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MALDI-TOF MS and MICRONAUT susceptibility test as recent bacteriological applications of *Klebsiella* spp isolated from large animals in Egypt.

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ABSTRACT

The purpose of this study is to assess the accuracy of the MALDI TOF technique in identifying *Klebsiella* spp. Plus, application of MICRONAUT susceptibility testing for detection of MDR *klebsiella* isolates. A total of 293 samples (233 nasal swabs, 45 milk sample, 10 anal swabs and 5 vaginal swabs) were collected from 258 clinically diseased animals. All samples were examined and all typical colonies were analyzed biochemically and confirmed by MALDI TOF method. All confirmed *klebsiella* spp. isolates were tested for antimicrobial susceptibility to 18 antimicrobials. Typical colonies were confirmed biochemically in 30 samples as *klebsiella* (10.2%). They were identified as *klebsiella* spp. (n=24), *k. pneumoniae* (n=3) and enterobacter spp. (n=3) by MALDI TOF. Antibiotic susceptibility testing of 27 *k. pneumoniae* strains by using MICRONAUT susceptibility testing discovered that all of the isolated strains were sensitive to 7/16 tested antibiotics (43.75%) amoxicillin/clavulanic acid, ceftiofur, cephalothin, enrofloxacin, gentamicin, spectinomycin, trimethoprim/sulfamethoxazol, complete resistance was observed to only 6 antibiotics, i.e., ampicillin erythromycin , tiamulin, tilmicosin, penicillin G, tulathromycin. All strains showed intermediate susceptible to florfenicol except only one strain. Eighty five percent of the isolates recorded sensitivity to colistin and the remaining show intermediate susceptibility. Ten strains were unsusceptible to tetracycline (6 resistance, 4 intermediate) and the other were susceptible to tetracycline.

1. INTRODUCTION

Klebsiella (K.) pneumoniae is a commensal bacteria that causes illnesses in animals such as pneumonia, mastitis, neonatal septicemia in calves, and bacteremia (Roberts et al., 2000). It has also been linked to substantial milk production losses, lower quality, and even high mortalities in infected cows (Gröhn et al., 2004). They cause serious hospital-acquired bacterial infections in people and animals (Paterson, 2006, Timofte et al., 2014). Moreover, *K pneumoniae* is the most common source of opportunistic healthcare-associated infections, which are exacerbated quickly by the presence of ESBLs and carbapenem resistance (Gorrie et al., 2022). The most often prescribed and utilized antibiotic class for treating bacterial infections produced by the Enterobacteriaceae family, which includes *Klebsiella* spp., is β -lactam antibiotics. Antimicrobial resistance in *Klebsiella*-producing wide range -lactamases, such as extended-spectrum -lactamases (ESBL) and Amp C -lactamases, is jeopardizing the future of β -lactam drug usage in humans and animals (Rubin and Pitout, 2014). As a result, several investigations studied antibiotic susceptibilities and genetic features of *Klebsiella* spp. generating ESBL and Amp C-lactamases (Stolle et al., 2013, Haenni et al., 2012). Increasing antimicrobial resistance has been documented in the previous decade, particularly to aminoglycosides, (fluoro) quinolones, third and fourth generation cephalosporin, cephamycins, and carbapenems (Paterson et al., 2003). *K. pneumoniae* was found to be the

most common member of the Enterobacteriaceae, with significant rates of resistance to antibiotics such as beta-lactams, aminoglycosides, quinolones, and folate-pathway antagonists (Edward et al., 2022a). The rise of multidrug-resistant (MDR) *K.pneumoniae* has posed a severe healthcare concern in Egypt. The rapid spread of blaCTX-M genes, notably blaCTX-M-14 and blaCTX-M-15, has been seen among these MDR *K. pneumoniae* (Edward et al., 2022b). For the complete genome sequencing, four *K. pneumoniae*ST627 were employed. The molecular analysis comprises 15 antimicrobial resistance genes and 65 virulence genes. Tetracycline, aminoglycoside, and fosfomycin resistance is predominantly caused by resistance genes such as *tet(D)*, *aph(3'')-Ib*, *aph(6)-Id*, *blaTEM-234*, *fosA*, and *fosA6* (Enany et al., 2022).

