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Antimicrobial Susceptibility Profile of *Klebsiella pneumoniae* Isolated as a Foodborne Pathogen from Some Dairy Products in Libya

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Abstract: *Klebsiella pneumoniae* is one of the most common causes of clinical and asymptomatic mastitis in dairy cattle, also in milk and dairy products affecting its quality. Mastitis caused by *K. pneumoniae* is even more serious due to its poor response to antibiotic therapy. This study was conducted to detect and identify the presence of *K. pneumoniae* in milk and dairy products. A total of 234 samples were randomly collected from various locations in Libya. Samples were examined for the presence of *K. pneumoniae* by conventional cultural techniques that included cultivation in violet red bile agar plus 4-methylumbelliferyl-ß-D-glucuronide (VRBA + MUG) broth, and CHROM agar followed by identification by PCR and partial sequencing of 16S rRNA. Out of the 234 samples of milk and dairy products collected, 16 (6.8%) of the isolates revealed mucoid colonies on agar media phenotypically suggested to be *K. pneumoniae*. The isolates identification was confirmed by molecular techniques (16S rRNA). Among examined samples, *K. pneumoniae* was recovered from she camel's milk, raw cow's milk, raw fermented milk, Maasora cheese, Ricotta cheese, soft cheese, full cream milk powder, milk powder infant formula, cereal baby food and growing up formula. From the 16 *K. pneumoniae* isolates; antibiotic susceptibility testing was performed on 12 isolates, the results showed that *K. pneumoniae* isolates were resistant to more than 8 antibiotics, interestingly, two isolates revealed MBL production. This study emphasized the relationship between *K. pneumoniae* and raw milk, cheese, milk powder and infant milk retailed in Libya, which is considered as a risk for human health as many of these products did not comply with microbiological criteria of international and/or Libyan standards. The necessary precautions have to be taken to carry out effective sanitary practices during the production in dairy plants, handling and distribution in the markets particularly at local small manufacture scale.

Keywords: *Klebsiella pneumoniae*; dairy products; foodborne; antibiotic sensitivity; Libya

1. Introduction

Klebsiella pneumoniae (*K. pneumoniae*) is a Gram-negative pathogen. It is an important member of the family Enterobacteriaceae that can infect humans through contaminated poultry, beef, fish and dairy products [1]. On agar medium, *K. pneumoniae* has a mucoid phenotype and is imparted by the polysaccharide capsule attached to the outer membrane of the cell, and lactose fermentation [2]. Due

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to its ability to sustain in different environments such as surfaces, human skin, respiratory and urinary tract, it has emerged as a clinically and epidemiologically important human pathogen. *K. pneumoniae* is easily transmitted between patients through surgery and has become one of the most common causes of outbreaks in intensive care units [3]. *K. pneumonia* is a common bacterial pathogen that accounts for a high proportion of nosocomial infections in pediatrics and can cause life-threatening invasive infections in young people [4].

Sources of *Klebsiella* species on dairy farms include organic bedding materials such as wood by-products and fecal excrement from cattle, which contains a wide variety of *K. pneumoniae* strains in their dairy products [5]. To avoid this problem, microbial agents are added to dairy cow feed in pastures to inhibit pathogenic bacteria [6].

Mastitis is the one of the most important diseases in dairy herds, despite the widespread use of management programs such as teat immersion, treatment of dry cows, and weaning of infected animals. Mastitis caused by *K. pneumoniae* can be particularly severe compared to mastitis caused by *E. coli*, due to its poor response to antibiotic therapy and its rapid progression to toxic shock and death [7]. Furthermore, this bacterium can cause severe clinical symptoms and major economic loses [8]. In earlier studies, *K. pneumoniae* ST1224 was isolated from neonates, while *K. pneumoniae* ST48 was reported from chicken meat in Western Algeria, one of the Middle East regions that was indicating these STs are not host specific and could be easily transmitted to humans from food animals and their products [9].

K. pneumoniae is an opportunistic pathogen for individuals with weakened immunity, because it can cause variety of infections, namely; pneumonia, urinary tract infection, bacteremia, and meningitis. In contrast to the "classical" *K. pneumoniae* (cKP), a new "hypervirulent" *K. pneumoniae* (hvKP) with hypermucoviscosity has emerged as a clinically important pathogen over the last two decades. It causes highly invasive infections such as liver abscesses in both healthy and immune compromised individuals [10]. Antibacterial resistance to some infectious pathogens has become a serious public health concern. Several studies have documented that food-producing animals are a possible source or reservoir for the spread of resistant bacterial strains or antibiotics resistant genes (ARGs) to humans [1].

Pseudomonas aeruginosa, Acinetobacter baumannii, and *K. pneumoniae* are well-known nosocomial pathogens; recently they have seen a worldwide surge in multi-drug (MDR) and pan-drug resistant counterparts on the WHO list of priority (serious) pathogens of antibiotic-resistance. Surveillance study reported that resistance of *K. pneumoniae* to 3rd generation cephalosporins peaked at (15–20%), and ciprofloxacin resistance was in the range (10–50%) [11].

Interestingly, *K. pneumoniae* population contain extraordinary numerous genes, comprising of unbiased phylogenetic lineages or 'clones' that fluctuate from each other. The majority of MDR sanatorium outbreaks are due to a small subset of *K. pneumoniae* clones with excessive incidence of received antimicrobial resistance (AMR) genes, at the same time as the bulk of community-received invasive infections are due to 'hypervirulent' clones that not often harbor received AMR genes, however, have excessive incidence of key virulence loci [10]. The empirical use of antibiotics and persistent exposure to a wide range of antimicrobials have led to the increased prevalence of MDR *K. pneumoniae* [4].

Resistance to most antimicrobial classes and the lack of new antimicrobial agents against Gram-negative bacteria potentiates the re-use of old antibiotics, particularly polymyxins such as colistin. Colistin is currently known as the last-resort antimicrobial agent for the treatment of infections caused by MDR Gram-negative bacteria. It binds to the negatively charged lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria, leading to disruption of the membrane, induces the cytoplasmic material to fade away, and eventually cell death.

