Research Article

Molecular Studies on Some Virulent and Multi-Drug Resistant Cattle Klebsiella Strains and their Heamatobiochemical Impacts

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Abstract | Multidrug resistant *Klebsiella* spp. (MDR) and the extended-spectrum β-lactamases (ESBL) producing strains are one of the dominantly pathogenic bacteria that involved in bovine respiratory infections. Its spreading problem is growing rapidly especially it could combine resistance with high-level of virulence traits that make it is more difficult to be treated. This study provides insight into the respiratory infections by *Klebsiella* spp. and its MDR profile in bovine cases, as well as the potential target genes that were involved in the links between virulence determinants and MDR. The bacterial examination of 200 nasal swabs and 30 lung tissue samples (taken from 30 emergency slaughtered cattle) indicated that *Klebsiella* spp. was recovered in 55/230 (24%) of all samples with a high proportion of *K. pneumoniae* (18.7%). In-vitro antibiogram exhibited a wide MDR phenotype. The virulence genes (*fimA*, *aerobactin*, *rmpA* and *magA*) were exhibited in 90%, 85%, 90% and 80% of the tested isolates, respectively. Moreover, ESBL-resistant markers (bla_{CTX-M}, bla_{TEM} and bla_{SHV}) were found in 100%, 85% and 80% of the same isolates, respectively. A significant positive correlation (R = 0.7) was reported between *aerobactine* and *magA* virulence genes and bla_{TEM} and bla_{SHV} ESBL resistant genes, respectively. In addition, the hemato-biochemical analysis of 100 blood samples (50 from naturally infected and 50 from apparently healthy cattle) revealed significant decreases in the haemobiogram parameters levels meanwhile, leucogram picture total proteins, globulins, urea, creatinine levels were significantly increased in klebsiella-infected animals. An alarming increase in MDR and ESBL klebsiella bovine infections necessitates a controlled use of antibiotics in cattle farms and warrants sustainable monitoring of antibiotic emergence events and further studies for their genetic phenotypic interrelation. Moreover, the haemato-biochemical alterations in the klebsiella infected cattle could be act as biomarkers for bacterial infections.

Keywords | *Klebsiella* spp, ESBL, Heamato-biochemical; antibiotics, MDR, Virulence, Resistant, Hemobiogram, Leucogram, Cattle farms

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INTRODUCTION

Klebsiella organisms are opportunistic nosocomial pathogens primarily associated with variety of infectious diseases including upper respiratory tract infections especially pneumonia, also, mastitis, blood stream infections and pyogenic liver abscesses in animals and mammals (Butaye et al., 2021; Xu et al., 2022).

Klebsiella spp. possesses a main crucial feature that is their ability to emerge multi antimicrobial resistance (AMR) phenotypes. They are well known with its high phenotypic and genetic diversity, in particular regarding antimicrobial-resistance genes (ARGs) (Wyres and Holt, 2018). Consequently, a particular concerning is given to the emerged MDR and hypervirulent *K. pneumoniae* (hvKP) strains due to their rapid geographic spread. Even though it is still unclear how exactly *K. pneumoniae's* virulence and antibiotic resistance work together (Wang et al., 2020).

MDR bacterial phenomena is growing concern due to the irrational indiscriminate use of many antibiotics in veterinary field especially in the absence of legislation regulating the antimicrobial agents use in countries like Egypt (Cheng et al., 2018). The spread and dissemination of antimicrobial resistance (AMR) is linked with the genetic mobile elements (MGS) like plasmids, integrons, transposons and insertion sequences (Wareth and Neubauer, 2021). Integrons are found to be widely disseminated in most clinical isolates of AMR Gram-negative bacteria. Integrons also, could capture the individual ARGs and enhance their transcription and expression limiting the chance to treat infectious diseases in humans and animals (Meshref et al., 2021). Also, MGS could pass the ESBL resistant genes through; into different animal species or humans or even to the surrounding environment via contaminated soil and water, especially in poor hygienic or bad sanitation conditions (Wareth and Neubauer, 2021).

ESBL resistance traits of the family *Enterobacteriacea* including *Klebsiella* spp. are of universe public health alarm in many livestock animals and human (Nossair et al., 2022). Cattle might act as the potential reservoir for resistant ESBL (Extended Spectrum Beta Lactamase) *Klebsiella* spp. that could exist naturally in their nostril or intestine, invading lung tissues in the presence of predisposing factors with subsequent infection (Montso et al., 2019; Shin et al., 2021; Wareth and Neubauer, 2021).

The data describing ESBL-mediated resistance in *Klebsiella* spp. in bovine or ESBL detection in veterinary medicine is still limited; however, ESBL-mediated resistance of *Klebsiella* spp. against β-lactam drugs were recorded (Lee et al., 2021). ESBL bacteria could produce extended-spectrum β-lactamases enzymes, making them survive longer than other Gram -ve bacteria in the environment. MDR ESBL *K. pneumoniae* strains could put human and animal health at risk since it limited the therapeutic choices prompting the usage of colistin antibiotic, which was no longer utilized owing to toxicity (Abdeltawab et al., 2022). ESBL strains could harbour (bla_{CTX-M}, bla_{TEM} and bla_{SHV}) genes. These genes were most frequently identified in animals and humans (Wareth and Neubauer, 2021). Moreover, ESBL genes could be mutated, creating novel enzymes with extended substrate proles. Indeed, over 300 distinct ESBL genes were recorded, which had been classified according to their amino acid sequence into nine different structural and evolutionary groups (Abdeltawab et al., 2022).

In addition, for the virulence factors of *Klebsiella* spp., they own several virulence traits encompassing fimbriae, capsular polysaccharides, lipopolysaccharides, and siderophores that had been encoded by *fimH*, *rmpA*, *maga*, *uge, wabg*, *iuc*, *iro*, and other virulence genes (Remya et al., 2019). Moreover, *K. pneumoniae's* infectivity and pathogencity was found to be correlated with the ability to express their virulence genes. A proficient pathogen is virulent, resistant to antibiotics, and epidemic. The interplay between resistance and virulence is poorly understood, and is receiving great (Xu et al., 2022).