Because *K. pneumoniae* is a multidrug resistance pathogen, many methods such as microdilution testing and Next-generation sequencing (NGS) were used to identify antimicrobial resistance (AMR), virulence-associated genes, and plasmid replicons of 24 *K. pneumoniae* strains isolated from milk powder samples. The majority of antibiotics tested were effective (14/18) against all isolates, while all of them were resistant to Colistin, fosfomycin, chloramphenicol, and piperacillin. All isolates tested positive for ambl class A β -lactamase and *blaSHV* variants, with *blaSHV-187* being the most prevalent (reported in 50% of isolates). All isolates had single-nucleotide variants of *oqxA* and *oqxB* giving resistance to phenicol/quinolone, with *oqxB17* being the most frequent (found in 46% of isolates). Despite presence

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of *fosA* genes in 67% of the isolates, only one was fosfomycin-resistant. Two isolates carried colistin resistance genes despite being susceptible. Iron absorption siderophores were found in the majority of virulence genes (Wareth et al., 2022).

MALDI-TOF MS is a useful method for detecting foodborne pathogens (Khater et al., 2021). In a short period of time, MALDI-TOF-MS can identify *k. pneumoniae* from a single colony to species level. Until recently, clinical diagnostic laboratories relied heavily on traditional phenotypic and gene sequencing methodologies for microbial identification. MALDI-TOF MS machines were introduced into clinical microbiology laboratories, revolutionizing routine microbe identification by delivering a simple, rapid, high throughput, low-cost, and efficient identification approach (Croxatto et al., 2012). The aim of this study is to investigate the efficiency of (MALDI-TOF MS) to identify *klebsiella* at species level and detect MDR *klebsiella* spp by MICRONAUT susceptibility testing.

2. MATERIAL AND METHODS

Sampling, isolation, and identification

Two hundred and ninety-three samples (233 nasal swabs, 45 milk sample, 10 anal swabs and 5 vaginal swabs) were collected from 258 clinically diseased cattle, buffaloes, and calves between March to December 2020 from different localities in Egypt (85 samples from Gharbia, 140 sample from Behera and 33 from Qalubia Governorate). Swabs were collected and kept in pepton water media then transferred a long with collected milk samples, in ice box, directly to the department of Bacteriology, Immunology and Mycology, faculty of Veterinary Medicine, Benha University for bacteriological examination. All samples (milk, nasal, anal and vaginal swabs) were incubated for 24hr, then cultivated onto MacConky's agar, blood agar and TSI media (Oxoid) (Manual, 1998). Pure bacterial isolates were identified based on morphological characters. Each isolate was biochemically characterized as *Klebsiella* spp. by (Indol, urease, oxidase, catalase and Citrate utilization test) (Effendi et al., 2018). Using Amies agar gel, putative pure colonies (n=30) of bacteria were collected and sent to the Institute of Bacterial Infections and Zoonoses (IBIZ, Jena, Germany) for confirmation and bio typing. MALDI-TOF MS with a log value greater than 2.300 was used to re-identify all isolates at the species level. Protein extraction was carried out from pure colonies of each sample as previously described (Khater et al., 2021). MALDI-TOF measurements were taken with a Microflex LT equipment (BrukerDaltonics, Bremen, Germany) in accordance with the MALDI Biotyper manufacturer's recommendation of a log score value of 0-3 for species identification. The species identification that was most likely scores between 2.300 and 3.000, 2.000 and 2.290 were classified as 'secure genus identification', while values between 1.700 and 1.990 were classified as 'probable genus identification' and values between 0 and 1.690 were not tested for identification.

Susceptibility testing

The Susceptibility test was performed by the broth microdilution method using an automated MICRONAUT-S system (Micronaut, MERLIN Diagnostics GmbH, Bornheim-Hersel, Germany) and MICRONAUT-S MDR MRGN-Screening MIC plates (Catalog Nr. E1-114-040) to detect the minimum inhibitory concentration (MIC) (Wareth et al., 2020). The MICRONAUTS software was used for classifying the strains to susceptible, resistant and intermediate by apply MIC value according to Clinical Laboratory Standards Institute (CLSI) for *K. pneumoniae*.

The susceptibility of the isolates was detected by using different concentration of 16 antibiotics: amoxicillin/clavulanic acid (AMC), ampicillin (amp), ceftiofur (cet), colistin (col), cephalothin (ctn), enrofloxacin (enr), erythromycin (ery), florfenicol (fll), gentamicin (gen), penicillin G (pen), spectinomycin (spt), trimethoprim/sulfamethoxazole (t/s), tetracycline (TET), tiamulin (tia), tilmicosin (tilm), tulathromycin (tul).