The indiscriminate use of antimicrobials has led to the development of multidrug-resistant (MDR) Gram-negative bacteria in raw milk, in particular *E. coli* O157:H7, *K. pneumoniae*, Aeromonas hydrophilia and Proteus mirabilis [9].

Over the past few years, Gram-negative bacteria have shown a significant increase in resistance against β-lactam antibiotics due to various plasmid-mediated extended spectrum β-lactamases

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(ESBL) genes found in Enterobacteriaceae especially in *E. coli* and *K. pneumoniae*. The emergence of resistant *K. pneumoniae* due to misuse of antibiotics in ranches and veterinary facilities is a serious public and livestock health problem. Ultimately, it can transmit to human through various environmental niches [1]. Several recent investigations reported the emergence of MDR bacterial pathogens from different origins, including humans, birds, cattle, and fish that necessitates the need for new potent and safe antimicrobial agents [12].

ESBL is an enzyme that hydrolyzes various β-lactam antibiotics and can confer resistance to penicillin's, 3rd and 4th generation cephalosporins. The genes encoding for these enzymes are common on both chromosomes and plasmids of species belonging to Enterobacteriaceae family. As a result, ESBLs are emerged among Enterobacteriaceae, especially *E. coli* and *Klebsiella* spp. Various groups of antibiotics are used in livestock management at both therapeutic and sub-therapeutic levels. β-lactam antibiotics are widely used in veterinary medicine due to their high specificity, complete selective toxicity and strong killing effect. Therefore, abuse of these antibiotics in veterinary medicine increased the emergence and spread of genetic determinants, especially in *E. coli* and *K. pneumoniae* spp. [13].

Interestingly, ESBL-producing *K. pneumoniae* was isolated from food handlers who consumed unpasteurized milk and raw meat [14]. Recently, *K. pneumoniae* was detected in human samples, which in turn emphasizes again the concept of transferring between animal/animal products including milk from/to human and animal during the last decade. Alternatively, MDR *K. pneumoniae* isolates were widely detected in milk samples [9]. Moreover, *K. pneumoniae* is considered to be a major transporter of resistance genes from environmental sources to clinically important bacteria and some isolates can carry acquired AMR genes or plasmids to move between environments, humans, and animals [12].

Previously, *E. coli*, *Cronobacter* spp and *Salmonella* enterica have been reported as foodborne pathogens associated with dairy products in Libya [15–17], Data on microbiological quality of Libyan dairy products related to *K. pneumoniae* are lacking. Previously, many information has been only recoded on the mastitic cases, although this is not a common food poisoning cause, it was known to be a causative agent of mastitis. Poor hygienic practices during manufacture of dairy products were occurred especially in locally made milk products as a result of lacking standard guidelines during manufacturing. All the aforementioned factors are considered the greatest public health risk in Libya. Therefore, this study was designed to investigate the presence of *K. pneumoniae* in milk and dairy products sold in Libya and to determine its antibiotics sensitivity profile.

2. Materials and Methods

2.1. Collection of samples

A total of 234 samples of milk and dairy products (Table 1) were randomly collected from various locations in Libya.

2.2. Isolation and identification of K. pneumoniae

Isolation of *K. pneumoniae* was performed according to the reference method described by Davis et al. [18] for detection of *K. pneumoniae* in dairy products. Briefly, 25 g/mL from each sample was aseptically transferred into sterile polyethylene stomacher bag and blended with 225 mL of buffer peptone water (Park Scientific, M 0063, Northampton Limited, UK), homogenized in a stomacher (Stomacher 400, Seaward Medicals, UK) at 230 rpm for 1 min, then incubated at 37°C for 18 ± 2 hrs. Afterward, 10 mL from above incubated samples were added to enrichment broth [violet red bile agar plus 4-methylumbelliferyl-ß-D-glucuronide (VRBA + MUG)] and incubated overnight at 44°C. Only 0.1 mL of the selective enriched broth was streaked onto (CHROM agar, Hardy Diagnostics, Santa Maria, California); the inoculated plates were then incubated at 37°C for 24 hrs. The presumptive colonies were selected and kept for further investigation [18].

2.3. Identification of K. pneumoniae by PCR and partial sequencing of 16S rDNA

Suspected colonies cultivated on CHROM agar were selected and purified several times. The procedure for extracting DNA from *K. pneumoniae* isolates was performed as previously described by Azwai et al. [19]. Partial 16S rDNA was amplified using universal oligonucleotides primers; Forward: S-D Bact-0341-b-S-17 and Reverse: S-D-Bact-0785-a- A-21. The amplified 16S rDNA PCR fragment (464 bp) was excised from the gel and the DNA was extracted from the gel using GF-1 AmbiClean kit (Cat. # GF-GC-100, Vivantis, Malaysia) as reported by Azwai et al. [19]. The purified 16S rDNA amplicons were then sequenced in IZSLER-Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy. BLAST search was performed on consensus reached by both NCBI (http://www.ncbi.nlm.nih.gov/ pubmed) and 16S bacterial cultures Blast Server for the identification of prokaryotes (http://bioinfo.unice.fr/blast/).

