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# Genotyping and antibiotic resistance profile of *Klebsiella pneumoniae* and *Corynebacterium bovis* isolates recovered from clinical and subclinical mastitis milk samples

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**ABSTRACT**

Mastitis is an inflammation of the mammary gland caused in dairy cows due to bacterial infections causing high economic losses. Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) is an effective genotyping tool for tracing the infection by different bacteria. One hundred milk samples were collected (50 from clinical mastitis and 50 from subclinical mastitis) from different dairy farms at different regions of El-Gharbia governorate in Egypt. The samples were examined bacteriologically for the isolation and identification of *Klebsiella pneumoniae* and *Corynebacterium bovis*. Antibiotic sensitivity testing for the isolates and genotyping by ERIC-PCR were performed. Our results showed that the prevalence of *K. pneumoniae* was 41% from total samples and *C. bovis* strains was18% from subclinical mastitis milk samples. All the examined isolates were multi drug resistant with higher resistance to ampicillin, amoxicillin-clavulanate and cefotaxime for *K. pneumoniae* and to penicillin, erythromycin and tetracycline for *C. bovis*. Discriminatory index of ERIC-PCR was 0.984 and 1 for *K. pneumoniae* and *C. bovis* isolates, respectively. The dendrogram analysis for *K. pneumoniae* showed three clusters and two separate isolates, while for *C. bovis* 1 cluster with 2 sub clusters and three separate isolates were observed. It was concluded that ERIC-PCR is proven to be effective genotyping technique with high discriminatory index and is a good epidemiological tool for mastitis in cows as there was a genetic relatedness between some strains collected from different regions at El-Gharbia governorate in Egypt. This indicated the possibility of infection transmission between these regions and necessitates the need to increase control measures.

## Introduction

Bovine mastitis is an inflammation of the mammary gland due to microbial infections or physical trauma. It is the most common disease of dairy cattle causing economic losses by reducing the milk yield and quality (Gomes and Henriques, 2016; Cheng and Han, 2020).

Mastitis reduces milk yield and causes culling of affected animals thus reducing the economic costs to about $147 per year per cow, these costs due to losses in milk production and culling of animals, which estimated per year as 11% to 18% of the gross margin per cow (Hogeveen *et al*., 2019).

*K. pneumoniae* is a common pathogen causing mastitis in cows, but its molecular epidemiology studies are lacking from many parts of the world (Podder *et al*., 2014). This pathogen causes economic losses because its infection lowers milk production and increases veterinary costs. *K. pneumoniae* infection is characterized by severe clinical signs and poor cure rates by antimicrobial agents (Fuenzalida and Ruegg, 2019; Haxhiaj *et al*., 2022). *K. pneumoniae* is the major Gram-negative pathogens that cause mastitis and shows no desirable treatments response to antibiotics (Oliveira *et al*., 2013; Schukken *et al*., 2012). Mastitis caused by *K. pneumoniae* is more severe than that caused by *E. coli* and its symptoms progressed faster than the *E. coli*, also the cows with *K. pneumoniae* mastitis are significantly more culled or died compared with cows with *E. coli* mastitis (Sugiyama *et al*., 2022).

*C. bovis* is another important pathogen in dairy cows causing subclinical mastitis (Gonçalves *et al*., 2014), it colonizes readily the teat canal of dairy cows, therefore, it is isolated from over 60% of quarter milk samples in herds that not used post milking teat antisepsis (Yimana and Bekele, 2022)

Polymerase chain reaction (PCR) is a molecular method which is used extensively for the identification of bacteria (Abrishami *et al*., 2015). The PCR assays are quicker than culture, taking less than 24 hours to complete, whereas traditional microbiological and biochemical techniques take more than 72 hours to identify bacteria to the species level. Therefore, a PCR-based approach serves as an effective tool for confirming diagnosis of mastitis pathogens (Gangwal *et al*., 2017).

Antibiotics are usually used to treat Mastitis (Xavier *et al*., 2017), however, the random and misuse of antibiotics result in increased multidrug resistance and residues in milk increasing the threat to humans and animals (Ganda *et al*., 2016; Yimana and Bekele, 2022). Hence, antimicrobial susceptibility to bacteria should be routinely monitored as recommended by The Office International des Epizooties (OIE) (Acar and Rostel, 2001).

A variety of genotyping techniques have been reported to examine the bacteria causing mastitis in dairy cattle at the species and subspecies levels, these techniques range from simple restriction digest or PCR-based methods to micro-arrays and whole genome sequencing, Molecular epidemiology by PCR-based methods, which uses DNA-based subspecies-level classification of microorganisms, are used to determine their sources, modes of transmission, and biological linkages (Zadoks *et al*., 2011). The ERIC-PCR genotyping technique has the practical benefits of being easier, faster, and less expensive and more sensitive (Adzitey *et al*., 2013).

