MOLECULAR CHARACTERIZATION OF SOME VIRULENCE GENES IN KLEBSIELLA PNEUMONIAE ISOLATED FROM BROILERS

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ABSTRACT

In the present study, a total of 160 samples (lung, air sacs, liver and kidneys, 40 samples each) were collected from clinically diseased broiler chickens. Clinical signs were weakness, gasping, ruffled feather, nasal mucoid discharge, poor growth, facial oedema and conjunctivitis, while postmortem findings were signs of septicemia, pneumonia, air sacculitis, nephritis, sinusitis and liver and lung abscesses. Isolation and biochemical identification of K. pneumoniae were done. Results of bacteriological examination revealed that K. pneumoniae isolates were recovered from 160 samples with overall prevalence (14.4%). The isolation rates from lung, air sacs, liver and kidneys were 30%, 12.5%, 10% and 5%, respectively. The isolates of K. pneumoniae were found to be virulent by using PCR assay incorporating magA, fimH and traT genes primers and were found to be resistant to some antibiotics by using PCR assay incorporating tetA(A), blaTEM and mphA genes primers. Antibiogram for 20 recovered K. pneumoniae isolates against 10 commercially used antibiotics in broiler chicken farms revealed that K. pneumoniae isolates were completey resistant to oxytetracycline (100%) and ampicillin (100%) followed by erythromycin (90%), streptomycin (80%), cefotaxim (70%) and gentamycin (65%) and moderate resistance to neomycin (45%) and chloramphenicol (30%). On the other hand K. pneumoniae isolates showed the lowest resistance to ciprofloxacin (20%) and norfloxacin (10%).

Key words: K. pneumoniae, PCR, Virulence genes, antibiotic resistance genes.

INTRODUCTION

*Klebsiella* species are gram-negative, encapsulated, non-motile, rod shape, lactose fermenting bacteria, belong to family *Enterobacteriaceae*. Members of this family are facultative anaerobic. This genus consists of 77 capsular antigens (K antigens), leading to different sero-groups (Janda and Abbott, 2006). The organism expresses both O-antigen (smooth lipopolysaccharide) and K-antigen (capsular polysaccharide) and both antigens contribute to its pathogenicity. A major virulence factor of *K. pneumoniae* is the capsule, which protects Klebsiella from lethal serum factors and phagocytosis (Fung et al., 2002 and Mizuta et al., 1983).

The genomic map of *K. pneumoniae* capsule contains gene clusters as follows: rmpA, rmpAl and rmpA2 (regulator of the mucoid phenotype A, Al and A2, respectively), magA (mucoviscosity associated gene A), cps (capsular polysaccharide synthesis), Wb (O-specific polysaccharide is directed by the Wb gene cluster) (Regue et al., 2005 and Seidler et al., 1975).

The rmpA and rmpAl genes regulate the synthesis of the Klebsiella polysaccharide capsule and they are conserved in most isolates of *K. pneumoniae*. The magA gene is a part of the *K. pneumoniae* serotype K1 capsular polysaccharide gene cluster and contributes to the bacterial virulence (Fang et al., 2004). The magA plays an important role in serious infection of Klebsiella such as septicemia, bacteremia, pneumonia and liver and lung abscesses (Chan et al., 2005 and Chung et al., 2007). The chromosomal magA gene causes increased levels of resistance to phagocytosis and has hyperviscous phenotype, which is characterized by forming a mucoviscous string during passing loop through a colony (Struve et al., 2005).

Saif et al. (2003) related the clinical signs of weakness, gasping, oxidative pneumonia, mucoid discharge and poor egg quality and decrease egg production, pleuritis and air sacculitis to *Klebsiella spp.* infection.
In humans, *Klebsiella spp.* causing infections are often multidrug resistant and an increasing proportion of strains produce extended-spectrum beta-lactamases (ESBLs). Extended-spectrum β-lactamases confer resistance to penicillins and cephalosporins. ESBLs are most commonly detected in *K. pneumoniae*, they are plasmid-mediated enzymes, and these plasmids also carry resistance genes to other antibiotics. Thus, Gram negative bacilli containing these plasmids were multidrug-resistant. In contrast, the prevalence of antimicrobial resistance in animal and poultry *Klebsiella* isolates was poorly documented (Jacoby, 1997).