Furthermore, bovine respiratory diseases (BRD) had been considered as one of the major serious health problems that commonly occurred in cattle farms around the world (Fararh et al., 2017). These respiratory diseases were found to be directly impaired lung functions and acid-base balance in the animal body. Also, it could relatively alter the normal ratio of other blood components (Hb, RBCs, PCV), total and differential leucocytic count (Kumar et al., 2018). Moreover, they were intimately bound up with marked deterioration in the liver and kidney functions and affect seriously on the blood proteins in a previous study in Košice, Slovak Republic (Metwally et al., 2017).

Hence, this study aimed to monitor the prevalence of MDR and ESBL-mediated resistant *Klebsiella* spp. in cattle farms in Egypt studying their ESBL resistance genotypic phenotypic relation pattern with a special regard to estimation of the modulatory changes on different haemato-biochemical indices in naturally infected cattle.

MATERIALS AND METHODS

Animals

This investigation was undertaken between August 2021 and June 2022. A total of two hundred animals from various private cattle farms in the province of Ismailia, Northern Egypt; were clinically assessed to be sure that they were free from any external, internal or any blood parasites. All

examined animals were aged between 1-3 years old. Then, these animals were divided into two groups based on the health condition and clinical signs; the first group (n=100) that exhibited respiratory symptoms such as fever (>39.5 °C), fast breathing, coughing, and nasal discharge, while the second group was control apparently healthy (n=100) and exhibited no symptoms.

The first part of this study discussed the bacterial isolation with a special regard to *Klebsiella* species, so, 200 nasal swabs from diseased cattle (n=100) and from apparently healthy (n=100) were gathered and also, about thirty lung tissue samples were collected from some emergency slaughtered cases of cattle. All these samples were then transferred to the bacteriological laboratory without delay for further investigations for of *Klebsiella* species under aseptic conditions.

In addition, the second part of the study included the estimations of haemato-biochemical alterations inside the animal body, hence, one hundred whole blood samples only were taken aseptically from the jugular veins from both diseased (N=50) and apparently healthy (N=50) animals. Each blood sample was instantly divided into two tubes; one tube contained EDTA anticoagulant for direct haematological studies, while the other was a plain tube without anticoagulant for serum isolation; used for biochemical analysis.

Isolation and identification of Klebsiella species

Nasal swabs and lung tissue samples were cultivated onto HiCrome Klebsiella selective agar media (Himedia, India) and incubated at 37°C for 24h. The suspected purple colonies were then transferred onto EMB (eosin methylene blue) (Oxoid, Uk) and MacConkey's agar (Oxoid, UK), then re-incubated at 37°C for 24h for further confirmation. The presumptive isolates were biochemically identified on triple sugar iron (TSI; Oxoid, UK) agar and IMVC (indole, methyl red, Voges-Proskauer and citrate) tests (Quinn et al., 2002). For screening of the recovered isolates for ESBL production; pure colonies of the identified Klebsiella species that were taken from HiCrome Klebsiella selective agar media were; sub-cultured on Brilliance ESBL agar plates (Montso et al., 2019) (Oxoid, UK) and then incubated aerobically at 37°C for 24 hours. All klebsiella isolates, which gave green colonies on Brilliance ESBL agar plates were selected also for further confirmation by the Modified Double Disc Synergy Test (MDDST) (Paterson and Bonomo, 2005).

Antimicrobial susceptibility testing

The Kirby-Bauer disk diffusion susceptibility test using Muller Hinton agar (Oxoid, UK) medium evaluated the resistance of all recovered *Klebsiella* spp. isolates. All the recovered isolates were cultured on Muller Hinton (MH) broth (Oxoid, UK), incubated for 24 hours at 37°C, then cultivated on MH agar (Oxoid, UK) medium and re-incubated. The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI 2022). A panel of fifteen used antibiotic discs (Oxoid, UK) including ampicillin, erythromycin, tetracycline, streptomycin, norfloxacin, cefepime, cefotaxime, ciprofloxacin, cefadroxil, amoxiclav, colistin, gentamicin, aztreonam, imipenem and amikacin.

PCR investigations of different recovered *Klebsiella* spp. isolates

Genomic DNA was extracted from presumptive isolates with a QIAamp DNA Mini kit (Qiagen, GmbH, Germany) according to the manufacturer's instructions. PCR identification gene (*16S-23S ITS* gene) was tested to confirm the identity of the recovered *K. pneumoniae* isolates. Also, *aerobactin*, *rmpA* and *fimA* virulence genes were assigned to detect the virulence traits of all isolates. In addition, based on phenotypic antimicrobial resistance profiles, MDR ESBL *Klebsiella* spp. isolates were screened for integron (Int_1) and ESBL genes (bla_{CTX-M}, bla_{TEM} and bla_{SHV}). PCR amplification of all genes was carried out using specific oligonucleotide primers and reaction cycling conditions, as listed in Table 1. The positive control reference strain (*K. pneumonia*; ATCC BAA- 1705) was kindly obtained from Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Giza, Egypt) and the negative control was (PCR reaction mixture without DNA).

Hematological analysis

EDTA collected blood samples were performed for estimation of total red blood cells count (RBCs) count, hemoglobin (Hb) concentration, packed cell volume (PCV), total leukocytic count (TLC) and differential leukocytic counts as previously described by (Feldman et al., 2000).

Biochemical analysis

The serum total proteins were evaluated by the spectrophotometric method mentioned by (Doumas et al., 1981). The calorimetric method using the dye-binding procedure with bromocresol green is used to measure albumin (Doumas et al., 1971). The serum globulin could be attained mathematically by subtracting albumin from total protein according to (Chernecky and Barbara, 2008). Albumin/globulin (A/G) ratio was obtained by dividing the albumin value by the globulin value as in (Fischbach and Dunning, 2009).Aspartate transaminase (AST), Alanine aminotransferase (ALT) were calculated according to (Reitman and Frankel, 1957).