ERIC-PCR genotyping of *K. pneumoniae* isolated from cow's milk has been previously reported and revealed heterogeneity of the isolates and high discriminatory power of the test (Koovapra *et al*., 2016). Moreover, ERIC-PCR primer was used for the detection of strain diversity of *C. bovis*


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in bovine lactating udder quarters (Lücken *et al*., 2022). It was also used for genotyping of *Corynebacterium pseudotuberculosis*, as well as other species of *Corynebacterium* rather than *C. pseudotuberculosis* (Ramos *et al*., 2022).

The aim of the current work was genotyping of *K. pneumoniae* and *C. bovis* causing mastitis in cows by ERIC-PCR fingerprinting.

## Materials and methods

*Sampling*

The study received approval from the ethical committee of Benha University Institutional Animal Care and Use Committee (BU-IACUC) under approval number BUFVTM 02-06-23. An informed verbal/written consent for participation in the study was obtained from farm owners.

One hundred mastitis milk samples were collected from 50 cows showing clinical signs (inflammation on the udder and change in the milk appearance and quantity) and 50 subclinical mastitis cows positive for California Mastitis Test (CMT) according to the method described by Quinn *et al*. (1999). Two ml of milk sample from each quarter were added in the four cups in the CMT paddle then an equal amount of CMT reagent was added on each cup, and mixtures were shacked gently in a horizontal plane for more than 15 seconds. The subclinical mastitis milk was detected by the viscosity of the mixture, coagulation, and gel formation.

The milk samples were aseptically collected from different dairy farms at different centers of El-Gharbia governorate in Egypt. From each milk sample, 15-20 ml were added in a sterile clean screw capped bottle kept in sterile cooled container and transported rapidly to the laboratory for the bacteriological examination (Radostits *et al*., 2007).

*Bacteriological examination*

Isolation of bacteria

Milk samples were inoculated into nutrient broth (Oxoid, CM0001B) that was aerobically incubated at 37°C for 18-24 h. To isolate *K. pneumoniae*, a loopful from the cultured broth was streaked onto the surface of nutrient agar (Oxoid, CM0003) and selective diagnostic agar media (MacConkey`s agar (Oxoid, CM0115) and Eosin Methylene blue (EMB) agar (Oxoid, CM0069) (Quinn *et al*., 2002). The inoculated plates were incubated aerobically at 37°C for 18-24-48 hours.

*C. bovis* was isolated for microbiological culture-based identification according to Oliver *et al*. (2004), a loopfull from the cultured broth was streaked onto the surface of nutrient agar plate which had1% Tween 80 and 5% defibrinated bovine blood. After 24, 48, and 72 h of aerobic incubation at 37°C, the plates were checked for bacterial growth in order to assess the bacteria colonies' morphology (shape, size, and color), hemolytic ability (presence and type), and potential contamination.

Identification of bacterial isolates

The suspected colonies were examined by microscopic examination of Gram-stained films (Quinn *et al*., 2002). *K. pneumoniae* and *C. bovis* suspected isolates were biochemically confirmed by indole test, methyl red test, Voges-Proskauer test, Citrate utilization test, Hydrogen sulphide production test, Triple Sugar Iron test (TSI), urease test, catalase test and oxidase test (Quinn *et al*., 2002; Koneman *et al*., 1997).

*Molecular identification of the isolates*

The DNA from the biochemically suspected isolates was extracted by QIAamp DNA Mini Kit (Catalogue no.51304) according to the manufactures' guidelines. *K. pneumoniae* isolates were confirmed by the amplification of the 16S-23S internal transcribed spacer sequence (16S-23S ITS) (Turton *et al*., 2010). *C. bovis* isolates were identified by the amplification of 16S rRNA (Lee *et al*., 2008). The primers were synthesized by Metabion (Germany).

*Antibiotic sensitivity test*

The Kirby-Bauer disc diffusion method was used to evaluate the isolates' antibiotic susceptibility in accordance with the standards established by the National Committee for Clinical Laboratory Standards (NCCLS). Antibiotic disks used for *K. pneumoniae* were cefotaxime (CRO, 30 µg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg), tetracycline (TE, 30 µg), gentamicin (CN, 10 µg), ciprofloxacin (CIP, 5 µg), streptomycin (S, 10 µg), ampicillin (AMP, 10 µg) and amoxicillin-clavulanate (AMC, 20/10 µg). The zones of inhibition were measured and interpreted based on the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI, 2021).

Antibiotic disks used for *C. bovis* were penicillin (P, 10 µg), tetracycline (30 µg), erythromycin (E, 15 µg), ceftriaxone (30 µg), gentamicin (10 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg) and ciprofloxacin (5 µg). The zones of inhibition were measured and interpreted based on the guidelines provided by EUCAST (2019) breakpoints for penicillin, tetracycline, gentamicin, and ciprofloxacin, while the results for erythromycin, ceftriaxone and trimethoprim- -sulfamethoxazole were interpreted according to CLSI (2021).

