Isolation and Molecular Identification of Klebsiella Microbe Isolated from Chicks

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ABSTRACT:
Klebsiella pneumoniae is one of the most common infectious disease affecting chicks, causing great economic losses. Also, possess food safety and antimicrobial resistance threats, as it may act as a contamination source of poultry meat and eggs. We aim to isolation and identification of klebsiella organisms causing death and loss in chicks. To perform this aim, it is necessary to full following points: Isolation of klebsiella causing respiratory manifestations and death in chicks purification and identification (morphologically, culturally, biochemically) of recovered klebsiella, antibiotic sensitivity test, PCR detection of Klebsiella genus specific gene (GyrA), PCR detection of klebsiella carbapenem resistance gene (kpc) and PCR detection of two of important virulence genes: Mucoviscocity attached gene (MagA) and Iron uptake system gene (Kfu). A total of 150 chicks from different locations were examined clinically for respiratory symptoms lung, liver, trachea, intestine and samples (dropping and oro-pharyngeal swab) were collected for bacteriological examination. The results of biochemical tests for detection of biochemical characteristics of isolated Klebsiella; where all the examined isolates cleared that Klebsiella, gave positive reaction for catalase test, vorges proskeure, urease and citrate test, meanwhile the isolates were negative for oxidase, Indole and Methyl red tests. Cultivation of isolate on macConkey gives lactose fermenting colonies, more or less dome shaped, 3-4 mm diameter after overnight incubation at 37°C. The sixteen klebsiella isolates subjected for PCR detection and the results for detection of Klebsiella genus specific gene (GyrA) were positive for all isolates. Also ten of sixteen subjected to PCR for detection of Klebsiella pneumonia carbapenamase gene (KPC) and the results were negative, ten of sixteen subjected to PCR for detection of Mucoviscocity attached gene (MagA) and the results were positive for seven out of ten isolates, eight out of sixteen isolates subjected to PCR for detection of Iron uptake system gene (Kfu) and the results were positive for eight out of ten isolates. The 16 Klebsiella isolates were tested for the resistance to antibiotics and the results indicates that Meropenem, Imipenem, Amikacin, Cefotaxime sodium, Cefazidime, Ciprofloxacin and Tobramycin had high effect on klebsiella isolates. Meanwhile, Gentamycin and Cefepime have moderate effect on Klebsiella. Moreover, antibiotics have less effect on the isolated Klebsiella were Azithromycin and Erythromycin.

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1. INTRODUCTION
Klebsiella spp. are Gram-negative, nonmotile, usually encapsulated rod-shaped bacteria, belonging to the family Enterobacteriaceae. Members of the Enterobacteriaceae family are generally facultative anaerobic, and range from to1.0 mm in width and 0.6 to 6.0 mm in length. Klebsiellasp. often occur in mucoid colonies. The genus consists of 77 capsular antigens (K antigens), leading to different serogroups (Janda et al. 2007). Weakness, gasping, pump-handled respiration, dyspnoea, mucous discharge and mortality, swelling of sinuses, facial oedema, tracheitis, exudative pneumonia, pleuritis, air sacculitis, pericarditis, sinusitis, drop in egg production and poor egg quality characterize the respiratory infection (Zorman et al., 2000; Canal et al. 2005). klebsiella organisms cause death and loss in chicks. The genus klebsiella was named by Trevisan (1885) to honor the clinical microbiologist Edwin klebs (1834-1913). The first klebsiella species ever described was a capsulated bacillus from patients with Rhinoschleoma (Von Frisch 1882; Sylvian et al. 2009).
2. MATERIAL AND METHODS

2.1. Collection of samples: Each suspected chick slaughtered, opened aseptically and internal organs (liver, lungs and trachea). Apparently healthy chicks samples are taken from droppings and oro-pharyngeal swabs.

2.1.2. Cultivation of the samples for isolation of Klebsiella:

Swabs were cultivated into nutrient broth and incubated aerobically at 37°C for 18-24 hours. A loopful of inoculated nutrient broth was streaked onto MacConkey’s agar medium. The inoculated medium were incubated aerobically at 37°C for 24-48 hours, and then examined for bacterial growth.

2.1.3. Purification of the isolates: Suspected colonies were subcultured onto MacConkey’s agar plates and incubated at 37°C for another 24 hrs.

2.1.4. Identification of the isolates:

A- Morphological characterization: Films were stained with Gram’s stain and examined microscopically for morphological characteristics of the isolates.

B- Cultural characteristics: The colonial morphology onto MacConkey’s agar was studied.

C- Biochemical characterization: Catalase test, Oxidase test, Indol test, Methyl red test, Vogas-proskauer test, Citrate utilization test and Urease test were performed.