Table 1: Oligonucleotide primer sequences, cycling conditions and predicted sizes of PCR products for detecting different gene Markers of *Klebsiella spp.*

Function of gene	Primers sequences	Amplified segment (bp)	Initial denaturation °C/ min	Actual cycles (35 cycles) °C/sec			Final extention °C/ min	Reference
				Denaturation	Annealing	Extension		
Confirmation gene for species	ATTTGAAGAGGTTGCAAACGAT	150	94/5	94/30	55/30	72/30	72/7	(Turton *et al* ., 2010)
	TTCACTCTGAAGTTTTCTTGTGTTC							
fimA Virulence gene	CGGACGGTACGCTGTATTTT	436	94/5	94/30	55/40	72/45	72/ 10	(Alcántar-Curiel *et al*., 2013)
	GCTTCGGCGTTGTCTTTATC							
Aerobactin virulence gene	GCATAGGCGGATACGAACAT	556	94/5	94/30	50/40	72/45	72/ 10	(Siu *et al*., 2011)
	CACAGGGCAATTGCTTACCT							
rmpA Virulence gene	ACTGGGCTACCTCTGCTTCA	535	94/5	94/30	50/40	72/45	72/ 10	(Yeh *et al*., 2007)
	CTTGCATGAGCCATCTTTCA							
magA Virulence gene	GGTGCTCTTTACATCATTGC	1282	94/5	94/30	50/40	72/60	72/ 10	
	GCAATGGCCATTTGCGTTAG							
Int1 Class 1 integron gene	CCTCCCGCACGATGATC	280	94/5	94/30	54/30	72/30	72/ 7	(Kashif *et al*., 2013)
	TCCACGCATCGTCAGGC							
bla_{CTX-M} ESBL resistance gene	ATGTGCAGYACCAGTAARGTKATGGC	593	94/5	94/30	54/40	72/45	72/ 10	(Archambault *et al*., 2006)
	TGGGTRAARTARGTSACCAGAAYCAGCGG							
bla_{TEM} ESBL resistance gene	ATCAGCAATAA-ACCAGC	516	94/5	94/30	54/40	72/45	72/ 10	(Colom *et al*., 2003)
	CCCCGAAGAAC-GTTTTC							
bla_{SHV} ESBL resistance gene	AGGATTGACTG-CCTTTTTG	392	94/5	94/30	54/40	72/45	72/ 10	
	ATTTGCTGATT-TCGCTCG							

Also, urea (Patton and Crouch, 1977) and creatinine (Aminlari and Vaseghi, 1987) concentrations were also evaluated.

Also, the serum concentrations of calcium (Ca, mmol/l), (Gindler and King, 1972) and magnesium (Mg, mmol/l) (Smith, 1955) were analyzed using atomic absorption spectrophotometer (M 2380; PERKIN-ELEMER, AHRI). Sodium and potassium values were tested by atomic absorption spectrophotometer. Also, zinc and iron were evaluated by atomic absorption spectrophotometer (A Analyst 100, Perkin Elmer).

Statistical analysis

The obtained data in this study were edited in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Before conducting the main statistical analysis, the data were checked for normality according to A Shapiro-Wilk test (Mohd Razali and Yap, 2011). T-Test (Proc test, (SAS, 2012) was used to compare between apparently healthy and diseased cattle for serum biochemical parameters and minerals concentrations. The results were expressed as means ± SE. Fisher's Exact Test was performed to detect the significant relationship between the cattle with respiratory disease and the other apparently healthy for the prevalence ratio of *Klebsiella* spp. Moreover, the significant differences between the antibiotic resistance profiles of different used antibiotics *in Klebsiella* spp. were calculated according to afore mentioned test. The correlation analyses among various bacterial features were calculated and visualized using *Heatamply_cor* function within heatmaply package in R software v. 4.2.1 (Galili et al., 2017). Figures were fitted by the Graph-Pad Prism software 5.0 (Graph Pad, USA). Statistical significance was set at *p-value* less than 0.05.

Ethical approvement

All research protocols were carried out in accordance with regulations and approved guidelines of the Institutional Ethics Committee on Animal Care and Use established by the Faculty of Veterinary Medicine, Zagazig University (ZU-IACUC/2/F/902/2022). This article does not contain any studies with human participants performed by any of the authors.

RESULTS AND DISCUSSION

Phenotypic characterization of the recovered *Klebsiella* spp. isolates

The recovered isolates were identified phenotypically as *Klebsiella* spp. The isolates gave the characteristic purple-magenta mucoid colonies on HiCrome Klebsiella selective agar base, lactose fermenting mucoid colonies on MacConkey's agar, and large mucoid pink-to-purple colonies on EMB agar medium. Moreover, biochemically they gave positive reactions for Voges-Proskauer, citrate utilization, lysine decarboxylase and urease tests. However, they were negative for indole and methyl red tests. On TSI agar media, *Klebsiella* isolates produced acid slant/ acid butt and no H2S production. In addition, ESBL-producing *Klebsiella* isolates were identified by the green colonies on Brilliance ESBL agar plates.

The prevalence and distribution of *Klebsiella* spp. in cattle farms

There was a significant relationship between the prevalence of *Klebsiella* spp. and different types of cattle whether diseased, apparently healthy, or dead (p=0.001). Also, Table 2 showed that 55 *Klebsiella* isolates were recovered from a total of 230 analyzed samples in 24%. Forty-three (43/230) positive *K. pneumoniae* were recovered from all samples (18.7%). *K. pneumoniae* were recovered from 100 nasal swabs from respiratory-diseased animals in 18%, while it was 11% from apparently healthy ones. However, 14 lung tissue samples from emergency slaughtered cattle that were positive for *K. Rhinoscleromatis* were recovered in (6% and 4%) from diseased and healthy animals, respectively.

Antibiotic susceptibility testing of different recovered *Klebsiella* spp. isolates

MDR strains of *Klebsiella spp.* were detected and confirmed with phenotypic antibiotic sensitivity test. As shown in Table 3 and Figure 1, the recovered isolates belonging to all analyzed spp. shared a maximum resistance (100%) to ampicillin, erythromycin,tetracycline and streptomycin. Other *Klebsiella* spp. showed differential phenotypic resistance to antimicrobials. In *K. pneumonia*, the resistance rate to cefepime, cefotaxime, ciprofloxacin and colistin antibiotics (range: 83.7-95.3%)

Table 2: Prevalence of different *Klebsiella* spp. in diseased and apparently healthy cattle.