The ratio of the number of antibiotics to which isolates showed resistance to the number of medications to which the isolates were screened was calculated as the multiple antibiotic resistance (MAR) index (Krumperman, 1983). The term "multidrug resistance" (MDR) refers to an isolate's resistance to at least one agent across three or more antibiotic classes. (Magiorakos *et al*., 2012).

*Genotyping of the isolated strains using ERIC-PCR*

Genotyping was performed on extracted DNA through fingerprinting PCR using ERIC primers to identify the genetic relationship between the isolates by a single amplification profile. The primers were synthesized by Metabion (Germany) and the sequencies are according to Versalovic *et al*. (1991).

Fingerprinting data were transformed into a binary code according to the absence or presence of each band. The dendrogram was generated by the unweighted pair group method with arithmetic average (UPGMA) and Ward,s hierarchical cluster technique. The dendrogram and clusters analysis were performed with SPSS version 22 (IBM Corp. 2013, Armonk, NY). ERIC-PCR discriminatory power was measured by Simpson's index of diversity (D), which indicates the average probability that a typing system will assign a different type to two unrelated strains randomly sampled from a population (Hunter, 1990). A D value of more than 0.9 indicates good differentiation.

## Results

*Prevalence of K. pneumoniae and C. bovis in milk samples*

After bacteriological examination of 100 milk samples, 41 (41%) *K. pneumoniae* isolates were identified, of which 13 (26%) isolates were recovered from clinical mastitis milk samples and 28 (56%) were identified in subclinical mastitis milk (Table 1). However, *C. bovis* was only detected in 9 (18%) subclinical mastitis milk (Table 1).

*Antibiotic sensitivity test*

Antibiotic sensitivity test was performed on representative *K. pneumoniae* (n=12) and *C. bovis* (n=9) isolates according to the localities where the samples were collected. For *K. pneumoniae* isolates, 100% resistance

was observed against ampicillin, amoxicillin-clavulanate and cefotaxime followed by ciprofloxacin (33.3%), while the strains were sensitive to gentamicin (83.3%), streptomycin (66.6%) trimethoprim-sulfamethoxazole (58.3%), and tetracycline (50%) as reported in Table 2. All the isolates showed multidrug resistance to 3 or more antibiotics (Table 3).

For *C. bovis* isolates, 100% resistance was observed against penicillin, erythromycin, and tetracycline, followed by gentamicin (66.6%), while the strains were sensitive to ciprofloxacin (100%), ceftriaxone (77.7%) trimethoprim-sulfamethoxazole (66.6%), as shown in Table 4. All the isolates showed multidrug resistance to 3 or more antibiotics as in Table 5.

*ERIC-PCR genotyping*

The fingerprinting patterns of isolates were investigated by ERIC-PCR using a single amplification profile. ERIC-PCR profiles were discriminated by the number and position of the amplified fragments.

*ERIC-PCR for K. pneumoniae*

The visual comparison of banding patterns revealed multiple DNA fragments, the size ranged between 139 and 1535 bp (Figure 1). ER

Table 1. Isolation rates of *K. pneumoniae* and *C. bovis* in clinical and subclinical mastitis milk samples.

| Bacterial isolates | *K. pneumoniae* | | *C. bovis* | |
|---|---|---|---|---|
| Type of mastitis samples | No | % | No | % |
| Clinical mastitis | 13 | 26% | 0 | 0 |
| Subclinical mastitis | 28 | 56% | 9 | 18% |
| Total | 41 | 41% | 9 | 9% |

Table 2. Antibiotic sensitivity of *K. pneumoniae* isolates recovered from clinical and subclinical mastitis milk samples.

| Antibiotic class | Antimicrobial (abbreviation) | *K. pneumoniae* isolates n=12) | | |
|---|---|---|---|---|
| | | R | I | S |
| Penicillins | ampicillin (AMP) | 12(100%) | 0% | 0% |
| β-lactam combination agents | amoxicillin-clavulanate (AMC) | 12(100%) | 0% | 0% |
| Cephalosporins | cefotaxime (CTX) | 12(100%) | 0% | 0% |
| Fluoroquinolones | ciprofloxacin (CIP) | 4(33.3%) | 6(50%) | 2(16.6%) |
| Folate pathway antagonists | trimethoprim-sulfamethoxazole (SXT) | 4(33.3%) | 1(8.3%) | 7(58.3%) |
| Aminoglycosides | Streptomycin (S) | 3(25%) | 1(8.3%) | 8(66.6%) |
| | Gentamicin (CN) | 0% | 2(16.6%) | 10(83.3%) |
| Tetracyclines | Tetracycline (TE) | 1(8.3%) | 5(41.6%) | 6(50%) |

Table 3. Antibiotic resistance patterns of *K. pneumoniae* isolates and their MAR index.