The present study aimed to determine some virulence and antimicrobial resistance genes associated with *K. pneumoniae* infection and to study antimicrobial resistance profile to prevent the spread of resistant *K. pneumoniae* among the diseased chickens via planning a proper control program.

**MATERIALS AND METHODS**

**Samples:**
A total of 40 clinically diseased broiler chickens were obtained from different private chicken farms in Dakahlia province and also from cases which were arrived to Mansoura Provincial Laboratory. Four samples consisting of lungs, air sacs, liver and kidneys were collected from each diseased bird. The samples were dispatched to the Laboratory without delay to be examined bacteriologically for isolation and identification of causative agent.

**Clinical and Postmortem examination:**
All chickens were examined clinically, then sacrificed and immersed in a disinfectant before being autopsied. Gross pathological changes were recorded, summarized and presented with results for both freshly dead and clinically diseased broiler chickens.

**Media:**
- Liquid media: Tryptose broth, peptone water and nutrient broth.
- Solid media: Sheep blood agar, MacConkey’s agar and Xylose lysine deoxycholate (XLD) agar (Oxoid).

**Isolation and identification:**
Bacterial isolation was carried out by inoculating aseptically collected samples from lungs, air sacs, liver and kidneys directly on sheep blood agar and MacConkey’s agar and incubated at 37°C for 24-48 hrs (Quinn et al., 1994). After incubation, colonies culture characters and morphological characters were studied. Biochemical tests including, catalase, oxidase, indole production, methyl red, Voges-Proskauer, citrate utilization, lysine decarboxylase, urea hydrolysis, lactose fermentation and H₂S production were used for *Klebsiella spp.* Identification (Trivedi et al., 2015).

**Molecular characterization of *Klebsiella pneumoniae* by PCR:**
Five *K. pneumoniae* isolates were subjected to PCR test in PCR unit in Animal Health Research Institute, AHRI according to Olivera et al. (2003).

**DNA extraction:**
Chromosomal DNA extraction from samples was performed using the QIAlamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer’s recommendations. Briefly, 200µl of the sample suspension was incubated with 10µl of proteinase K and 200µl of lysis buffer at 56°C for 10 min. After incubation, 200µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer’s recommendations. Nucleic acid was eluted with 100µl of elution buffer provided in the kit.