2.4. Antimicrobial susceptibility profile

2.4.1. Selected Antibiotics Discs

K. pneumoniae isolates confirmed by PCR were subsequently tested against a variety of 32 antibiotics using disc-diffusion method described in the Clinical and Laboratory Standards Institute (CLSI) guidelines as described by Davis et al. [18]. Antibiotics used included Gentamycin (Bioanalyse 10µg), Streptomycin (Oxoid 10µg), Amoxicillin (Oxoid 10µg), Bacitracin (Oxoid 10µg), Oxytetracyclin (Oxoid 30µg), Doxycycline (Oxoid 30µg), , Penicillin G (Oxoid 10µg), Erythromycin (Oxoid 15µg), Amoxicillin/ Clavulanic Acid (Oxoid 30µg), Ampicillin (Oxoid 10µg), Methicillin (Bioanalyse 5µg), Kanamycin (Oxoid 30µg), Lincomycin (Oxoid 10µg), Tobramycin (Oxoid 10µg), Levofloxacin (Oxoid 5µg), Clindamycin (Oxoid 2µg), Cefotaxime (Oxoid 30µg), Ciprofloxacin (Oxoid 5µg), Cloxacillin (Oxoid 5µg), Nitrofurantoin (Oxoid 300µg), Tetracycline (Oxoid 30µg), Chloramphenicol (Oxoid 30µg), Sulphamethoxazole/Trimethoprim (Oxoid 25µg), Pipercillin/Tazobactam (100/10µg), Cephoxitin (30µg), Cefoperazone/ Sulbactam (75/30µg), Cefotaxime (30µg), Ceftriaxone (30µg), Ceftazidime (30µg), Aztreonam (30µg), Imipenem (10µg), Ertapenem (10µg), and Amikacin (30µg).

2.4.2. Antibiogram Assay

A single colony of the tested organism was picked up by a disposable loop, emulsified into 5 ml of Brain Heart Infusion Broth (Liofilchem, Italy), and incubated overnight at 37°C to reach 0.5 McFarland's standard turbidity. The suspension was spread on the surface of Mueller-Hinton Agar (Oxoid, England) with a cotton swab, a suitable test antibiotic discs were placed on the agar surface and the plates were incubated at 37 °C for 24 hrs. The diameter of inhibition zone was measured in millimeters and recorded.

3. Results

The current study results showed that out of 234 samples of milk and dairy products, only 16 (6.4%) of the isolates revealed mucoid colonies on agar media that phenotypically suggested to be *K. pneumoniae* (Table 1).

Table 1. Total number of samples produced *Klebsiella pneumoniae* on agar media.

Type of Samples	Total Number of Samples	Number of Positive Samples
Raw cow's milk	46	3 (6.5%)
Raw she camel's milk	5	1(20%)
Raw fermented milk	28	1(3.5%)
UHT milk	8	0
Yoghurt	5	0
Maasora cheese	21	4(19%)

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Ricotta cheese	13	1(7.6%)
Imported soft cheese	6	1 (16.6%)
Ice cream	6	0
Full cream milk powder	10	2(20 %)
Skimmed milk powder	6	0
Powder infant formula	36	1 (2.7 %)
Growing up formula	18	1 (5.5 %)
Ready to feed baby milk	10	0
Cereal baby food	16	1(6.25%)
Total	**234**	**16 (6.8%)**

The isolates identification was confirmed by molecular techniques (16S rRNA) which was identified that 16 (6.4%) mucoid isolates as *K. pneumoniae* Table (2). Among examined samples, *K. pneumoniae* was recovered from the she camel's milk, raw cow's milk and fermented milk as 1 (20%), 3 (6.5%) and 1(3.5%) respectively, while no *K. pneumoniae* was detected in UHT milk samples. In cheese samples, *K. pneumoniae* was detected in Maasora cheese, Ricotta cheese and imported soft cheese as 4 (19%) and 1 (7.6%) and 1(16.6%) respectively.

Table 2. Bacterial identification of 12 isolates confirmed to be *K. pneumoniae* by using the PCR-16S rDNA technique.

No.	Blast NCBI 16S rDNA	Nucleotide Identity (%)	Type of samples
1	*K. pneumoniae*	99	Raw cow's milk
2	*K. pneumoniae*	99	Raw cow's milk
3	*K. pneumoniae*	99	Raw cow's milk
4	*K. pneumoniae*	99	She-camel's milk
5	*K. pneumoniae*	98	Raw fermented milk
6	*K. pneumoniae*	99	Ricotta cheese
7	*K. pneumoniae*	99	Maasora cheese
8	*K. pneumoniae*	99	Maasora cheese
9	*K. pneumoniae*	99	Maasora cheese
10	*K. pneumoniae*	100	Maasora cheese
11	*K. pneumoniae*	99	Imported soft cheese
12	*K. pneumoniae*	100	Full cream milk powder
13	*K. pneumoniae*	99	Full cream milk powder
14	*K. pneumoniae*	100	Infant formula
15	*K. pneumoniae*	99	Fortified milk powder
16	*K. pneumoniae*	99	Cereal baby food

Although, *K. pneumoniae* was not recovered from skimmed milk powder, whereas it was isolated from full cream milk powder 2 (20 %). In baby food samples, *K. pneumoniae* was detected in powder infant formula, cereal baby food and growing up formula (fortified milk powder) as 1 (2.7%), 1(6.25%) and 1 (5.5%) respectively. In contrast, no *K. pneumoniae* was observed in ready to feed baby milk and cereal baby food samples. In the investigated samples, ice cream and yoghurt, no *K. pneumoniae* was detected.

Furthermore, no zone of inhibition was observed against the Amoxicillin, Ampicillin, Bacitracin, Penicillin, Lincomycin, Clindamycin and Cloxacillin. In contrast, these isolates were sensitive to

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Levofloxacin, Ciprofloxacine, Amikacin, Gentamycin, Ertapenem and Chloramphenicol. All isolates were resistant to Erythromycin except one isolate was intermediate. As well as, some of these isolates exhibited different degree of susceptibilities to some antibiotics such as, Kanamycin, Tobramycin, Nitrofurantoin, Streptomycin, Tetracycline, Oxytetracycline, Pipercillin Cefoprazone/ Sulbactin, Cefriaxone, Cephoxitin and Sulfamethoxazole/ Trimethoprim, Ceftazidime and Cefotaxime. Furthermore, only one isolate was sensitive to Methicillin while the remaining isolates were resistant. Additionally, all isolates were sensitive to Aztreonam except one isolate appeared intermediate. Moreover, all isolates were sensitive to Doxycycline and Imipenem except two isolates were resistant. In addition, all isolates were sensitive to Amoxicillin/ Clavulanic acid, except three isolates were intermediate (Tables 3 and 4).