| Isolates | Resistance profile | MAR index |
|---|---|---|
| 1 | AMP-CTX-AMC-CIP-SXT-TE | 0.75 |
| 2 | AMP-CTX-AMC-CIP –TE | 0.63 |
| 3 | AMP-CTX-AMC | 0.38 |
| 4 | AMP-CTX-AMC-CIP-SXT-TE | 0.75 |
| 5 | AMP-CTX-AMC-CIP-SXT-TE | 0.75 |
| 6 | AMP-CTX-AMC-CIP | 0.5 |
| 7 | AMP-CTX-AMC-CIP | 0.5 |
| 8 | AMP-CTX-AMC- SXT-TE | 0.63 |
| 9 | AMP-CTX-AMC-CIP-SXT-TE-S | 0.88 |
| 10 | AMP-CTX-AMC-CIP-CN-S | 0.75 |
| 11 | AMP-CTX-AMC-CIP-CN-S | 0.75 |
| 12 | AMP-CTX-AMC-CIP –S | 0.63 |

MAR index: multiple antibiotic resistance index; AMP: ampicillin; AMC: amoxicillin-clavulanate; CTX: cefotaxime; CIP: ciprofloxacin; S: Streptomycin; TE: Tetracycline; SXT: trimethoprim-sulfamethoxazole.

Table 4. Antibiotic sensitivity of *C. bovis* isolates recovered from clinical and subclinical mastitis milk samples.

| Antibiotic class | Antimicrobial (abbreviation) | *C. bovis* strains (n=9) | | |
|---|---|---|---|---|
| | | R | I | S |
| Penicillins | penicillin (P) | 9(100%) | 0% | 0% |
| Macrolides | Erythromycin (E) | 9(100%) | 0% | 0% |
| Tetracyclines | Tetracycline (TE) | 9(100%) | 0% | 0% |
| Aminoglycosides | Gentamicin (CN) | 6(66.6%) | 0% | 3(33.3%) |
| Folate pathway antagonists | trimethoprim-sulfamethoxazole (SXT) | 3(33.3%) | 0% | 6(66.6%) |
| Cephalosporins | ceftriaxone (CRO) | 2(22.2%) | 0% | 7(77.7%) |
| Fluoroquinolones | ciprofloxacin (CIP) | 0% | 0% | 9(100%) |

C-PCR primers produced 11 profiles (referred to as E1 to E11). The discriminatory power of the ERIC-PCR was 0.984. The dendrogram analysis of the isolates examined (n = 12) showed three clusters and two separate isolates (Figure 2). Some strains collected from different regions were present in the same cluster, for example: two strains (1 and 5) collected from the same area and three strains (3-10-12) collected from different areas were located in the same cluster (cluster I). However, some strains collected from the same area and also the same farm were located in different clusters such as strain 2 in cluster III and 9 in cluster II.

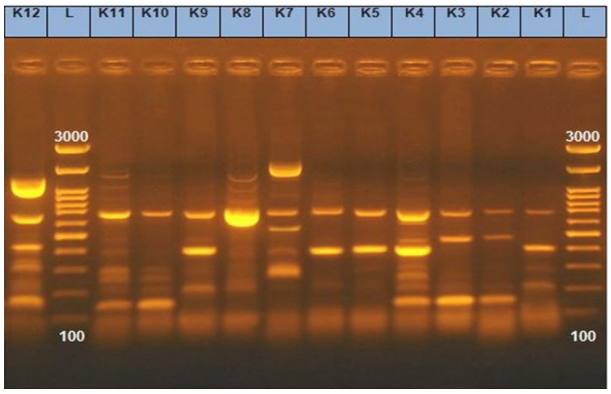


Fig. 1. ERIC-PCR fingerprinting of *K. pneumoniae* isolates in a 1 % agarose gel. Lanes L, 100-bp ladder (Range: 100-3000 bp) and lanes k1-k12 *K. pneumoniae* isolates.

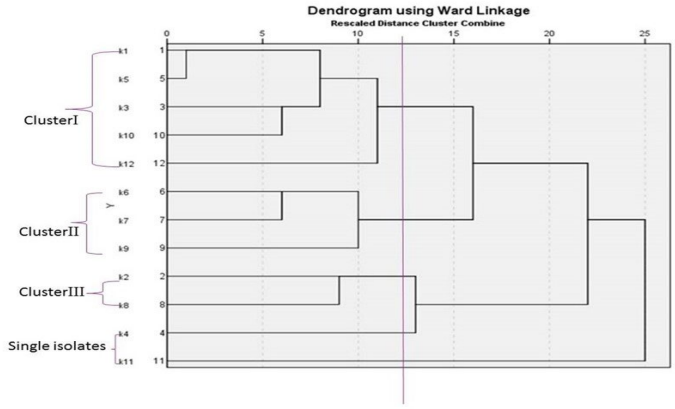


Fig. 2. Dendrogram showing the relatedness of *K. pneumoniae* samples isolated from different dairy farms at different centers of El-Gharbia governorate(Isolates 1-5-11 (tanta center), 3-8 (zefta center), 10 (elmahalla center), 12 (basuon center), 6 (samanood center), 7 (kotoor center), 2-9 (el santa center), and 4 (kafer elzayat center)) as determined by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) fingerprinting using the SPSS computer software program.