Sample and animal health	Diseased animals (Nasal swab N=100)	App. Healthy animals (Nasal swab N=100)	Dead animals (lung N=30)	Total (230)	p value
	+ve (%)	+ve (%)	+ve (%)	+ve (%)	
K. Pneumoniae	18/100 (18%)	11/100 (11%)	14/30 (46.7%)	43/230 (18.7%)	0.001
K. Rhinoscleromatis	6/100 (6%)	4/100 (4%)	0	10/230 (4.3%)	0.516
K. Ozaenae	1/100 (1%)	0	1/30 (3.3%)	2/230 (0.9%)	0.362
Total recovered *Klebsiella* isolates	25/100 (25%)	15/100 (15%)	15/30 (50%)	55/230 (24%)	0
p-value	0.001	0.096	0.0001	0	0.001

Table 3: Antibiotic resistance profile of recovered *Klebsiella* spp. isolates.

Antibiotic disc	Antibiotic group	Abbreviation	Disc conc. (µg)	*K. pneumoniae* recovered isolates											
				K. Pneumoniae (N=43)				*K. Rhinoscleromatis* (N=10)				*K. Ozaenae* (N=2)			
				S	I	R	R%	S	I	R	R%	S	I	R	R%
Ampicillin	Aminopenicillin	AMP	10	-	-	43/43	100%	-	-	10/10	100%	-	-	2/2	100%
Erythromycin	Macrolides	ERY	10	-	-	43/43	100%	-	-	10/10	100%	-	-	2/2	100%
Tetracycline	Tetracycline	TE	30	-	-	43/43	100%	-	-	10/10	100%	-	-	2/2	100%
Streptomycin	Aminoglycosides	ST	10	-	-	43/43	100%	-	-	10/10	100%	-	-	2/2	100%
Norfloxacin	Fluoroquinolone	Nor	10	-	1/43	42/43	97.7%	-	-	10/10	100%	-	-	2/2	100%
Cefepime	4th generation cephalosporin	FEB	30	2/43	-	41/43	95.3%	2/10	1/10	7/10	70%	2/2	-	-	-
Cefotaxime	3rd generation cephalosporin	CTX	30	--	3/43	40/43	93%	1/10	1/10	8/10	80%	-	-	2/2	100%
Ciprofloxacin	Fluoroquinolone	CIP	10	1/43	2/43	40/43	93%	2/10	-	8/10	80%	-	-	2/2	100%
Cefadroxil	1st generation cephalosporin	CFT	30	-	4/43	39/43	90.7%	-	-	10/10	100%	-	-	2/2	100%
Amoxiclav	Aminopenicillin	AMC	20	-	5/43	38/43	88.4%	-	-	10/10	100%	-	-	2/2	100%
Colistin	Polymyxin	C	30	4/43	3/43	36/43	83.7%	7/10	1/10	2/10	20%	2/2	-	-	-
Gentamicin	Aminoglycosides	GEN	10	11/43 (25.6%)	5/43	27/43	62.8%	-	1/10	9/10	90%	-	-	2/2	100%
Imipenem	Carbapenem	IP	10	32/43 (81.4%)	-	11/43	25.6%	2/10	-	8/10	80%	-	-	2/2	100%
Aztreonam	Monobactam	AZM	30	34/43 (79%)	-	9/43	21%	-	-	10/10	100%	-	-	2/2	100%
Amikacin	Aminoglycosides	AK	30	26/43 (60.5%)	5/43	10/43	23.3%	1/10	-	9/10	90%	-	-	2/2	100%

R%: resistance percentage.

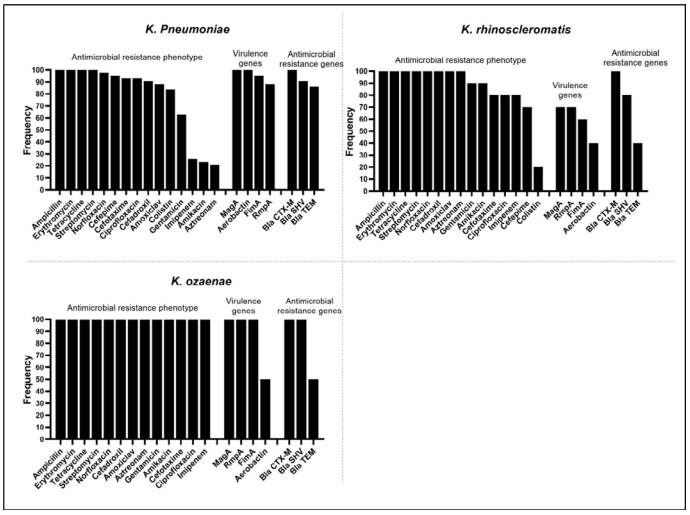

Figure 1: Frequency of antimicrobial resistance profile, relation between resistance and virulence genes in the analyzed *Klebsiella* spp.

was higher than that in *K. Rhinoscleromatis* (range 20-80%). on the other hand, *K. pneumonia* showed variable resistance percentages to norfloxacin (97.7%), cefadroxil (90.7%), amoxiclav (88.4%) and gentamicin (62.8%), imipenem (25.6%), amikacin (23.3%) and aztreonam (21%), 100% resistance to all analyzed antibiotic. Moreover, ESBL resistance traits of isolated *klebsiella* strains were positive by the phenotypic results of Modified Double Disc Synergy Test (MDDST).

Molecular identification of virulence and resistant genes of the recovered *Klebsiella* spp. isolates

The recovered *klebsiella* isolates were confirmed with PCR technique for the species specific (16S-23S ITS) gene of *K. pneumoniae* as illustrated in (Supplementary Figure 1). However, the virulence (*fimA*, *aerobactin*, *rmpA* and *magA*) genes were detected in 90%, 85%, 90% and 80% of all examined *klebsiella* isolates, respectively. The pattern of occurrence of resistance genes was consistent among isolates. Moreover, class 1 integron (*Int1*) gene was detected in 100% of the tested isolates (Supplementary Figure 2). Furthermore, ESBL resistant genes (Bla_{CTX-M}, bla_{TEM} and bla_{SHV}) were detected in 100%, 85% and 80% of the same isolates (Figure 2). In both *K. pneumonia* and *K. Rhinoscleromatis*, Bla_{CTX-M} was top detected gene followed by Bla_{SHV} and bla_{TEM} genes (Figure 1). However, Bla_{CTX-M} and Bla_{SHV} showed similar presence, which was higher than Bla_{TEM} gene. Regarding virulence genes, *MagA* was the top detected gene in *K. pneumonia* and *K. Rhinoscleromatis*. *RampA* was the least detected gene in *K. pneumonia* and *Aerobactin* was the least detected gene in *K. Rhinoscleromatis*. *K. Ozaenae* showed similar presence pattern for virulence genes as *K. Rhinoscleromatis*.