**Oligonucleotide Primer:**
Primers used were supplied from Metabion (Germany) are listed in table (1)

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

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

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers sequences (5' → 3')</th>
<th>Amplified segment (bp)</th>
<th>Primary denaturation</th>
<th>Amplification (35 cycles)</th>
<th>Final extension</th>
<th>Reference</th>
</tr>
</thead>
</table>
| *K. pneumonia* 16S-23S ITS | F: ATTTGAA GAGGTGTC AAACGAT  
R: TTTCAC CTGAAAGTTT TCTTGTTG TC | 130 | 94°C 5 min. | 94°C 30 sec.  
55°C 30 sec.  
72°C 30 sec.  
72°C 7 min. | Turton et al., 2010 |
| magA             | F: GGGGTCTC TTACATC ATTGC  
R: GCAATGT GCATTGT CGTTAG | 1282 | 94°C 5 min. | 94°C 30 sec.  
50°C 40 sec.  
72°C 1.2 min.  
72°C 12 min. | Yeh et al., 2007 |
| fimH             | F: TGGCAGA ACGGATAA GCCTGGG  
R: GCAATGT ACCTGCCC TCCGTA | 508 | 94°C 5 min. | 94°C 30 sec.  
50°C 40 sec.  
72°C 45 sec.  
72°C 10 min. | Ghanbarpour and Salehi, 2010 |
| TraT             | F: GATGCG TGAACGCT ATTTATGC  
R: CACAGC GTCTGGGT ATTTATGC | 307 | 94°C 5 min. | 94°C 30 sec.  
55°C 30 sec.  
72°C 30 sec.  
72°C 7 min. | Kaipain-en et al., 2002 |
| mphA             | F: GGTGAGG AGGAGC TCTGGAG  
R: TGCCGC AGGACTC GGAAGTC | 403 | 94°C 5 min. | 94°C 30 sec.  
58°C 40 sec.  
72°C 40 sec.  
72°C 10 min. | Nguyen et al., 2009 |
| TetA(A)          | F: GGTTCAC TGAAAC GACGTC  
R: CTGTCC GAACGT TGCACTA | 576 | 94°C 5 min. | 94°C 30 sec.  
50°C 40 sec.  
72°C 45 sec.  
72°C 10 min. | Randall et al., 2004 |
| blaTEM           | F: ATCACG AAATAAA CCAGC  
R: CCCCGA AGAACG TTTTC | 516 | 94°C 5 min. | 94°C 30 sec.  
54°C 40 sec.  
72°C 45 sec.  
72°C 10 min. | Colom et al., 2003 |

In vitro Antibiotic Susceptibility Test:
Twenty *K. pneumoniae* isolates were subjected to antibiotic sensitivity test against 10 commonly used antibiotics in chicken farms. The antimicrobial susceptibility profile against oxytetracycline, ampicillin, erythromycin, gentamycin, streptomycin, neomycin, cefotaxim, chloramphenicol, ciprofloxacin and norfloxacin were tested by disk diffusion methods according to Clinical and Laboratory Standards Institute (CLSI, 2012).

RESULTS

Results are illustrated in tables (2-4) and figures (1-7)
Table (2): Prevalence of *K. pneumoniae* in examined broiler chickens:

<table>
<thead>
<tr>
<th>Organs</th>
<th>No. of examined samples</th>
<th>No. of positive samples</th>
<th>Percentage of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>40</td>
<td>12</td>
<td>30%</td>
</tr>
<tr>
<td>Air sacs</td>
<td>40</td>
<td>5</td>
<td>12.5%</td>
</tr>
<tr>
<td>Liver</td>
<td>40</td>
<td>4</td>
<td>10%</td>
</tr>
<tr>
<td>Kidney</td>
<td>40</td>
<td>2</td>
<td>5%</td>
</tr>
<tr>
<td>Total</td>
<td>160</td>
<td>23</td>
<td>14.4%</td>
</tr>
</tbody>
</table>

Twenty *K. pneumoniae* isolates were subjected to antibiotic sensitivity test against 10 commonly used antibiotics in chicken farms. Results are shown in table (3).

Table (3): Antibiotic sensitivity and resistance pattern for (20) *K. pneumoniae* isolates.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Sensitive No. (%)</th>
<th>Intermediate No. (%)</th>
<th>Resistant No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Oxytetracycline</td>
<td>0 0 0 0 20 100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Ampicillin</td>
<td>0 0 0 0 20 100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Erythromycin</td>
<td>1 5 1 5 18 90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Streptomycin</td>
<td>1 5 3 15 16 80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 Cefotaxim</td>
<td>2 10 4 20 14 70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 Gentamycin</td>
<td>2 10 5 25 13 65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 Neomycin</td>
<td>5 25 6 30 9 45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 Chloramphenicol</td>
<td>8 40 6 30 6 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 Ciprofloxacin</td>
<td>11 55 5 25 4 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 Norofloxacin</td>
<td>13 65 5 25 2 10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table (4): Results of PCR assay for detection of *K. pneumoniae* virulence and antimicrobial resistance genes.