Tables (5 and 6) showed ARI index and MDR of all isolates against 32 antibiotics that were used in this study.

Table 3. Antibiogram of *Klebsiella pneumoniae* isolates.

No.	No. of Isolates	1	2	3	4	5	6	7	8	9	10	11	12
1	Amoxycillin (10µg)	R	R	R	R	R	R	R	R	R	R	R	R
2	Ampicillin (10µg)	R	R	R	R	R	R	R	R	R	R	R	R
3	Bacitracin (10µg)	R	R	R	R	R	R	R	R	R	R	R	R
4	Penicillin G (10µg)	R	R	R	R	R	R	R	R	R	R	R	R
5	Methicillin (5µg)	R	R	R	R	R	S	R	R	R	R	R	R
6	Erythromycin (15µg)	R	R	R	I	R	R	R	R	R	R	R	R
7	Kanamycin (30µg)	I	S	I	R	R	S	I	I	S	S	I	I
8	Lincomycin (10µg)	R	R	R	R	R	R	R	R	R	R	R	R
9	Tobramycin (10µg)	I	I	R	I	R	S	I	R	S	I	R	I
10	Levofloxacin (5µg)	S	S	S	S	S	S	S	S	S	S	S	S
11	Clindamycin (2µg)	R	R	R	R	R	R	R	R	R	R	R	R
12	Doxycycline (30µg)	S	R	S	S	S	S	S	S	S	R	S	S
13	Cloxacillin (5µg)	R	R	R	R	R	R	R	R	R	R	R	R
14	Nitrofurantoin (300µg)	S	S	S	S	I	S	R	I	I	I	I	R
15	Oxytetracyclin (30µg)	I	R	R	I	I	I	R	R	I	R	R	I
16	Streptomycin (10µg)	I	I	S	S	R	S	I	I	S	R	I	R
17	Tetracycline (30µg)	S	S	S	I	S	S	S	S	S	R	S	I
18	Chloramphenicol (30µg)	S	S	S	S	S	S	S	S	S	S	S	S
19	Sulphamethoxazole/ Trimethoprim (25µg)	S	I	R	S	S	S	S	S	S	R	S	S
20	Amoxycillin/ Clavulanic acid (30µg)	S	I	S	I	S	S	S	S	S	I	S	S
21	Pipercillin/Tazlactm (100/10µg)	S	I	I	S	S	S	I	S	S	S	I	I
22	Cephoxitin (30µg)	I	R	I	I	S	I	R	I	S	R	I	R
23	Cefoperazone/ Sulbactin (75/30µg)	S	I	I	S	S	S	I	S	S	I	I	S
24	Cefotaxime (30µg)	S	R	R	I	S	S	I	S	I	R	S	I
25	Ceftriaxone (30µg)	S	I	I	S	S	S	I	S	S	S	S	I
26	Ceftazidim (30µg)	R	R	R	R	I	R	R	R	R	R	R	R
27	Aztreonam (30µg)	S	S	S	S	S	S	S	S	S	-	S	I
28	Imipenem (10µg)	S	R	R	S	S	S	S	S	S	S	S	S
29	Ertapenem (10µg)	S	S	S	S	S	S	S	S	S	S	S	S
30	Amikacin (30µg)	S	S	S	S	S	S	S	S	S	S	S	S

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31	Gentamicin (10µg)	S	S	S	S	S	S	S	S	S	S	S	S
32	Ciprofloxacin (5µg)	S	S	S	S	S	S	S	R	S	S	S	S
	IEH (10/750 µg)	-	MBL	MBL	-	-	-	-	-	-	-	-	-
Sensitivity %		51.5	27.2	36.3	45.4	51.5	63.6	36.3	45.4	57.5	30.3	42.4	33.3
Intermediate %		15.1	21.2	15.1	21.2	9	6	21.2	12.1	9	12.1	18.1	24.2
Resistance %		33.3	48.8	48.4	33.3	39.3	30.3	42.4	42.4	33.3	54.5	39.3	42.4

Table 4. Showed the resistance, intermediate and sensitivity percentage of *K. pneumonia* isolates.

No	Name of Antibiotic	Resistance No. (%)	Intermediate No. (%)	Sensitive No. (%)
1	Amoxycillin (10µg)	12 (100)	0 (0)	0 (0)
2	Ampicillin (10µg)	12 (100)	0 (0)	0 (0)
3	Bacitracin (10µg)	12 (100)	0 (0)	0 (0)
4	Penicillin G (10µg)	12 (100)	0 (0)	0 (0)
5	Methicillin (5µg)	11 (91.7)	0 (0)	1 (8.3)
6	Erythromycin (15µg)	11 (91.7)	1 (8.3)	0 (0)
7	Kanamycin (30µg)	2 (16.7)	6 (50)	4 (33.3)
8	Lincomycin (10µg)	12 (100)	0 (0)	0 (0)
9	Tobramycin (10µg)	4 (33.3)	6 (50)	2 (16.7)
10	Levofloxacin (5µg)	0 (0)	0 (0)	12 (100)
11	Clindamycin (2µg)	12 (100)	0 (0)	0 (0)
12	Doxycycline (30µg)	2 (16.7)	0 (0)	10 (83.3)
13	Cloxacillin (5µg)	12 (100)	0 (0)	0 (0)
14	Nitrofurantoin (300µg)	2 (16.6)	5 (41.7)	5 (41.7)
15	Oxytetracyclin (30µg)	6 (50)	6 (50)	0 (0)
16	Streptomycin (10µg)	3 (25)	5 (41.7)	4 (33.3)
17	Tetracycline (30µg)	1 (8.3)	2 (16.7)	9 (75)
18	Chloramphenicol (30µg)	0 (0)	0 (0)	12 (100)
19	Sulphamethoxazole/ Trimethoprim (25µg)	2 (16.7)	1 (8.3)	9 (75)
20	Amoxycillin/ Clavulanic acid (30µg)	0 (0)	3 (25)	9 (75)
21	Pipercillin/ Tazlactm (100/10µg)	0 (0)	5 (41.6)	7 (58.3)
22	Cephoxitin (30µg)	4 (33.3)	6 (50)	2 (16.7)
23	Cefoperazone+ Sulbactin (75/30µg)	0 (0)	5/ (41.7)	7 (58.3)
24	Cefotaxime (30µg)	3 (25)	4 (33.3)	5 (41.7)
25	Ceftriaxone (30µg)	0 (0)	4 (33.3)	8 (66.7)
26	Ceftazidim (30µg)	11 (91.7)	1 (8.3)	0 (0)
27	Aztreonam (30µg)	0 (0)	1 (9.1)	10 (90.9)
28	Imipenem (10µg)	2 (16.7)	0 (0)	10 (83.3)
29	Ertapenem (10µg)	0 (0)	0 (0)	12 (100)
30	Amikacin (30µg)	0 (0)	0 (0)	12 (100)
31	Gentamicin (10µg)	0 (0)	0 (0)	12 (100)
32	Ciprofloxacin (5µg)	1 (8.3)	0 (0)	11 (91.7)