2.1.5. Detection of the genus klebsiella specific gene (GyrA gene), virulence genes of Klebsiella isolates (magA, Kfu ) and Kpc gene using polymerase chain reaction (PCR):

2.2. Extraction of bacterial DNA: DNA templet was prepared from Klebsiella cells according to Bridge (1996). 1ml distilled water was added to Klebsiella growth on slope agar then shacked well. The bacterial suspension was centrifuged and the pellet was resuspended in distilled water by using vortex. The genomic DNA was extracted by boiling of the suspension for 10 minutes in water bath to ensure lysis of cells and for complete extraction of DNA then the supernatant was used as a template for polymerase chain reaction.

2.2.5.2. Oligonucleotide primers:

1. Oligonucleotide primers set GyrA: 25μL of of master mix, 200 nm of each primer (forward and reverse),100 ng of template , and water to make final volume of 50μL (Yogesh and Kevan, 2011). The melting temperature TM of each oligonucleotide using the formula Tm = 4(G+C) + 2 (A+T), where G, C, A and T indicate the number of corresponding nucleotides in the oligonucleotide.

2. Oligonucleotide primers set KPC: PCR was performed with a final volume of 20 μL in 0.2 mL thin-walled tubes (AccupowerTM HotStart PCR PreMix; Bioneer, Daejeon, Korea).Total volume : 18μl Master Mix + 2μl of template = 20μl reaction, Pipet 18 μl of the final Master Mix add 2μl of template. Mix up and down. Cover with optical adhesive fil (Sang et al.2012).The melting temperature TM of each oligonucleotide using the formula Tm = 4(G+C) + 2 (A+T), where G, C, A and T indicate the number of corresponding nucleotides in the oligonucleotide.

3. Oligonucleotide primers set MagA:Template DNA (3μl),forward and reverse primers (1μl), 12.5μl of mastermix (2 times) (Fermentas,India) and 7.5μl of DNase free water (Fermants, India) in atotal volume of 25 μl. (Aher et al. 2012).The melting temperature TM of each oligonucleotide using the formula Tm = 4(G+C) + 2 (A+T), where G, C, A and T indicate the number of corresponding nucleotides in the oligonucleotide.

4. Oligonucleotide primers set Kfu: Template DNA (3 μl),forward and reverse primers (1μl), 12.5 μl of mastermix (2 times) (Fermentas,India) and 7.5μl of DNase free water (Fermants, India) in atotal volume of 25 μl. (Ather et al. 2012). The melting temperatur TM of each oligonucleotide using the formula Tm = 4(G+C) + 2 (A+T), where G, C, A and T indicate the number of corresponding nucleotides in the oligonucleotide.

2.2.5.3. Polymerase chain reaction protocol:

1. The reaction was included in a total volume of 50μL in 0.5 ml eppendorf tube containing 25μ L Master mix, 10μ l templet DNA, 5μ L up stream, 5μL downstream primer and 5μL nuclease free water.
2. The reaction mixture was overlaid with 50 µL nuclease free mineral oil to prevent evaporation during thermocycling.
3. The tubes were placed in the thermal cycler previously programmed.
4. At the end of cycling the tubes were stored at -20°C until needed for electrophoresis.

2.2.5.4. Programming thermal cycler:
• Programming thermal cycler for detection of GyrA gene:

The thermal cycler was programmed as follows:(i) one cycle for 15 minutes at 95°C to denaturate the template DNA followed by (ii) 35 cycles of denaturation, annealing and extension at 95°C for 1 min and, 55°C for 1 min and 72°C for 2 min. The 35 cycles were followed by a final cycle of extension at 72°C for 10 minutes to ensure that the entire PCR product was double strand DNA.

• Programming thermal cycler for detection of KPC gene:

The thermal cycler was programmed as follows: one cycle for 5 mints at 94°C to denature the template DNA followed by (ii) 25 cycles of denaturation, annealing and extension at 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 7 mins. The 25 cycles were followed by a final cycle of extension at 72°C for 6 minutes to ensure that the entire PCR product was double strand DNA.

• Programming thermal cycler for detection of Mag gene: One cycle for 1 minute at 94°C to denature the template DNA followed by (ii) 30 cycles of denaturation, annealing and extension at 94°C for 30 seconds, 59°C for 45 seconds and 74°C C for 2 min. The 30 cycles were followed by a final cycle of extension at 72°C for 6 minutes to ensure that the entire PCR product was double strand DNA.

• Programming thermal cycler for detection of Kfu gene:(i) one cycle for 5 mints at 94°C to denature the template DNA followed by (ii) 30 cycles of denaturation, annealing and extension at 94°C for 60 seconds, 54°C for 45 seconds and 72°C C for 60 seconds . The 35 cycles were followed by a final cycle of extension at 72°C for 7 minutes to ensure that the entire PCR product was double strand DNA.