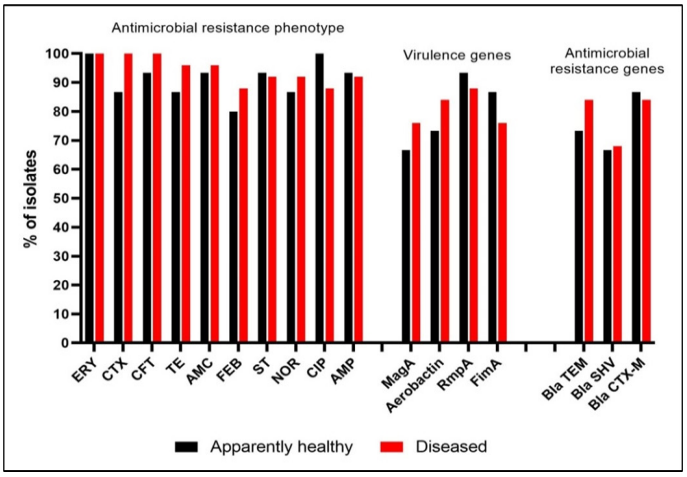

Figure 2: Frequency of antimicrobial resistance profile, resistance and virulence genes in isolates recovered from animal under different conditions. The axis shows the frequency of presence of certain trait (i.e. resistance phenotype, resistance genes and virulence genes) in isolates recovered from all animals representing each condition.

Association between antimicrobial resistance, virulence traits and disease status of cattle

The distribution of antimicrobial and virulence traits in bacterial isolates differed according to the animal status, from which the bacteria were isolated (Figure 2). We used variance as a measurment of how much of the difference is the occurrence of one trait between the appraently healthy and diseased animals. Regarding antimicrobial resistance phenotypes, the rate of resistance to CTX and CIP was the mosty different. Resistance to 60% (6/10) of the analyzed traits were of higher rate in isolates from diseased aninmals compared to those from appraently healthy animals, whereas only 30% (3/10) of these were more frequent in isolates from appranetly healthy animals, and only resistance to ERY was similar in both condition. With regard to antimicrobial resistance genes, bla_{TEM} showed the most variable rate of resistance between isolates from appraently healthy and diseased animals. Two of the analyzed genes (bla_{TEM} and bla_{SHV}) were more frequent in isolates from diseased than those from healthy cattle. Regarding virulence genes, rampA, aerobactin and magA were more enriched in isolates from diseased animals compared to those from apparently healthy animals, whereas resistance to fimA was more prevalent in isolates from appraently animals.

Correlation analyses of antimicrobial resistance and virulence traits

As shown in Figure 3, we observed significant high positive correlation among antimicrobial resistance phenotypes. For instance, streptomysin (ST) and amoxiclave (AMC) (R = 0.46, p-value = 0.0002), tetracyclin (TE) and cefadroxil (CFT) (R = 0.48, p-value = 0.0001). Lower correlation was detected between norfloxacin (NOR) and cefotaxime (CTX) (R = 0.2, p-value = 0.03) and also between erythromycin (ERY) and amoxiclave (AMC) (R = 0..3, p-value = 0.01). The phenotypic resistance against cefadroxil (CFT) was correlated weakly positive, but it was significantly with both bla_{CTX-M} and bla_{TEM} ESBL resistant genes (R= 0.28, p-value = 0.02, each).

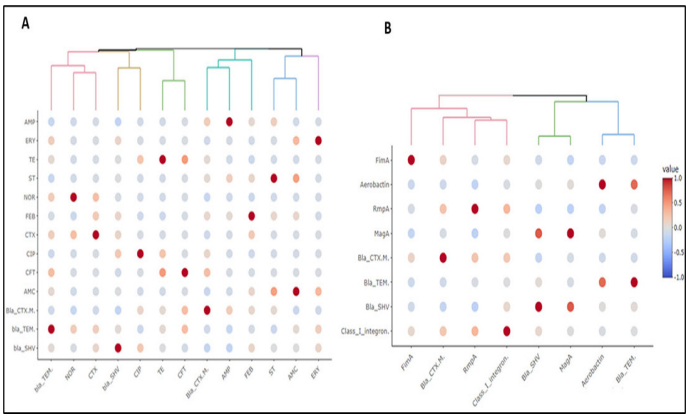

Figure 3: Heatmap showing the correlation coefficient (R) between pairs of (A) antimicrobial resistance phenotypes and antimicrobial resistance genes and (B) antimicrobial resistance genes and virulence genes. The color denotes R value as indicated on the side color scale. The upper dendrogram illustrate the clustering pattern of feature's correlation according to their R value. Each colored branch of dendrogram represents a single cluster.

Regarding the correlation between antimicrobial resistance and virulence genes, the aerobactine virulance and bla_{TEM} resistant genes exhibited the highest positive signficiant correlation (R= 0.7, p-value < 0.0001). Similarly, magA and bla_{SHV} were found to be correlated highly positively and significantly (R = 0.7, p-value < 0.001). The presence of class I integron (int1) showed also a weak positive but significant correlation with rmpA (R = 0.3, p-value = 0.01) (Figure 3).

Haematological finding

Infection of *Klebsiella* spp. in diseased cattle induced a significant reduction (P<0.05) in the RBCS, Hb, PCV% and lymphocytes count; as well as a significant (P<0.05) rise in the total leucocytic count (TLC), neutrophils, monocytes and eosinophils counts compared to the control group (apparently healthy cattle) (Table 4).

Biochemical findings

The diseased cattle showed a significant rise (P<0.05) in the total protein, globulin, AST, ALT, urea and creatinine levels, alongside with a significant reduction in both albumin levels and A/G ratio compared to the control group (Table 5). In addition, the diseased cattle showed a significant plummet (P < 0.05) in the serum concentration of Mg, P, Zn and Fe relative to the control healthy group (Table 6).