Table 5. Showed ARI& MDR of *K. pneumonia* isolates.

No	Name of Antibiotic	Resistance isolates	ARI	MDR

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1	Amoxycillin (10µg)	12/100	0.03	36.3
2	Ampicillin (10µg)	12/100	0.03	36.3
3	Bacitracin (10µg)	12/100	0.03	36.3
4	Penicillin G (10µg)	12/100	0.03	36.3
5	Methicillin (5µg)	11/91.6	0.02	33.3
6	Erythromycin (15µg)	11/91.6	0.02	33.3
7	Kanamycin (30µg)	2/16.6	0.005	6
8	Lincomycin (10µg)	12/100	0.03	36.3
9	Tobramycin (10µg)	4/33.3	0.01	12.1
10	Levofloxacin (5µg)	0/0	0	0
11	Clindamycin (2µg)	12/100	0.03	36.3
12	Doxycycline (30µg)	2/16.6	0.005	6
13	Cloxacillin (5µg)	12/100	0.03	36.3
14	Nitrofurantoin (300µg)	2/16.6	0.005	6
15	Oxytetracyclin (30µg)	6/50	0.01	18.1
16	Streptomycin (10µg)	3/25	0.007	9
17	Tetracycline (30µg)	1/8.3	0.002	3
18	Chloramphenicol (30µg)	0/0	0	0
19	Sulphamethoxazole/ Trimethoprim (25µg)	2/16.6	0.005	6
20	Amoxycillin/ Clavulanic acid (30µg)	0/0	0	0
21	Pipercillin/ Tazlactm (100/10µg)	0/0	0	0
22	Cephoxitin (30µg)	4/33.3	0.01	12.1
23	Cefoperazone+ Sulbactin (75/30µg)	0/0	0	0
24	Cefotaxime (30µg)	3/25	0.007	9
25	Ceftriaxone (30µg)	0/0	0	0
26	Ceftazidim (30µg)	11/91.6	0.02	33.3
27	Aztreonam (30µg)	0/0	0	0
28	Imipenem (10µg)	2/16.6	0.005	6
29	Ertapenem (10µg)	0/0	0	0
30	Amikacin (30µg)	0/0	0	0
31	Gentamicin (10µg)	0/0	0	0
32	Ciprofloxacin (5µg)	1/8.3	0.002	3

MDR: Multi Drug Resistance; ARI: Antibiotic Resistance Index.

Table 6. Multi antibiotics resistant index (MARI) of *K. pneumoniae*.

No. of Isolates	No. of Resistant Antibiotics (Total)	MARI
1	11 (32)	0.33
2	16 (32)	0.48
3	16 (32)	0.48
4	11 (32)	0.33
5	13 (32)	0.39
6	10 (32)	0.3
7	14 (32)	0.42
8	14 (32)	0.42
9	11 (32)	0.33
10	18 (32)	0.54
11	13 (32)	0.39

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12	14 (32)	0.42

MARI: Multi Antibiotic Resistance Index.

4. Discussion

Klebsiella pneumoniae is commonly associated with infections in humans and animals, causing nosocomial infections worldwide with high morbidity and mortality. The gastrointestinal tract and hands of hospital medical staff are recognized as sources of contamination by *Klebsiella*. In a recent study, *K. pneumoniae* has been identified as a nosocomial pathogen responsible for a large-scale outbreak that occur throughout the hospital food supply chain [20]. Antimicrobial resistance is a new global healthcare crisis that has significant implications for human health and the economy. This crisis is exacerbated by the lack of new antibiotics, especially against Gram-negative pathogens, and the proliferation of high-risk MDR clones [21].

The results in Table (1) showed that *K. pneumoniae* has been recovered from three samples (6.5%) of raw cow's milk and this finding were slightly similar with that obtained by Tartor et al. [9] (7.2%) and Gaffer et al. [22] (8%). For instance the result was less than that reported by (Fu et al., 2022) (35.9%), Chaudhry et al. [1] (10.52%), Osman et al. [23] (9.6%), (Garedew et al. [24] (16. 7%) and Jayarao and Wang [25] (9.5%). Interestingly, Nobrega et al. [26] detected *K. pneumoniae* in most samples of milk from 25 lactating cows, indicating that raw milk may serve as a risk of contamination by pathogenic bacteria. Their results also emphasis the importance of quality and safety monitoring and biological control of raw milk. Moreover, *K. pneumoniae* was detected only in one sample (20%) of she-camel's milk. This finding was similar with that recorded by Njage et al. [27] (33%). Interestingly, to the best of our knowledge, this is the first report that describes the isolation and identification of *K. pneumoniae* from She camel's milk in Libya. Presences of *K. pneumoniae* in raw milk come from the environments of dairy herd or dairy herd mastitis might be due to the lack of hygiene in some dairy farms in Libya. According to Garedew et al. [24], the presence of *K. pneumoniae* in raw milk at various important critical points is associated with pre-milking udder preparation, milking treatment, milking and storage equipment. Among all important control points, it was suggested that poor hygiene practices during transportation of containers at milk collection points and processing plants are the most critical risk factors. On the other hand, *K. pneumoniae* was not detected in eight samples of UHT milk. This may be due to the heat treatment during the manufacturing.