Table 5. Antibiotic resistance patterns of *C. bovis* isolates and their MAR index.

| Isolates | Resistance profile | MAR index |
|---|---|---|
| 1 | P-TE-E | 0.42 |
| 2 | P-TE-E-CRO-CN | 0.71 |
| 3 | P-TE-E -CN | 0.57 |
| 4 | P-TE-E-CN-SXT | 0.71 |
| 5 | P-TE-E-CN-SXT | 0.71 |
| 6 | P-TE-E-CRO-CN | 0.71 |
| 7 | P-TE-E -CN | 0.57 |
| 8 | P-TE-E -SXT | 0.57 |
| 9 | P-TE-E | 0.42 |

MAR index: multiple antibiotic resistance index; P: penicillin; CRO: ceftriaxone; E: Erythromycin; CN: Gentamicin; TE: Tetracycline; SXT: trimethoprim-sulfamethoxazole.

*ERIC-PCR for C. bovis*

The visual comparison of banding patterns revealed multiple DNA fragments its size ranging from 142 and 1573 bp (Figure 3). ERIC-PCR primers produced 9 profiles (referred to as E1 to E9). The discriminatory power of the ERIC-PCR was 1. The dendrogram analysis of the isolates examined (n = 9) showed one cluster (2 sub clusters) and three separate isolates (Figure 4). Some strains collected from different regions were present in the same cluster, for example1: strains 2, 4 and 6 collected from different regions and located in cluster I and example2: strains 8, 6 and 9 from different regions were located in cluster I. Some strains collected from the same area and from the same farm were located in different clusters such as strain1 and strain 2 collected from the same region but strain 2 present in cluster I and strain1 present as separate isolate not in the same cluster.

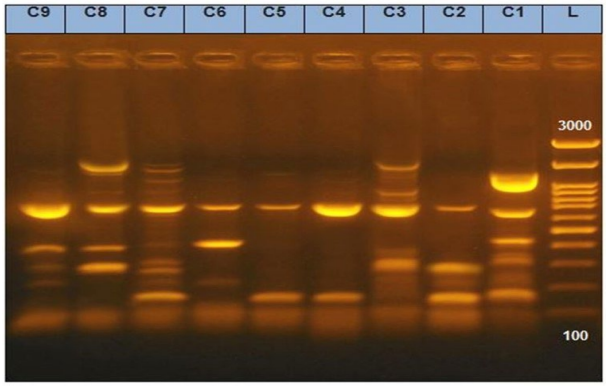


Fig. 3. ERIC-PCR fingerprinting of *C. bovis* isolates in a 1% agarose gel. Lanes L, 100-bp ladder (Range: 100-3000 bp) and lanes C1-C12 *C. bovis* isolates.

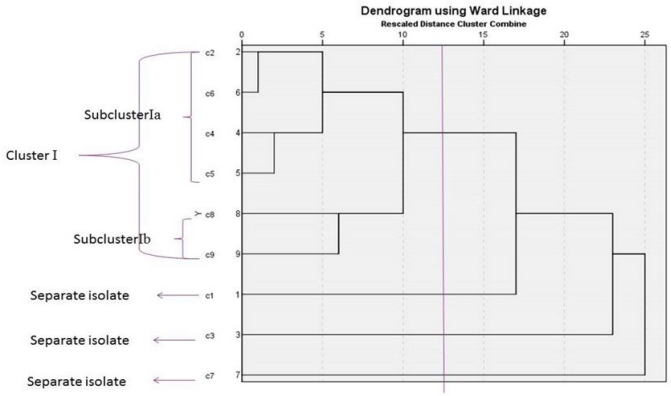


Fig. 4. Dendrogram showing the relatedness of *C. bovis* samples isolated from different dairy farms at different centers of El-Gharbia Governorate (Isolates 4-7- 8- el santa center, 3- tanta center, 6- kotoor center, 1-2-5- 9- zefta center), as determined by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) fingerprinting using the SPSS computer software program.

## Discussion

Mastitis is a very important disease that affects dairy cows and leads to sever economic losses due to the decrease in milk production, disposal of the milk and decrease its sale price, and animals culling from the herd (Demir and Eşki, 2019). Mastitis can cause $159 per cow annual loss (Dalanezi *et al*., 2020). Despite the efforts to overcome this disease, it remains a very important problem in dairy farms that affects milk quality and quantity (Guimarães *et al*., 2017; He *et al*., 2020; Lücken *et al*., 2022). Mastitis has several causes, but bacteria are the most predominant causative agent (Dalanezi *et al*., 2020). Therefore, it has a very serious zoonotic risk because of the presence of bacterial toxins in the affected milk (Abebe *et al*., 2016).