3.2.6. In vitro antibiotic sensitivity test:

Procedures were performed according to (Chess brough, 2000). The sensitivity of microorganisms to different antibiotic discs were measured by the diameter of inhibitory zones and compared with antibiotic susceptibility testing sheet (table 7) to get the result (sensitive, intermediate or resistant).This testing sheet is according to (Antimicrobial Susceptibility Test Discs As per CLSI (Clinical Laboratory Standards Institute) Guidelines (Formerly NCCLS)).

3. RESULTS

3.1. Results of clinical examination of tested chicks:

Results of examination of 150 chicks samples revealed 16 isolate of Klebsiella with percentage of 10%.

3.2. Identification of suspected isolate:

3.2.1. Results of morphological characteristics of Klebsiella: gram negative bacilli, non-spore forming, capsulated, non-motile and arranged singly.

3.2.2. Culture character: on macConkey: lactose fermenting colonies, more or less dome shaped,3-4 mm diameter after overnight incubation at 37°C.

3.2.3. Biochemical identification:

Gave positive reaction for catalase test, vogues proksaure test, citrate test and urease test. Meanwhile the isolates were negative for indole, oxidase and methyl red tests.

3.3. Results of PCR:

3.3.1. Result of polymerase chain reaction for detection of klebsiella genus specific gene (Gyra A):

Sixteen isolates gave positive result (Fig.1, 2).

Figure (1): Electrophoretic analysis of PCR amplified DNA of Klebsiella genus specific gene (Gyra A). Lane M,
3.3.2. Result of PCR for detection of Klebsiella KPC gene:
Seven Klebsiella isolates gave negative results for KPC gene (Klebsiella Pneumoniae Carbapenamase gene) responsible for carbapenems resistance (Fig.3).

3.3.3. Result of PCR for detection of Klebsiella Mag A gene:
Ten Klebsiella isolates were tested for detection of Mag A gene using PCR. Seven isolates gave positive result for Klebsiella Mag A gene (Mucoviscocity attached gene) responsible for mucoviscosity and attachment to host cells (Fig.4).

3.3.4. Result of PCR for detection of Klebsiella Kfu gene:
Ten Klebsiella isolates were tested for detection of Kfu gene using PCR. Eight isolates gave positive result for Klebsiella Kfu gene (Iron uptake system gene) this gene is responsible for virulence of Klebsiella (Fig.5).

3.5. Results of antibiogram:
The 16 Klebsiella isolates were tested for the resistance to antibiotics. The results were the antibiotic of high effect on isolated Klebsiella were Meropenem, Impienem, Amikacin, Cefotaxime sodium, Ceftazidime and Ciproflaxin. Meanwhile, Gentamycin and Cefepime has moderate effect on Klebsiella. Moreover, antibiotics have less effect on the isolated Klebsiella were Azithromycin and Erythromycin. Since isolated Klebsiella are sensitive to Carbapenems (Meropenem and Impienem); Therefore isolated strains are non carbabenem resistant as demonstrated in above PCR negative result for detection of klebsiella pneumonia carpabenamase gene (KPC).