As recorded in Table (1), *K. pneumoniae* was recovered from four samples (19%) of Maasora cheese, one sample (7.6%) of Ricotta cheese and one sample (16.6%) of imported soft cheese. These results were similar to somehow to that reported by Gaffer et al. [22] who found *K. pneumoniae* in Damietta cheese (12%) and Kariesh cheese (8%). While Massa et al. [28] found that 90% of mozzarella cheese samples were contaminated by *K. pneumoniae*. Remarkably, Massa et al. [28] indicated that *K. pneumoniae* is one of the most pathogens that cause spoilage of cheese. Additionally, in Libya most of handling workers at small milk parlor, locally manufactured dairy products as milk and cheese, do not follow the proper hygienic guidelines at preparation.

As shown in Table (3) *K. pneumoniae* was recovered from one sample of raw fermented milk (3.5%), these findings are not consistent with that obtained by Abushaala and Alwoshesh [29] (12.9%) and Njage et al. [27] (33%) who found *K. pneumoniae* in natural fermented milk. In contrast, no *K. pneumoniae* was found in yoghurt samples 5 (0%), this is may be due to that *K. pneumoniae* could not tolerate the high acidity in this product.

In this study, *K. pneumoniae* was isolated from one sample of powder infant formula (PIF) (2.7%), cereal baby food 1(6.25%) and growing up formula (fortified milk) 1 (5.5%), these results were nearly similar to those reported by Yao et al. [30] (4.3%), Sani and Lim [31] (6.6%, (7.14%), Oonaka et al. [32] (4.6%) and Estuningsih et al. [33] (4%) Table (1). On other hand, the results of our study are not consistent with that obtained by Zhou et al. [34] (26.7%) and Sani and Lim [31] (6.6%) Muytjens et al. [35] (9.2%) respectively.

Sani and Lim [31] found that K. pneumoniae was detected in one normal infant formula (6.25%) and one special infant formula milk (7.14%) In contrast, no *K. pneumoniae* was recovered from

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ready to feed baby milk (0%). Interestingly, Pereira and Vanetti [20] detected *K. pneumoniae* in milk-based and food supplement (20%) (Modular Enteral Diets) used in two public hospitals in Brazil.

Baby milk powder may contain *K. pneumoniae* due to the addition of heat-sensitive ingredients such as vitamins, minerals, dried rice and fruits. In addition, considerable attention has been paid to the microbiological safety of PIF in recent years. This is mainly due to neonatal infections by Enterobacteriaceae, including Cronobacter Sakazakii and *K. pneumoniae* associated with contaminated PIF. These products are not sterile, but are expected to meet international microbiological standards [34].

The major microorganisms associated with PIF contamination were *C. sakazakii*, *Salmonella enteritidis*, *Enterobacter agglomerans*, *Hafnia alvei*, *K. pneumoniae*, *Citrobacter koseri*, *C. freundii*, *K. oxytoca*, *Enterobacter cloacae*, *E. coli*, *Serratia* spp., *Acinetobacter* spp., *Bacillus cereus*, *Clostridium difficile*, *C. perfringens*, *C. botulinum*, *Listeria monocytogenes*, *Staphylococcus aureus*, and coagulase-negative *Staphylococci* [36].

The current study proved that the presences of *K. pneumoniae* in PIF is considered as major culprit of infections among infants that came from the improper preparation and conservation of these products especially at hospitals and home which could cause a health risk for infants. Moreover, as previously documented by Giammanco et al. [37], there are several Enterobacteriaceae spp., for example *K. pneumoniae*, *E. cloacae*, and *E. coli*, have been associated with necrotizing enterocolitis in infants. These species have been classified as category B organisms, and intended to cause infections in infants. Additionally, as reported by Pereira and Vanetti [20], *K. pneumoniae* was recovered from modular industrialized enteral diets and from milk-based and food supplement that used in public hospitals. As documented by Wareth et al. [14], *K. pneumoniae* was among the most frequently isolated bacterial species in milk substitution formulas for infants collected from 35 countries. A high incidence of MDR strains has been reported in pasteurized milk and whole milk powder samples collected from retail shops in Mexico, representing a public health hazard.

In this study, *K. pneumoniae* was not recovered from ice cream samples, this might be due to the inability of *K. pneumoniae* to survive in freezing temperatures. However, Gaffer et al. [22] detected only one isolate (4%) of *K. pneumoniae* in ice cream samples Table (1).

As exhibited in Table (1), *K. pneumoniae* was isolated from two samples (20 %) of full cream milk powder. For instance, Wareth et al. [14] had obtained 24 isolates of *K. pneumoniae* from milk powder producer companies. Interestingly, to our knowledge, this is the first report that describes the presences of *K. pneumoniae* in milk powder in Libya; this could be a result of post processing contamination. In contrast, no *K. pneumoniae* was found in 6 (0%) of skimmed milk powder.

The results shown in Table (3), revealed that *K. pneumoniae* was highly resistant to Amoxicillin (100%), these findings are similar to that reported by Hayati et al. [38], Montso et al. [13] and Pereira and Vanetti [20]. On other side, Xu et al. [39] reported (67.6%) of *K. pneumoniae* isolates resistant to Amoxicillin. Moreover, this study reported that (75%) of isolates susceptible to Amoxycillin/ Clavulanic acid while the remaining isolates were intermediate (25%), this finding was slightly similar to that documented by Wu et al. [12], Gaffer et al. [22] and Pereira and Vanetti [20] respectively.

