **(Singh, 2000).** However, reduced TP values in *Klebsiella pneumoniae* mastitic cows were observed by (**Zaki *et al.*, 2008; Zaki *et al.*, 2010; Krishnappa *et al.*, 2016 and Sarvesha *et al.*, 2017).** Increased alpha globulin and gamma globulin in the blood of *Klebsiella pneumonia* mastitic cows indicates an activation of the immune response following infection of the mammary gland. These proteins are mainly immunoglobulins that are implicated in udder defense mechanisms **(Tsenkova *et al.*, 2001 and Azza and Ebtisam, 2013)**. Higher levels of globulin and total protein were reported by **Dwivedi *et al.* (2004)** in the serum of mastitic cows.

Interleukin-6 (IL-6) is a key cytokine that was originally from B-cell differentiation factor; it is acted as multifunctional as immune response, inflammation, bone metabolism and hematopoiesis **(Mi and Zeng, 2008).** IL-6 is control of the transport of serum iron and zinc so, involved in the regulation of serum iron and zinc levels. IL-6 induces hepcidin production, and blocks the action of the iron transporter ferroportin 1 on gut so leads to reduce serum iron levels **(Nemeth *et al.*, 2004).** This means that the IL-6-hepcidin axis is responsible for hypoferremia and anemia associated with chronic inflammation. IL-6 also enhances zinc importer ZIP14 expression and so induces hypozincemia seen in inflammation **(Liuzzi *et al.*, 2005).** When IL-6 reaches the bone marrow, it promotes megakaryocytic maturation, thus leading to the release of platelets **(Ishibashi *et al.*, 1989)**. However, in some pathological conditions, the continuous addi-

tion of IL-6 has been shown to increase antibody production, making significant increase to the inflammatory response and accelerating the progression of autoimmune diseases **(Wei *et al.*, 2013).**

In the present study, IL-6 and TNF-α were significant increases in *Klebsiella pneumoniae* mastitis cows as compared to control group. These results agreed with **Nakajima *et al.* (1997)** who reported that TNF-α levels were higher in the sera of mastitic animals. IL-6 was increased in the serum of clinical mastitic animals, but was low in animals that died and in healthy controls. Furthermore, the presence of IL-6 and TNF-α in the serum indicate severe clinical condition of coliform mastitis. **Slebodziński *et al.* (2002)** showed that the TNF-alpha was significant increased in infected milk, and IL-6 was unchanged. The decreased triiodothyronine (T3) content in mammary secretions during mastitis is associated with the severity of inflammation, and increased TNF-α .

In conclusion, The higher prevalence of *Klebsiella pneumoniae* organism in mastitis cow milk in percentage of 13.3% that's indicated *Klebsiella spp.*, infect mammary glands through environmental contact. Also, The identification of some virulence genes in *Klebsiella pneumoniae* strains causing mastitis will be helpful in the report of this infectious disease. The positive resistance of *klebsiella* to bla $_{TEM}$ that's indicated the more resistance of *Klebsiella pneumoniae* to the third generation of antibiotic. On the other hand, this organism leads to disturbance in the general health condition of cow by producing anemia, increased enzyme of liver, minerals as Ca, Na, K, Cl and interleukin-6 (IL-6) and tumor necrotic factor (TNF-α). Therefore, the control procedure for reducing *Klebsiella* infections are avoiding the use of sawdust and recycled manure, bedding and maintaining a clean and dry environment for bedding cows is of at most importance and vaccinating animal. Also, the periodic analysis of blood of cows.

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