<table>
<thead>
<tr>
<th>Isolate</th>
<th><em>K. pneumoniae</em> 16S-23S ITS</th>
<th>Virulence genes</th>
<th>Antimicrobial resistance genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>magA</td>
<td>fimH</td>
<td>traT</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure (1): Agarose gel electrophoresis showing amplification of 130 bp fragment using 16S-23S ITS primer of *K. pneumoniae*.
L: 100 - 600 bp ladder
Lane (1-5): Positive samples

Figure (2): Agarose gel electrophoresis showing amplification of 1282 bp fragment using *magA* primer.
L: 100 - 1500 bp ladder
Lane (1, 2, 4 and 5): Positive samples
Lane (3): Negative sample

Figure (3): Agarose gel electrophoresis showing amplification of 508 bp fragment using *fimH* primer.
L: 100 - 600 bp ladder
Lane (1-5): Positive samples
Figure (4): Agarose gel electrophoresis showing amplification of 307 bp fragment using TraT primer. 
L: 100 - 1000 bp ladder 
Lane (1-5): Positive sample 

Figure (5): Agarose gel electrophoresis showing amplification of 576bp fragment using tetA (A) primer. 
L: 100 - 600 bp ladder 
Lane (1-5): positive samples 

Figure (6): Agarose gel electrophoresis showing amplification of 516 bp fragment using blaTEM primer. 
L: 100 - 600 bp ladder 
Lane (1-5): positive samples
DISCUSSION

Bacterial pathogens play an important role in causing respiratory disease in domestic poultry species (Glisson, 1998). In many cases, the bacterial pathogens colonize the respiratory system as a secondary bacterial invasion only after a primary viral or environmental insult. *Klebsiella pneumoniae* has been frequently recovered from birds in which it functioned as a primary pathogen and was associated with respiratory tract disease (Sandra and Duarte, 1998). *Klebsiella pneumoniae* infection of young poultry increased the severity of respiratory disease (Saif et al., 2003).

In the present study, the clinical findings of *K. pneumoniae* infected broilers were weakness, gasping, ruffled feather, nasal mucoid discharge, poor growth, facial oedema and conjunctivitis. These findings were similar to that observed by Popy et al. (2011).

Regarding the postmortem lesions of *K. pneumoniae* infected broilers, there were signs of septicemia, pneumonia, air sacculitis, nephritis, sinusitis and liver and lung abscesses. These findings agreed with that observed by Chung et al. (2007).

In general, the investigation of 160 samples collected from clinically diseased broiler chickens revealed that the prevalence rate of *K. pneumoniae* was (14.4%) as shown in (Table 2). Nearly similar results were recorded by Aly et al. (2014) and Khalda et al. (2000) who recorded that the prevalence rate of *K. pneumoniae* in broiler chickens was (10%) and (10.2%), respectively. On the other hand, Turkylmaz (2005) recorded a higher prevalence rate (47.1%). Meanwhile, Dashe et al. (2013) and Abdulrazzaq et al. (2014) reported that the prevalence of *K. pneumoniae* in broiler chickens was (8%) and (7%), respectively.

In the present study, the isolation rate of *K. pneumoniae* from lungs (30%) was higher than that of the other internal organs (air sacs, liver and kidneys, 12.5, 10 and 5%, respectively) as shown in (Table 2). It was in the same direction with Younis et al. (2016).

PCR detection based on 16S-23S rDNA internal transcribed spacer (ITS) of *K. pneumoniae* was carried out in the present study. Five isolates of *K. pneumoniae* were positive to the PCR detection (Figure 1). This agree with that reported by Yin Liu et al. (2008).