Although *K. pneumoniae* is the most important environmental opportunistic pathogen causing mastitis, few studies described *K. pneumoniae* isolated from cows with mastitis (Massé *et al*., 2020). Milk samples from cows with clinical and subclinical mastitis contained considerably (P = 0.006) more isolated *Klebsiella* spp. than milk samples from healthy cows (Koovapra *et al*., 2016).In the current study, *K. pneumoniae* isolates were detected in 41% from total collected milk samples, Lower isolation rate was detected by Singh *et al*. (2018) in India and Saddam *et al*. (2023) in Pakistan who reported 20.16% and 25.7% *K. pneumoniae* from cow milk samples, respectively. In clinical mastitis milk samples, *K. pneumo-*

*niae* was isolated from 26% samples, higher isolation rate was recorded by Cheng *et al*. (2021) who reported the same pathogen in 36.3% (129/355) of milk samples from two large Chinese dairy. Moreover, Sugiyama *et al*. (2022) detected it in 38.8% in Ehime, Japan. Lower isolation rate of *K. pneumoniae* (10%) in clinical mastitis cases in cows was recorded by Gangwal *et al*. (2017) in Bikaner city, Rajasthan .In subclinical mastitis milk samples, *K. pneumoniae* was identified in 56% of the examined samples in our study. Lower rate of 13.7% (77 /561) was recorded by Cheng *et al*. (2021) in two large Chinese dairy farms and 17.79 % was reported by Anueyiagu *et al*. (2022) in Nigeria.

The distribution of microorganisms causing mastitis varied significantly between different nations, regions, and farms and differed according to mastitis management and bedding materials used, geography and season (Gao *et al*., 2017). Different herds, countries and host species have different mastitis pathogen strain distributions (Amer *et al*., 2018). The various cleanliness and management practices used in each herd can account for the variations in mastitis pathogen prevalence (Dos Reis *et al*., 2011). *K. pneumoniae* is an environmental pathogen (Massé *et al*., 2020) transmitted to the animal from the surrounding environment and during the milking process (Langoni *et al*., 2015). The relative frequency of environmental bacteria suggests that udder hygiene must be prioritized (Gangwal *et al*., 2017). The presence of acquired Lac Operon and Fec iron-enterobactin Operon in *K. pneumoniae* results in excessive growth of this pathogen due to the utilization of lactose in cow milk and consequently facilitates the invasion of the udder and proliferation in the mammary epithelial cells (Koovapra *et al*., 2016).Therefore, from our point of view in this research, the higher prevalence of *K. pneumoniae* isolates could be due to lower management and bad hygiene in the examined farms during the study.

The presence of *K. pneumoniae* in the surroundings of animals and in places like bulk tanks and hind limbs of the animal can cause intramammary infection in cows by entering the udder through the lactiferous duct, which can result in decreased milk production and quality as well as public health issues due to the presence of these agents in milk intended for human consumption. The potential appearance of strains that are multi-resistant to antibiotics given to both men and animals is another important factor for public health (Langoni *et al*., 2015)

Many researchers gave less importance for *C. bovis* but there are many reasons to increase its participation as Nowadays, it is an important etiological agent of mastitis, because of the decrease in milk production and increase of Somatic Cell Count (SCC) (Joaquim *et al*., 2017).

Nine *C. bovis* isolates were identified in the present study by the percentage of 18% from subclinical mastitis milk samples and this result to certain extent agreed with Gonçalves *et al*. (2016) who isolated *Corynebacterium* spp. from cows with subclinical mastitis by 15.79% at species level and recorded that 92.78% of them were *C. bovis* at the quarter level in Mid-west area of São Paulo State, Brazil. Beloti *et al*. (1997) isolated *C. bovis* from 18.98% subclinical cases of mastitis in in northern Paraná, Brazil.

Lower percentage of *C. bovis* 13.40%, was detected by Dereje *et al*. (2018) in Holleta, Ethiopia. Moreover lower isolation rate was reported by Abd El-Tawab *et al*. (2020) in Egypt as isolated 8 *C. bovis* from total 150 subclinical mastitis milk samples in cows by 5.3% and Sombo *et al*. (2021) isolated *C. bovis* by 5.88% in Sebeta Town, Central Ethiopia from subclinical mastitis milk samples in cows. However, higher isolation rate of *C. bovis* in subclinical mastitis cases in cows (33.9%) was reported by Lücken *et al*. (2021) in Germany.

*Corynebacterium* spp. are detected in subclinical mastitis cases with significantly increase in somatic cell counts and *C. bovis* is a contagious microorganism isolated from subclinical mastitis cases frequently (Gonçalves *et al*., 2016).

*C. bovis* causes moderate forms of cow mastitis and can be transmitted easily through deficient milking techniques and lack of cleaning the milking equipment (Tarazona-Manrique *et al*., 2019). The bacteria colonize at the teat canal and are transmitted in inadequate cleaned milking equipment in herds that not used post milking teat antisepsis and frequently causes subclinical mastitis (Gonçalves *et al*., 2014; Yimana and Bekele, 2022). Therefore, from our point of view in this research, the prevalence of this pathogen depends on the hygienic measures and differs according to different geographical countries.