The results in Table (3) show that *K. pneumoniae* isolated in this study were 100 % resistant to Ampicillin, and this result is consistent with other published studies [9,12,40,38,22,18,20,23,34,27].

As presented in Table (3), all isolates were 100 % resistant to Bacitracin, Lincomycin, and Clindamycin. Whereas, one isolate (8.3%) was sensitive to Methicillin whereas the remaining isolates were resistant (91.6%).

Furthermore, *K. pneumoniae* isolates revealed different susceptibility to Tobramycin, only two isolates (16.6%) were sensitive, six isolates were intermediate (50%) and the remaining isolates were resistant (33.3). Despite that, Fu et al. [40] has observed that most isolates were sensitive (75%) to Tobramycin, only (15%) were resistant and (10%) of isolates had intermediate susceptibility to Tobramycin. According to WHO, the intermediate susceptibility is going to be changing to resistant, due to *K. pneumoniae* had carrying multi drug resistance genes. In agreement with Wareth et al. [14] all *K. pneumoniae* isolates were sensitive to Levofloxacin (100%) Table (3).

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In the current study, all isolates were resistant to penicillin G (100%) and this was closely similar to that reported by Gaffer et al. [22], Osman et al. [23] and Timofte et al. [41]. Furthermore, Xu et al. [39] has found that (85.3%) of *K. pneumoniae* isolates were resistant to Penicillin G.

As documented in Table (3), all *K. pneumoniae* isolates were resistant to Erythromycin (91.6%) except one isolate that was intermediate (8.3%), these results are similar to that obtained by Fu et al. [40] and Osman et al. [23]. In agreement with Hayati et al. [38], Pereira and Vanetti [20], Osman et al. [23] and Zhou et al. [34], all *K. pneumoniae* isolates (100%) were sensitive to Gentamycin. Furthermore, these results were similar to that reported by Xu et al. [39] (s=94%), Wu et al. [12] (s=75%) and Nobrega et al. [26] (s=98%). In contrast Davis et al. [18] and Timofte et al. [41].

On other hand, Xu et al. [39] reported 5.9% of *K. pneumoniae* isolates were resistant to gentamycin and most of isolates sensitive to gentamycin.

Regarding sensitivity to kanamycin, the current study showed that 50 % of *K. pneumoniae* isolates recorded intermediate sensitivity to this antibiotic, whereas resistant isolates were 16.6% and 33.3% were recorded as sensitive to kanamycin. These results are similar to the findings of Pereira and Vanetti [20] and Wu et al. [12] who reported (14.3%) and (15%) of *K. pneumoniae* kanamycin resistance. In contrast, Pereira and Vanetti [20] (85%) and Wu et al. [12] (85%) had observed that most of *K. pneumoniae* were sensitive to Kanamycin.

Table (3), showed that most of *K. pneumoniae* isolates were susceptible (83.3%) to Doxycycline except two isolates were resistant (16.6%), these results were nearly similar with that reported by Wu et al. [12], (S=50%, I, =30%,R=20%). Despite that, Hayati et al. [38] found that (54%) of isolates were resistant to doxycycline, whereas Fu et al. [40] reported that 60 % of isolates appeared resistant to doxycycline, 29% were sensitive and only 11% were resistant. Moreover, 91.6% of *K. pneumoniae* were sensitive to Ciprofloxacin, and only one isolate was resistant (8.3%), these findings are similar with that reported by Wareth et al. [14], Wu et al. [12], Xu et al. [39], Nobrega et al. [26], Hayati et al. [38], Zhou et al. [34] and Davis et al. [18]. In contrast, Chaudhry et al. [1] only had remarked that most of *K. pneumoniae* was resistant to Ciprofloxacin (R= 76.9%, S= 23%).

Wu et al. [12], Nobrega et al. [26] and Hayati et al. [38] recorded susceptibility of most *K. pneumoniae* to Ciprofloxacin.

This study showed that different susceptibility of *K. pneumoniae* to Nitrofurantoin were noticed. Five isolates (41.6%) were sensitive to Nitrofurantoin, two isolates were resistant (16.6%) and the remaining isolates were intermediate (41.6%). Despite, Osman et al. [23] who observed that most isolates of *K. pneumoniae* were susceptible to Nitrofurantoin with exception of one isolate that was resistant, Xu et al. [39] found that all *K. pneumoniae* isolates were sensitive to Nitrofurantoin. In addition, 75% of isolates were sensitive to Tetracycline, (8.3%) were resistant and (16.6%) were intermediate, these results were similar to that reported by Xu et al. [39], Wu et al. [12], Nobrega et al. [26] and Pereira and Vanetti [20]. On other hand, Chaudhry et al. [1] reported that (54%) of *K. pneumoniae* were sensitive and (46%) were resistant. In contrast, Davis et al. [18] only noticed that *K. pneumoniae* was resistant to Tetracycline.

As recorded in Table (3), 50 % of isolates had intermediate sensitivity to oxytetracycline, the other 50% were resistant. These results are similar to somehow to that reported by Hayati et al. [38] (one isolate was sensitive and remaining isolates were resistant). However, Osman et al. [23] who illustrated (30%) of *K. pneumoniae* were resistant to Oxytetracycline whereas the remaining isolates were sensitive.

Table (3), revealed different susceptibility of *K. pneumoniae* to Streptomycin, 25% were resistant, 33.3% were sensitive and 41.6% were intermediate. The results agree with that recoded by Wu et al. [12], Nobrega et al. [26], Osman et al. [23], Timofte et al. [41] who reported that some of *K. pneumoniae* were resistant to Streptomycin. In contrast, Xu et al. [39] and Wu et al. [12] and Nobrega et al. [26] reported the sensitivity of most *K. pneumoniae* isolates to Streptomycin.