Table 4: Hematological parameters in apparently healthy and diseased cattle. (Values are shown as mean±SE).

Parameters	Control (apparently healthy) group	Diseased group (showed respiratory signs)	p value
RBCs (10^6 /µl)	7.9±1.9	6.3±2.02	0.001
Hb (gm/dl)	10.5±2.3	8..9±1.87	0.001
PCV (%)	36.45±1.39	27.32±2.41	0.001
TLC (10^3 /µl)	8.39±1.3	12.15±2.3	0.001
Lymphocytes (10^3 /µl)	4.87±0.72	3.56±0.51	0.006
Neutrophil (10^3 /µl)	3.75±0.12	5.27±0.19	0.001
Monocytes(10^3 /µl)	0.72±0.09	1.21±0.07	0.019
Eosinophils (10^3 /µl)	0.32±0.02	0.52±0.03	0.047

Table 5: Alterations of serum biochemical parameters of both control and diseased cattle (Mean±SE).

Parameters	Control (apparently healthy) group	Infected group (showed respiratory signs)	p value
Total Protein (g/dl)	6.13±1.21	6.75±1.45	0.028
Albumin (g/dl)	3.41±0.4	3.08±1.02	0.036
Globulin (g/dl)	2.82±0.52	3.49±0.72	0.048
A/G ratio	1.13±0.09	0.85±0.07	0.031
AST(U/L)	75.86±2.03	115.96±1.8	0.001
ALT(U/L)	16.32±1.51	33.25±1.32	0.001
Urea(mg/dl)	10.19±0.82	17.35±0.21	0.001
Creatinine(mg/dl)	0.98±0.08	1.43±0.06	0.026

Table 6: Concentration levels of some serum minerals in control and diseased cattle (Mean ± SE; n=50).

Parameters	Control (apparently healthy) group	Infected group (showed respiratory signs)	p value
Ca (mmol/l)	2.85±0.21	2.68±0.16	0.142
Mg (mmol/l)	0.98±0.04	0.72±0.01	0.044
P (mmol/l)	2.34±0.13	1.53±0.18	0.018
Fe (µmol/l)	20.56±1.22	12.54±1.46	0.001
Zn (µmol/l)	16.87±1.37	11.47±1.1	0.001

Bovine respiratory diseases (BRD) could be regarded as the main obstacle for animal farm owners worldwide as it negatively impacts animal performance causing high mortality rate and significant economic losses (Lee et al., 2020). Because of the limited data on *Klebsiella* species and their association with BVD, *K. pneumoniae* especially the hypervirulent (hvKp) strains had been reiterated periodically in acute and chronic respiratory infections in cattle (Darniati et al., 2021).

In the current study, Table 2 showed that the prevalence of *Klebsiella* spp. in diseased cattle that suffered from respiratory signs was high (25%) with a high recovery rate of *K. pneumoniae* (18%). Previous study done on apparently healthy cattle in Shandong province, China revealed similar infection rate of 18% in *Klebsiella* spp. from nasal swabs (Arbab et al., 2023) Also, cows recorded the most prevalent isolation rate of *K. pneumoniae* among different examined animal species (25.6%) in KSA, in which it was isolated with higher rate from cow nasopharyngeal swabs than from other samples. On the other side, a lower isolation rate of *K. pneumoniae* strains was reported from nasal swabs samples of cattle in a percentage of (15.5%), of which 12 isolates were hyper virulent strains (Cheng et al., 2018). This variation in the prevalence of *Klebsiella* spp. might be attributed to several factors such as hygienic and sanitary measures in the examined area, animal age, sex, breed, immunity, geographical distribution and also the availability and diversity of different culture and identification techniques.

K. pneumoniae in pneumonic lungs was regarded as the second most prevalent pathogen after *Staphylococcus aureus* in mixed bacterial respiratory infection in cattle (Darniati et al., 2021). In the present study, *K. pneumoniae* was found in almost half (46.7%) of the lung tissue of the analyzed 30 slaughtered cows. This was in parallel with (Darniati et al., 2021), who isolated hvKp from all examined lung tissue of Aceh cattle in Indonesia exhibiting acute and chronic upper respiratory tract infection. In addition, *K. pneumoniae* was also isolated in 25% of total 150 pneumonic lungs in a slaughterhouse in Nigeria. Our results regarding the low prevalence of *Klebsiella rhinoscleromatis* and *K. ozaenae* is in agreement with previous studies, which detected *K. ozaenae* 14 (5.7%) and *K. rhinoscleromatis* (1.6%) in diseased animals.

Most common MDR pathogens were gathered in the word ESKAPE, which encompassed six microbial species (*Klebsiella pneumoniae, Enterococcus faecium, Staphylococcus aureus, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species). These MDR organisms could degrade the drug enzymes, inactivate antimicrobial compounds, change the membrane permeability or modify the target site of antimicrobial compounds by mutation of bacterial proteins (Wareth and Neubauer, 2021). MDR patterns could arise due to the horizontal or vertical transfer of plasmid encoding antimicrobial resistance genes among bacterial pathogens from animals to humans (Ammar et al., 2020). ESBL *K. pneumoniae* is one of the MDR bacteria, which inhibit β-lactam (penicillin, cephalosporins, and carbapenems) drugs (Wareth and Neubauer, 2021).

The 3rd generation of cephalosporin resistance of *K.*

pneumoniae in food animals was previously reported, especially against cefotaxime, ceftazidime, and ceftriaxone. This resistance could arise from the bacterial ability to produce penicillinase enzymes that break down beta-lactam rings, converting penicillin into penicilloid acid that is not so active. In Gram-positive bacteria, the enzyme is liberated in the medium and destroys antibiotics before it reaches the cell, while in Gram-negative, it is located on the route where antibiotics proceed to reach the target (Effendi et al., 2018).