Our results showed that all *K. pneumoniae* strains were multidrug resistant (100%) and the highest resistance was to ampicillin, amoxicillin-clavulanate and cefotaxime. These results agreed with Yadav *et al*. (2021) who detected that all the *K. pneumoniae* isolates were resistant to at least two or more antibiotics with 100% resistance to ampicillin. Osman *et al*. (2014) and Alekish *et al*. (2013) recorded 100% resistance of *K. pneumoniae* strains to ampicillin. High resistance to amoxicillin-clavulanic (100%) acid and ampicillin (98.46%) was reported by Fu *et al*. (2022). Koovapra *et al*. (2016) recorded 100% resistance of *K. pneumoniae* strains to cefotaxime. Moreover, lower resistance to these antibiotics (β-lactams) (27.34%) was reported by Liu *et al*. (2022).

Resistance to β-lactams antibiotics may be related to the natural existence of β-lactamase in *K. pneumoniae*, an enzyme that inactivates penicillin and closely related β-lactams antibiotics such as 3rd and 4th generation cephalosporins (Russo and Marr, 2019). Extended spectrum β-lactamases (ESBLs) in *K. pneumoniae* are the plasmid mediated enzymes that confer resistance to β-lactams antibiotics (Koovapra *et al*., 2016)

The high sensitivity to gentamicin (83.3%) was in accordance with Ali *et al*. (2021) who recorded sensitivity to gentamicin by 92%. However, lower sensitivity rates of 50% and 55.60% were reported by Alekish *et al*. (2013) and Yadav *et al*. (2021), respectively.

Multidrug resistance (MDR) in bacteria could be attributed to the over and miss use of antibiotics in treatment of mastitis (Abd El-Tawab *et al*., 2020). Especially when the treatment is performed by non-veterinarians who use these antibiotics without antibiotic sensitivity tests that lead to an increase in the resistance of the bacteria to the widely and miss used antibiotics.

In our research, all *C. bovis* strains were multidrug resistant and the highest resistance was to penicillin, erythromycin, and tetracycline by100%. These results are in agreement with Yimana and Bekele (2022) who recorded poor inhibitory effect (71.4% resistance) of tetracycline against *C. bovis* strains in bishoftu, central Ethiopia and Alekish *et al*. (2013) who reported 88% resistance of *Corynebacterium* spp. to oxytetracycline, penicillin, and erythromycin in bovine mastitis in northern Jordan. Yajj (2006) reported 100% resistance to penicillin against *C. bovis* strains in subclinical bovine mastitis samples in kuku area, khartoum state, sudan. However, lower resistance rates to tetracycline (62.5%) and ampicillin (62.5%) were recorded by Abd El-Tawab *et al*. (2020), while lower resistance to erythromycin (50%) was reported by Yajj (2006).

The wide utilization and misuse of antimicrobials initiates the development of resistance in veterinary medicine and elevate incidence of MDR in bacteria (Abd El-Tawab *et al*., 2020), this resistance increases the threat in human and animals and monitoring of antibiotic sensitivity to bacteria in animals and human generates important data for treatment decisions and provides information in resistance that might be a cause for interventions regarding antimicrobial use (Yimana and Bekele, 2022). The usage of vitally needed human antibiotics in farm animals results in the growth of novel MDR bacteria (Lalruatdiki *et al*., 2018).

The high sensitivity of *C. bovis* strains to ciprofloxacin (100%) was in accordance with Yajj (2006) who reported 100% sensitivity of the same microbe to ciprofloxacin. While lower sensitivity (13%) was detected by Alekish *et al*. (2013).

Different techniques have been used for typing of bacteria causing mastitis, including Randomly Amplified Polymorphic DNA (RAPD), Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR), Pulsed-Field Gel Electrophoresis (PFGE) and Repetitive Extragenic Palindromic PCR (REP-PCR). The ERIC PCR assay is a frequently used straightforward and economical method for genotyping (Bobbadi *et al*., 2020). Veterinary diagnostic laboratories use comparative typing techniques based on electrophoretic banding patterns more frequently, dairy veterinarians and farm advisors can now apply molecular epidemiology for outbreak- and farm-related investigations (Zadoks *et al*., 2011). Practical benefits of the ERIC-PCR genotyping technology include its simplicity, speed, and affordability (Adzitey *et al*., 2013).

Molecular epidemiology, which uses DNA-based subspecies-level classification of microorganisms, are used to determine their sources, modes of transmission, and biological linkages, The majority of *Klebsiella* molecular epidemiology research concentrate on measuring *K. pneumoniae* heterogeneity within samples or herds (Zadoks *et al*., 2011).