PCR assay was conducted for detection of some virulence genes of *K. pneumoniae*. PCR assay could identify *magA*, * fimH* and *traT* genes by using specific primer sequences which yielded product sizes of 1282bp, 508bp and 307bp, respectively. Out of the tested isolates, four isolates were positive and one isolate was negative for *magA* gene (figure 2), five isolates were positive for * fimH* gene (Figure 3) and five isolates were positive for *traT* gene (Figure 4). Detection of these genes may indicate the virulence potential of *K. pneumoniae* isolates. Struve et al. (2005) described *magA* as a novel virulence factor responsible for the increased virulence of certain *K. pneumoniae* strains. They provided evidence that the *magA* gene, so far believed to be a specific virulence factor in highly virulent Klebsiella strains. El Fertas et al. (2013) concluded that * fimH* gene is the most common virulence gene of *K. pneumoniae* and *traT* gene was detected at a lower prevalence rate in *K. pneumoniae* isolates.

*K. pneumoniae* is an important multidrug-resistant (MDR) pathogen affecting both humans and animals. PCR assay was conducted for detection of some antimicrobial resistance genes of *K.
pneumoniae. PCR assay could identify tet(A), blaTEM and mphA genes by using specific primer sequences which yielded product sizes of 576bp, 516bp and 403bp, respectively. Out of the tested isolates, five isolates were positive for tet(A) gene (figure 5), five isolates were positive for blaTEM gene (Figure 6) and five isolates were positive for mphA gene (Figure 7). Detection of these genes may indicate the high multiple antibiotic resistances of K. pneumoniae isolates. WeiXia Wang et al. (2014) found that the class A tet determinants tet(A) and tet(A)-1 could confer high-level tetracycline resistance. Ojdana et al. (2014) found that the prevalence of blaTEM genes was responsible for the production of broad-spectrum β-lactamases among K. pneumoniae. Soge et al. (2006) found that all the large CTX-M plasmids of K. pneumoniae carried several drug resistance genes including blaTEM-lgenes (ampicillin resistance) and tet(A) gene (tetracycline resistance) while 65 % of plasmids carried mph(A) gene (macrolide resistance).

Twenty K. pneumoniae isolates were subjected to antibiotic sensitivity test against ten commonly used antibiotics in chicken farms. All isolates were resistant to ampicillin and oxytetracyclines. This was agreed with Gundogan and Avci (2013) who reported that klebsiella species showed 100 % resistant to ampicillin and Rasool et al. (2003) who found that K. pneumoniae was resistant to tetracyclines. Brisse et al. (2006) discussed that klebsiella species were resistant to ampicillin as a result of chromosomal class-A β-Lactamase production. Also, K. pneumoniae isolates showed 90% resistance rate against erythromycin. It was in agreement with that reported by Kilonzo et al. (2007). These results were supported by PCR assay which detected tet(A) (A), blaTEM and mphA antimicrobial resistance genes against tetracyclines, ampicillin and erythromycin, respectively. These results run parallel with that reported by Guo et al. (2016) and Hou et al. (2015). On the other hand, K. pneumoniae isolates showed moderate resistance rate (65% and 45%) against gentamycin and neomycin, respectively. This might run parallel with Chang et al. (2000) who recorded that K. pneumoniae were moderately susceptible to aminoglycosides. Also, K. pneumoniae isolates showed lower resistance rate (20% and 10%) against ciprofloxacin and norfloxacin, respectively. It was agreed with Gundogan and Avci (2013) who reported 23.8% resistance rate against ciprofloxacin. While, Olufemi et al. (2012) reported (54.5% and 63.6%) resistance rates against ciprofloxacin and norfloxacin, respectively.

REFERENCES


Seidler, R.J; Knittel, M.D. and Brown, C. (1975): Potential pathogens in the environment: cultural reactions and nucleic acid studies on Klebsiella pneumoniae from clinical and


The article is about the detection of specific genes associated with the virulence of Klebsiella pneumoniae. The study involved the analysis of 495 isolates from various sources, focusing on the variable number tandem repeat (VNTR) and virulence genes. The research was aimed at understanding the genetic diversity and resistance mechanisms of Klebsiella pneumoniae, which is a common pathogen associated with various infections. The isolates were characterized using PCR and typing methods, providing insights into the epidemiology and potential resistance patterns of this pathogen.