4- DISCUSSION
Results demonstrated by examination of 150 chicks according to clinical observation and isolation revealed the percentage of klebsiella infection among the examined chicks was found to be 10%. The obtained results agree with that of Khalda et al. (2000) (10.2%), Dashe et al. (2013); (8.8%) Dashe et al. (2008), N. Popy et al. (2011), and are differed with the result received by Burtram C. Fielding et al. (2010), Rajaa, et al. (2011) who recorded higher isolation rate of Klebsiella pneumoniae (40.4), Botchris et al. (2012): klebsiella isolated associated with bile and intestinal content of slaughtered chickens (63%) of samples. Also lower prevalence rate was recorded by Hajieh (2008) results who isolated klebsiella form (1%) only of samples tested, Dashe et al. (2008) detected klebsiella in (1.5%) of samples (Aher et al 2012) isolated klebsiella in (6.5%) of collected samples.
The selected isolation media used in this test was MacConkeys agar media after incubation 24 hrs colonies appear: lactose fermenting colonies, more or less dome shaped, 3-4 mm diameter after overnight incubation at 37°C. This result agree with that of Aher et al. (2012). In the present study, magA, kfu and kpc genes were detected by PCR using specific primer sequences which yielded product sizes of 1280 bp, 797 bp and 638 bp, respectively. Out of total 10 isolates, 7 isolates (70%) was positive for magA gene (Figure 4), 8 isolates (80%) were positive for Kfu gene (Figure 5) and all isolates were negative for KPC gene (Figure 3). This result concised with result of Chi-Tai Fang et al. (2004) who reported that magA (mucoviscosity-associated gene A), was present in 52 out of 53 invasive strains (98%; the sole strain negative for magA was from the patient with liver cirrhosis) and in 15 out of 52 noninvasive strains, with Carsten Struve et al. (2005) who recorded that all 39 magA-positive isolates were of the K1 capsule serotype, indicating a close relationship between magA and the K1 capsule serotype and with Sara K Sobirk (2010) who recorded that the isolates were positive for all four virulence genes mmpA, aerobactin, kfu and allS as revealed by polymerase chain reaction using specific primers. Thus the isolates exhibited similar characteristics as the highly virulent K. pneumoniae isolates that have so far mainly been observed. On the other hand; The results of the present study are in contrast with reports of Fang et al. (2004), Ma et al. (2005), Turton et al. (2007) and Aher et al. (2012). Who detected the prevalence of virulence associated genes magA and kfu genes were found at the rate of 12.5% and 25%, respectively. And Yu et al. (2006) who detected prevalence at the rate of 29% (Mag A gene) and 35% (kfu ). The negative result for detection of KPC gene is concised with result of Vered Schechner et al. (2009) who recorded that Five of the nine specimens that grew CRE but were blaKPC negative (Nine specimens grew CRE (all isolates were K. pneumoniae) but were blaKPC negative), also with Woodford N et al. (2008) who reported that the prevalence of carbapenem-resistant organisms in other parts of Europe is generally confined to imported cases and with Tsakris A et al. (2012) who confirmed that KPC-producing organisms rarely manifest as community onset infections in non-endemic regions without any prior health care contact. Probably this is due to the reduced fitness of organisms that lose their major porin. In the present study Since isolated Klebsiella are sensitive to Carapenems (Meropenem and Impienem). Therefore isolated strains are non-carapenem resistant as demonstrated in above PCR negative result for detection of klebsiella pneumonia carapenamase gene (KPC) as shown in fig. (2). But this result is in contrast with the results of Kitchel et al. (2009), Urban C et al. (1994), Leavitt A et al. (1997) and with Won SY (2011). Who recorded high prevalence of Klebsiella Pneumoniae harbouring KPC gene.

The result of antibiotic sensitivity test nearly conferred with those of (David et al. 2004) who treated Forty-nine episodes of bacteremia due to ESBL-producing K. pneumonia with monotherapy active in vitro (carapenems were used in 27 cases; ciprofloxacin was used in 11; cephalosporins were used in 5; β-lactam/β-lactamase inhibitor combinations were used in 4; and amikacin was used
in 2). And with results of Iroha et al. 2011 who analyzed all samples and organisms isolated using standard Microbiology techniques, antibiotic susceptibility testing was carried out as described in the manual of antibiotic susceptibility testing. Clonal relatedness of resistance strains of K. pneumoniae from different samples was determined by randomly amplified polymorphic DNA (RAPD). Antibiotic susceptibility studies revealed that Klebsiella pneumoniae strains from wound samples were the most susceptible strains followed by HVS, sputum and urine. The overall susceptibility profile is as follows; imipenem (100%), amikacin (100%), cefoxitin (99.4%), aztreonam (98%), ceftazidime (98%), cefotaxime (96.7%), amoxicillin/clavulanic acid (96%), ciprofloxacin (96%), tobramycin (93.3%), kanamycin (90%), cefuroxime (86.7%), gentamicin (76%), sulphonmethoxazole/trimethoprim (22%), chloramphenicol (15.4%) and ampicillin (5%). But the current antibiotic sensitivity test differs from that of G L French et al. (1996) who observed an aminoglycoside- and cephalosporin-resistant strain of Klebsiella pneumoniae K2 producing the extended-spectrum beta-lactamase SHV-5 infected or colonized 14 pediatric patients. Also differs with Jan IS et al. (1998) who noticed that K. pneumoniae collected from December 1995 through March 1997 were found to be resistant to at least one of the third-generation cephalosporins (cefotaxime and ceftazidime) or aztreonam using the routine disk diffusion method.

5- Conclusions

Incidence of Klebsiella depends on hygienic condition of the farm, age, season and breed. Mucoviscosity attaches gene (Mag A) gene and Iron uptake system gene Kfu genes are considered, important virulent genes. Isolated klebsiella strains are non Carbapenem resistant strains. Klebsiella infection to be treated we should do antibiotic sensitivity test because there is change in resistance due to long use of specific antibiotic and bad use of the other.

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7- REFERENCES


French, G. L., Shannon, K. P., Simmons, N. 1996. Hospital outbreak of Klebsiella pneumoniae resistant to broad-spectrum cephalosporins and beta-lactam-beta-