In agreement with Pereira and Vanetti [20] all *K. pneumoniae* isolates showed sensitivity to the Chloramphenicol, also Wareth et al. [14] and Nobrega et al. [26] detected that most of *K. pneumoniae* were susceptible to Chloramphenicol. However, Osman et al. [23] only had find out the resistance of

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K. pneumoniae to Chloramphenicol, Wareth et al. [14] and Nobrega et al. [26] who reported that (16.6%) and (8.3%) of isolates were resistant to Chloramphenicol.

It has been found that (75%) of the isolates exhibited sensitivity to Trimethoprim/Sulphamethoxazole/, as well as (16.6%) were resistant and (8.3%) of isolates were intermediate , these findings were nearly similar to that reported by Xu et al. [39], Pereira and Vanetti [20] and Osman et al. [23] respectively. On other side, Davis et al. [18] who recognized the variation in resistance and sensitivity of *K. pneumoniae* to Trimethoprim/Sulphamethoxazole/. However, Fu et al. [40], Tartor et al. [9] and Timofte et al. [41] noticed that *K. pneumoniae* was resistant to Trimethoprim/Sulphamethoxazole.

As documented in Table (3), (58.3%) of *K. pneumoniae* isolates exhibited sensitivity to the Pipercillin/Tazobactam, whereas (41.6%) were intermediate susceptibility to the Pipercillin/Tazobactam, these results were slightly similar to that recorded by Wareth et al. [14], Xu et al. [39], Gaffer et al. [22] and Zhou et al. [34]. In contrast, Tartor et al. [9], Montso et al. [13] had documented that *K. pneumoniae* isolates were resistant to Pipercillin/Tazobactam.

Furthermore, only five isolates (41.6%) had intermediate susceptibility to the Cefoperazone/Sulbactam, while the remaining isolates were sensitive (58.3%), this was similar with that found by Zhou et al. [34] were obtained only one isolate was resistant to Cefoperazone.

As reported by Tartor et al. [9], Montso et al. [13], Gaffer et al. [22], Timofte et al. [41] all *K. pneumoniae* isolates showed resistance to Ceftazidime (91.6%) except one isolate was intermediate (8.3%). Also, Njage et al. [27], Davis et al. [18] had observed different susceptibilities of *K. pneumoniae* to Ceftazidime. For instance, Pereira and Vanetti [20], Zhou et al. [34] had reported only one isolate of *K. pneumoniae* resistant to Ceftazidime. In contrast, Wareth et al. [14] and Nobrega et al. [26] had noticed no resistance of *K. pneumoniae* to ceftazidime was recorded.

Only five isolates were sensitive to Cefotaxime while the remaining isolates were resistant. These findings agree with that reported by Davis et al. [18], Pereira and Vanetti [20], Njage et al. [27] who displayed different susceptibilities of *K. pneumoniae* to Cefotaxime.

In this study, most of *K. pneumoniae* isolates were sensitive to the Ceftriaxone (66.6%), while the remaining isolates were intermediate (33.3%). These results are similar to Wu et al. [12] and Zhou et al. [34]. On other side, Njage et al. [27] and Davis et al. [18] reported that *K. pneumoniae* demonstrated varied susceptibilities to the Ceftriaxone. In contrast, Timofte et al. [41] reported resistance of *K. pneumoniae* to Ceftriaxone. All isolates were sensitive (83.3%) to Imipenem except two isolates were resistant (16.6%), Wareth et al. [14], Gaffer et al. [22], Davis et al. [18] and Pereira and Vanetti [20] reported that no resistance was found against Imipenem.

As shown in table (3), all isolates (90.9 %) were susceptible to Aztreonam except only one isolate was intermediate (9%), these results are similar with that reported by Gaffer et al. [22] (R=28%, S=71.4%) and Pereira and Vanetti [20] Despite that, Montso et al. [13] illustrated that *K. pneumoniae* was resistant to Aztreonam. Furthermore, as found by Wareth et al. [14], all isolates exhibited the susceptibility to Ertapenem (100%). Additionally, all *K. pneumoniae* isolates were sensitive to Amikacin, this was parallel to that obtained by Wareth et al. [14], Davis et al. [18] and Zhou et al. [34]. As well as, Pereira and Vanetti [20] detected that most of *K. pneumoniae* isolates were susceptible to Amikacin except only one isolate (4.8%) was resistant.

Recently, *K. pneumoniae* was detected in human samples, which in turn emphasizes again the concept of transmission between animal/animal products including milk from/to human and animal during the last decade. Alternatively, MDR *K. pneumoniae* isolates were widely detected in milk samples [9]. Moreover, *K. pneumoniae* is considered a major vehicle of resistance genes from environmental sources to clinically important bacteria and some isolates can carry acquired AMR genes or plasmids between environment, human, and animals [12]. Moreover, in this presented study two isolates were MBL producer as mentioned in Table (3).

Liang et al. [6] isolated *K. pneumoniae* from different sources; human, environment, and animal supporting the direct/indirect transmission between human and animals and this in tune with the recent global concept of dissemination of mobilized colistin resistance genes from animals to humans.

Followingly, this result emphasizing "the One Health" concept; human health is connected to the health of animals and the environment.

5. Conclusions

Klebsiella pneumoniae is now recognized as an urgent threat to human health because of the emergence of multidrug-resistant strains associated with hospital outbreaks and hypervirulent strains associated with severe community-acquired infections. *K. pneumoniae* is ubiquitous in the environment and can colonize and infect both plants and animals. This study emphasized the relationship between *K. pneumoniae* and raw milk, cheese, milk powder and infant milk retailed in Libya which is considered as a risk for human health as many of these products did not comply with the international microbiological criteria and/or Libyan standards. The necessary precautions have to be taken to carry out effective sanitary practices during the production in dairy plants, handling and distribution in the markets especially at locally small manufacture scale. Moreover, our results showed that high-level MDR existed among most of isolates against different classes of antibiotics. Interestingly, this is first study has been documented on transmission of MDR *K. pneumoniae* through food samples as milk and milk products, which constitutes a huge threat on food safety, especially on the public health for adults and neonates.

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