3. RESULTS

Incidence of *klebsiella* Spp. in cattle, buffaloes and calves

The bacteriological examination of 293 collected samples revealed that 30 isolates (10.2%) showed the characteristic *klebsiella* colonies. they appeared as large, regular, round, smooth, convex, moist, and cream yellow colonies. Within the animal spp, these 30 isolates were isolated from clinically diseased cattle (20/ 160 (12.5 %), / clinically diseased buffaloes 5/60 (8.33%) and clinically diseased calves 5/38(13.1%). On blood agar, 14 / 30 isolated *klebsiella* showed sticky and non hemolytic colonies.

Biochemical identification of *klebsiella* isolates

The putative *klebsiella* isolates showed positive catalase, urease, citrate utilization test and negative results for oxidase and indole test.

MALDI TOF identification results

Score values influence MALDI TOF results. Values between 2.300 and 3.000 were regarded as extremely plausible for species identification; values between 2.000 and 2.290 were regarded as 'secure genus identification'; values between 1.700 and 1.990 were regarded as 'probable genus identification'; and values between 0 and 1.690 were not evaluated for identification. MALDI TOF detected 24/30 strains as genus *klebsiella*, 3/30 strains as *Peumoniae* (score ≥ 2.3) and 3/30 strains as *entrobacter* spp.

AMR determinants in *k. pneumoniae* isolates

MICRONAUT susceptibility testing was used to determine the antibiotic susceptibility of the twenty. four *klebsiella* spp and the 3 *k. pneumoniae*. All strains were sensitive to 43.75 % of the antibiotic used (7/16) amoxicillin/clavulanic acid, ceftiofur, cephalothin, enrofloxacin, gentamicin, spectinomycin, trimethoprim/sulfamethoxazole, Complete resistance was observed to only 6 antibiotics, i.e., ampicillin, erythromycin, tiamulin, tilmicosin, penicillin G, tulathromycin. All strain showed intermediate susceptible to florfenicol except only one strain. 85% of the isolates record sensitivity to colistin and the remaining show intermediate susceptibility. Ten strains were unsusceptible to tetracyclin (6 resistant, 4 intermediate) and the other were susceptible to tetracycline (Table1).

Table 1 Antibiotic susceptibility of the *klebsiella* spp and *k. pneumoniae* by the micronutedilution test (n=27).

	Sensitive	Resistant	Intermediate
AMC	27(100%)	-	-
AMP	-	27(100%)	-
CET	27(100%)	-	-
COL	23(85%)	-	4(15%)
CTN	27(100%)	-	-
ENR	27(100%)	-	-
ERY	-	27(100%)	-
FLL	1(3.5%)	-	26(96.5%)
GEN	27(100%)	-	-
PEN	-	27(100%)	-
SPT	27(100%)	-	-
T/S	27(100%)	-	-
T/SH	27(100%)	-	-
T/SS	27(100%)	-	-
TET	17(63%)	6(22.2%)	4(14.8)
TIA	-	27(100%)	-
TILM	-	27(100%)	-
Tul	-	27(100%)	-

R: resistance, S: sensitive, I: intermediate. No: number of (sensitive, resistant, or intermediate strains) from total number of strains. %: percentage of (sensitive, resistant, or intermediate strains) from total number of strains.

4. DISCUSSION

Nosocomial infections are frequent and continue to be a major source of death and morbidity across the world. *Klebsiella pneumoniae*, a bacterium with a high level of antibiotic resistance, is one of the major causative agents of these illnesses. As a result, research into these microbes and their resistance to antibiotics is critical (Cadavid and Echeverri, 2019). *K. pneumoniae* is to blame for The majority of animal digestive and respiratory disorders These disorders (which include mastitis, gastritis, enteritis, hepatitis, and pneumonia) can be acute, subacute, or chronic, resulting in increased animal mortality (Lenchenko et al., 2014). *K. pneumoniae* is well known for causing serious lung damage. Furthermore, as a first infection, pneumonia usually results in host bacteremia (Holmes et al., 2021). *Klebsiella* has a complex antigenic structure that includes capsular and somatic antigens as well as endotoxin; certain strains can also produce exotoxin (Lenchenko et al., 2020a). In lambs, these bacteria can cause pneumonia, acute intestine infections, urogenital infections, conjunctivitis, meningitis, and sepsis (Lenchenko et al., 2020b).