Recent advancements in genotyping techniques have been used to research mastitis-causing bacteria in dairy cow at the species, subspecies, and strain levels Therefor, creation of a fingerprint database through this quick and accurate molecular technique (ERIC-PCR) is useful for tracing the origin of the epidemic causing the disease, and conducting studies to produce vaccines supplying crucial information for creating efficient mastitis prevention measures (Arslan and Mutlu, 2016).

ERIC-PCR genotyping of *K. pneumoniae* isolated from cow's milk revealed heterogeneity of the isolates and high discriminating capacity of ERIC-PCR (Koovapra *et al*., 2016). Moreover, ERIC-PCR has shown a superior ability to discriminate *K. pneumoniae* isolates as compared to (GTG)5-PCR (Zhang *et al*., 2018). For these previously mentioned reasons, we applied ERIC-PCR for genotyping of the bacteria in the present study.

In our study, ERIC-PCR primers for the 12 tested *K. pneumoniae* isolates produced 11 profiles or genotypes and high discriminatory power of the ERIC-PCR (0.984) which indicates high genetic diversity of the strains. Our results are consistent with Bobbadi *et al*. (2020) who revealed that genetic fingerprinting of ß-lactamase-producing *K. pneumoniae* using ERIC-PCR was able to distinguish between all the strains with a discriminatory value of 1 and high genetic variation across the strains. Koovapra

*et al*. (2016) reported that ERIC-PCR had a high level of discriminatory power for *K. pneumoniae* strains from cow's milk, and that the isolates were heterogeneous. On the same aspect, it's probable that the *K. pneumoniae* genotypes identified in the herd were impacted by management techniques, environmental hygiene standards, and regional variable (Paulin-Curlee *et al*., 2008)

Previous studies on genotyping *Corynebacterium* spp. other than *C. bovis* have reported that ERIC-PCR fingerprinting technique offers a number of advantages over other DNA based genotyping methods and it may be helpful for identifying genetic differences, establishing epidemiological links among bacterial isolates and establishing connections between clinical *C. pseudotuberculosis* isolates and infection sources (Taha, 2022).

ERIC-PCR for examining mainly *C. pseudotuberculosis*, the reaction was effective and had good repeatability, typeability, and resolution (Dorneles *et al*., 2012; Dorneles *et al*., 2014; Guimarães *et al*., 2011; Oliveira *et al*., 2016). However, for *C. amycolatum* and *C. testudinoris*, RAPD PCR was used and showed successful results for strain diversity (Lücken *et al*. 2022). No studies have reported the use of ERIC-PCR for genotyping *C. bovis* isolates, however, one of the ERIC primers was used in a RAPD-PCR reaction for examining the strains diversity (Lücken *et al*. 2022). Therefor, our results are promising in using ERIC-PCR for genotyping and tracing the source of infection by *C. bovis* causing mastitis with high discriminatory power of the reaction.

Our ERIC-PCR Results for *C. bovis* strains revealed 9 profiles or genotypes and high discriminatory power of the ERIC-PCR, these results indicate high genetic diversity of the strains. This agrees with Ramos *et al*. (2022) and Haas *et al*. (2017) who reported high discriminatory power (0.932 and 0.91, respectively) for *C. pseudotuberculosis* with high genetic diversity. A high degree of genomic heterogeneity showing the existence of several clones that may originate from different regions and animals arriving from various locations has also been reported (Taha, 2022).

In our study, the presence of some *K. pneumoniae* and *C. bovis* strains collected from different areas in the same cluster and some strains collected from the same area and the same farm in different clusters indicates the possibility of infection transmission between the studied areas and lack of control measures in the farms under investigation. The animals transported from various locations may have helped in spreading a wide variety of bacterial genotypes and a high genetic diversity among them (Guimarães *et al*., 2011; Sellyei *et al*., 2017).

Our results are in accordance with another study reported that ERIC-PCR genotyping of *K. pneumoniae* isolated from cow's milk showed genetic interrelationship between bacteria isolated from various places because the majority of the isolates from Jharkhand were grouped together with two samples each from West Bengal and Mizoram, demonstrating their clonal relatedness despite being isolated from various geographical locations (Koovapra *et al*., 2016).

According to Montso *et al*. (2019), the high degree of genetic relatedness between *K. pneumoniae* isolates recovered from various collection sites suggests cross contamination and this further demonstrates the necessity of raising farming management standards in the region. Moreover, the majority of the clusters in ERIC cluster analysis contained *K. pneumoniae* isolates from comparable sources and/or origins, indicating the probability of cross-contamination (Bobbadi *et al*., 2020).

## Conclusion

ERIC-PCR is an effective genotyping method for *K. pneumoniae* and *C. bovis* isolates from mastitis in cows with high genetic diversity and high discriminatory power. There was genetic relatedness between some strains collected from different regions at El-Gharbia governorate in Egypt indicating the possibility of infection transmission between the regions under investigation. Therefore, we recommend increasing the control measures and management practices in these regions.

## Conflict of interest

The authors declare that they have no conflict of interest.

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