The current study showed that *Klebsiella* spp. have moderate to high range of antimicrobial resistance against the panel of used antibiotics (Table 3). Other antibiotics (e.g., imipenem, aztreonam, amikacin) showed also high sensitivity rates. In the same trend, similar studies declared that MDR *Klebsiella* spp. was resistant to tetracycline, ampicillin, cefuroxime, amoxicillin-clavulanic acid and gentamycin in percentages of 95%, 85%, 80% and 62%, respectively (Arbab et al., 2023). Also, other reports had found that MDR ESBL resistance-mediated *Klebsiella* spp. was detected against β-lactams antibiotics (cefoxitin, cefotaxime, cefoperazone, ceftazidime, ceftriaxone and aztreonam), amoxicillin and ampicillin. However, imipenem, and ciprofloxacin, showed 100% activity against *K. pneumoniae* isolates (Mansour et al., 2014). In addition, a resistance against ampicillin and cefazolin by *K. pneumoniae* isolates was recorded, with higher sensitivity to amikacin and meropenem drugs (Nirwati et al., 2019). Moreover, *K. pneumoniae* isolates were highly resistant to ciprofloxacin and relatively low resistant to ceftazidime and Amikacin (Cheng et al., 2018). Corresponding results of the MDR pattern of *K. pneumoniae* in cattle isolates were reported against ampicillin and amoxicillin-clavulanic acid (100%), cefepime (72.72%) and tetracycline (54.54%); meanwhile, imipenem, aztreonam, amikacin and azithromycin were sensitive drugs in the ratio of (82%, 55%, 45% and 45%), respectively (Ammar et al., 2020). In addition, most animals that recovered *K. pneumoniae* isolates were susceptible to imipenem (IMP) and meropenem (MEM) (Yang et al., 2019). The observation that antimicrobial resistance rate differ according to the involved spp. reflects potential differences in fitness and survivability of different spp. This also suggests that treatment options of various spp. of *Klebsiella* should be reconsidered accordingly.

Analyzing antimicrobial resistance phenotypes should not be the sole evaluator criteria for resistance pattern of certain spp., and identification of genetic elements associated with this should be simultaneously determined. Indeed, many of the genetic determinants of ESBL *Klebsiella* spp. were developed in recent years due to the uncontrolled use of beta-lactam drugs in animal treatment. ESBL strains could hydrolyze beta-lactam antibiotics due to gene mutations, especially bla_{TEM} gene (the active site of the enzyme), causing higher enzymatic activity (Effendi et al., 2018). Moreover, bla_{CXT-M}, bla_{TEM}, bla_{SHV} resistance genes were documented as the most common determinants of ESBL-mediated resistance traits of *K. pneumoniae* isolates. The beta-lactamase enzyme (bla_{TEM}) was firstly identified in *K. pneumoniae* and Gram-negative bacteria (Tshitshi et al., 2020). Mutation of TEM genes could occur if a single amino acid or multiplier were substituted around the active site of its enzyme (Dantas and Ferreira, 2020)

The mobile genetic elements (MGE) could transfer the resistant genes in beta-lactamase-producing enzymes bacteria (Eka and Pinatih, 2017). Mutations of plasmid CTX (Cefotaximases enzymes) and chromosomally encoded SHV β-lactamase genes have been discovered within the family *Enterobacteriaceae,* including *K. pneumoniae* species (Elmowalid et al., 2018; Rahman et al., 2018). To date, integron of classes 1 was identified as the most prevalent in the group of Gram-negative bacteria out of nine known classes of integrons (Meshref et al., 2021). Furthermore, ESBL-resistant genes (bla_{CTX-M}, bla_{TEM} and bla_{SHV}) were also detected in 100%, 85% and 80% of different examined *Klebsiella spp*. isolates (Figure 2).

Furthermore, our data indicated that major virulence traits of the recovered *Klebsiella spp* were *fimA* (90%), *aerobactin* (85%), *rmpA* (90%) and *magA* (80%) (Figure 1). Similar findings of *fimA*, *rmpA*, and *aerobactin* genes were also detected in previous studies (Xu et al., 2022). It is documented that the most common virulence factors of *Klebsiella* spp. include capsular antigens, adherence factors, the O-lipopolysaccharide, and siderophores promoting infectivity (Wareth and Neubauer, 2021). The *rmpA* virlance gens is a plasmid regulator gene of the mucoid phenotype of *K. pneumoniae* (extracapsular polysaccharide capsule synthesis) meanwhile *magA* is a chromosomally encoded hyper mucoviscosity gene that encodes the mucoviscous serotype of *K. pneumoniae*. In addition, the *fim* gene is an essential factor for pili formation and attachment to cellular surfaces forming biofilms increasing the virulence of this species (Yang et al., 2019).

For a successful infection, bacterial agent, notably *Klebsiella* spp. would simultaneously be able to resist antimicrobial agents and express virulence genes (Abd-El-Hamid et al., 2022; Ramadan et al., 2018; Tartor et al., 2021). This is expected to substantiate bacterial fitness, evolution and their persistence in infection sites. The current study allowed us to get insights into possible association between both fitness traits (Figure 3). We found significantly positive correlation between *aerobactine* and and bla_{TEM} as well as between *magA* and bla_{SHV} genes. It could be possible that correlated genes might be carried on the same MGE (e.g., integron, plasmid or transposons, etc.), and that horizontal gene transfer of these elements among

bacteria could be responsible for such co-occurrence. The co-existence of virulence and carbapenem-resistance genes in *K. pneumoniae* was previously reported and was attributed to the presence of both genes on *K. pneumoniae*-derived outer membrane vesicles (OMVs), which mediates the horizontal gene transfer among isolates (Wang et al., 2022). Future studies are warranted to analyze this association on larger number of isolates, and how this occurs differentially in different infection sites.

In the current study, different *Klebsiella* spp. were isolated from both apparently healthy as well as diseased cattle, with these isolates exhibiting varied profile of antimicrobial resistance and virulence traits (Figure 3). In general, more isolates that express these traits was found in diseased cattle than that in apparently healthy ones. This initially suggests an association between disease outcome and presence of more fitted bacteria, in which case, disease appearance and possibly severity could be in part attributed to higher number of high-fitness isolates. While previous reports indicated lack of correlation between resisto-type of *Klebsiella* and the clinical outcome of the infected human neonates (Hassuna et al., 2020), this particular point remained to be deeply investigated in the animal world, in pursuit of using antimicrobial resistance or virulence profile as predictor of disease outcome or progression.

In an attempt to further analyze the ramifications of infection with *Klebsiella* spp. that have various antimicrobial resistance or virulence profile and linking this with disease outcome, we compared clinical parameters in diseased and apparently healthy cattle ads done previously (Hassuna et al., 2020).