In this study a total of 293 clinically different samples (160 from clinically diseased cattle, 60 from diseased buffaloes and 38 from diseased calves). All these samples were analyzed for detection of *k.pneumoniae* using MALDO TOF identification method and detection of antimicrobial resistant activity by using antibiotic resistance testing. The results of conventional methods identified 30 *Klebsiella* isolates with the highest isolation rate was detected in calve (13.1% (5/38) followed by cattle and buffaloes (12.5% and 8.3% respectively. These results were confirmed by MALDI TOF which presents twenty-seven strains from 30 strains as following 24 form thirty strains were *klebsiella* spp. (80%), three strains *k. pneumoniae* at score ≥ 2.3 and 3 strains appeared as *enterobacter* spp. So, The MALDI TOF MS approach largely matched the results of biochemical identification. This outcome was similar to (Rodrigues et al., 2017). The MALDI-TOF MS method correctly identified 92.9% (n = 171) of the biochemical identification findings. Other studies have found a strong relationship between these two techniques, with (Ratcliffe et al., 2013) achieving 95.2% consistency in 1116 previously identified bacterial strains in clinical routine and (Dhiman et al., 2011) achieving 95.1% species identification and 3% genus identification of 1278 strains. One of the key benefits of employing this technology for bacterial identification is that it reduces the time to result from 1 to 6 days to less than an hour. Furthermore, MALDI-TOF MS enabled excellent bacterial identification of a wide range of microorganisms. Another author stated that during the research period, isolates from 29 small dairy herds were processed by the VITEK system and were recognized as *K. pneumoniae* with 98% certainty. MALDI-TOF validated the identification as *klebsiella* species with a certainty of 100% (Silva-Sanchez et al., 2021).

MALDI-TOF MS was judged by (Nonnemann et al., 2019) to be a rapid and reliable approach for identifying the most frequent pathogenic bacteria in humans and animals.

The results of antibiotic susceptibility testing (table 1); Complete resistance were detected against 6 antibiotics, i.e., ampicillin, erythromycin, tiamulin, tilmicosin, tulathromycin and penicillin G. plus, 6 strains were resistance to tetracycline. Which indicated isolation of MDR *Klebsiella* spp. The result nearly agreed with that represented by (Xu et al., 2022), who discovered that 85.3% and 67.6% of *K. pneumoniae* were resistant to penicillin and amoxicillin, respectively. Furthermore, -lactams, such as

cephalothin and piperacillin, elicited similar levels of resistance in *K. pneumoniae* isolates, with 11.8% and 10.3% resistance, respectively. Notably, a rate of 41.2% for streptomycin resistance was discovered among the isolates recovered in this investigation, which is greater than the rate for gentamycin (5.9%). Furthermore, 17.6% of the *K. pneumoniae* isolates (12/68) were tetracycline resistant. Meropenem, nitrofurantoin, and ciprofloxacin were all effective against all 68 *K. pneumoniae* isolates. Thirteen *K. pneumoniae* isolates (19.1%) exhibited acquired resistance to several antibiotic classes, including tetracyclines, aminoglycosides, and -lactams.

(He et al., 2022) reported that KPHN001 was only sensitive to polymyxin B and furazolidone and was resistant to penicillin, cefoperazone, ofloxacin, gentamicin, kanamycin, and 28 other antibiotics. Likewise, (Tartor et al., 2021) demonstrated that *K. pneumoniae* was resistant to cephalothin, cefazolin, ceftiofur, ampicillin, amoxicillin-clavulanic acid, and bacterial protein analysis. Ceftazidime, piperacillin-tazobactam (96%), tigecycline (88%), cefotaxime and ceftriaxone (76%), sulfamethoxazole-trimethoprim (56%), and colistin (56%) were all used.

5. CONCLUSION

This study concluded that *klebsiella* considered one of MDR microorganism that showed resistance to more than antibiotic. MALDI-TOF MS is a precise, dependable, and quick technology for identifying bacteria at the species level. So, its preferred to depend on MALDI-TOF for bacteriological identification as a recent method also its not available in Egypt but it's one of essential methods for bacterial identification.

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