Regarding the haemobiogram estimation, the blood picture of group of cattle that suffered from respiratory signs recorded a significant descent in red blood cells count (RBCs), heamoglobin concentrations, packed cell volume and lymphocytes levels (Table 4). This finding was along with significant rises in the levels of TLC, neutrophil, monocyte and eosinophils. This change in erythrogram and leukogram pictures might be attributed to in appetence and iron and other nutrients deficiency that are required to blood element forming besides the adverse effect of bacterial toxins (Kumar et al., 2018). Additionally, a noticeable increase in TLC and neutrophils, monocytes and eosinophils count was attributed to severe inflammatory process during respiratory infections, acute microbial inflammation, stress factor and also, the body defense mechanism against this bacterium (El-Zahar et al., 2021; Lindholm-Perry et al., 2018). Similar results were reported in which alterations of the hematological parameters due to *Klebsiella* spp. in diseased buffaloes were stated and inhibited the erythropoiesis process (Anwar et al., 2019).

The observed remarkable reduction of the lymphocyte count could be due to the high concentrations of endotoxins that were released from the inflammatory sites leading to lymphocytes lysis and, consequently, lymphopenia, or it could be attained due to the immunosuppression that could result from the stress factor during the respiratory infections (Ramadan et al., 2019). The neutrophilia, monocytosis could compensate the lymphopenia results in the current study which was thought to be due to the production of bacterial endotoxins. This finding agreed with (Kumar et al., 2018), who mentioned that change could in turn interfered with granulopoieses, the haemoglobulin synthesis and erythropoiesis processes.

Furthermore, the protein profile in our investigation revealed a significant hike in total protein and globulin levels meanwhile a significant drop in albumin level and A/G ratio in the diseased group (Table 5). These alterations were thought to be caused by anorexia and failure of liver functions. Same results were mentioned previously (Su et al., 2019). The infection, inflammatory processes and liver damage might increase the acute phase of protein formation resulting in hyperproteinemia because microbial infection could make the body neglecting the synthesis of proteins leading to hypoalbuminemia (El-Zahar et al., 2021; Ramadan et al., 2019) but hyperglobinemia and consequently, a decline in the level of A/G ratio was recorded owing that immunoglobulins could be synthetized by the antigens via antigen antibody reaction (Ahmed, 2021; Anwar et al., 2019; Kumar et al., 2018).

For estimation of AST, ALT, urea and creatinine levels in the diseased group, the recording data expressed an obvious plummet in their levels than the healthy group of cattle that was suggested to be caused by liver and kidney cells damage. This finding was in harmony with previous results obtained by (Anwar et al., 2019; Hossain et al., 2018; Metwally et al., 2017). An impairment of liver functions of group with respiratory infections was mainly related to high respiration rate, muscle activity and inflammatory process (Abd-El-Hamed and Ibrahim, 2017). Moreover, significant increases in the urea and creatinine levels due to the microbial infection and inflammation were reported and that result was similar to a previous study by (Metwally et al., 2017). High serum levels of urea and creatinine might be caused by post bacterial infection or inflammation leading to renal dysfunction and massive body protein catabolism (Abd-El-Hamed and Ibrahim, 2017; Lung et al., 2022).

Eventually, the serum mineral profile in Table 6 showed that the infected group of cattle recorded a significant descend in the serum minerals concentrations such as calcium (Ca), magnesium (Mg), potassium (K), iron (Fe) and zinc (Zn) levels when compared with the control

healthy group. Resemble findings were also obtained by (Abd-El-Hamed and Ibrahim, 2017). Hypocalcemia was associated with hypoalbuminemia since it could hinder Ca absorption resulting into low levels of albumin bounded calcium (Ramadan et al., 2019). These changes in the levels of trace elements due to bacterial infection in pneumonic calves might be related to decrease of food consumption, low animal food intake, malabsorption, malnutrition and fever with subsequent disturbances of all metabolic processes (El-Zahar et al., 2021; Kumar et al.,2018; Yang et al., 2019).

We acknowledge that this study is limited by some factors such as small sample size that should be increased in future investigations. The small number of isolates made it difficult, for instance, to analyze the diversity of such isolates. It is worth noting that the blood indices measured in the current study is not an indication of *Klebsiella* infection alone given the nature of the field infection, where cattle could have other confounding infection. It is therefore important to conduct future validation studies for these parameters as biomarker for *Klebsiella* spp. in a controlled, pathogen-free *in-vivo* model.

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, monitoring the spread of ESBL producing strains of *Klebsiella* spp. are recommended to lower the emergence of BVD diseases and their potential sever economical losses and also to limit the bacterial spread of MDR and ESBL resistance traits in various animal species, which could lead to the failure of the antibiotics treatment strategy at the farm level. While the clinical and hemato-biochemical indices that were measured in filed cases with potential other confounding infections, it did indicate that they could be predictive aids for evaluation of the health condition of the animals. Due to the observed high positive correlation between some of the antimicrobial resistance genes and virulence genes in this study, our recommendations for further studies to validate this correlation on a large scale focusing on how this link could differentially present in different infection sites. Moreover, for both animal and human public health and to reduce the possibility of enhanced antibiotic resistance and MDR phenomena; new treatment regime such as the use of natural products and probiotics might be suggested.

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NOVELTY STATEMENT

This study sheds light for the first time on the correlation between the virulence and resistant traits of MDR and ESBL Klebsiella strains in cattle with respiratory infections and how this reflects on the heamato-biochemcial indices. This add benefits as to the utility of using these indices to pinpoint cattle suffering from such highly fitted bacteria.

AUTHOR'S CONTRIBUTION

GAI, with some inputs from MS, conceptualized the study. GAI performed the bacteriological analyses. AMM collected the samples and performed the microscopic analysis. MSH run the biochemical analyses. NAEH, MFMF performed and supervised the clinico-pathological part. GAI and MHS (the biochemical analyses part) wrote the initial draft of the manuscript. MS performed the statistical analyses and generated the drafts and final versions of the figures. GAI and MS edited the final version of the manuscript. All authors revised and approved the final version.

SUPPLEMENTARY MATERIAL

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.aavs/2023/11.3.485.498

Conflict of interest

The authors have declared no conflict of interest.

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