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Molecular Detection of Genus Klebsiella and Genotypic Identification of *Klebsiella pneumoniae* and *Klebsiella oxytoca* by Duplex Polymerase Chain Reaction in Poultry

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Abstract: Bacteriological examination of 360 internal organs (lung, liver, spleen and intestine) collected from 90 diseased chicken suffered from respiratory manifestation revealed isolation of Klebsiella species with an incidence 22.78%. The highest percentage of isolation was in liver 24% followed by spleen, intestine and lung with percentage rate (22%, 19% and 17%) respectively which indicate that Klebsiella could be probably affected birds by concurrent extra intestinal infection. Biochemical identification of *Klebsiella* species revealed isolation of *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Klebsiella rhinscleromatous* with percentage rate (7.78%, 10.83% and 4.17%) respectively. It was noticed that the highest percentage of isolation of *Klebsiella pneumoniae* was in lung sample 10%. The goal of this study was to compare between the results of culture method in isolation and identification of Klebsiella species and polymerase chain reaction. The distinctive identification of *Klebsiella* to species level is often difficult, time consuming and laborious. Twenty seven out of thirty-three samples were positive by PCR using genus specific primer (*gyr*A). Seven samples could amplify a region of 441bp which it was negative by culture method this could be attributed to misidentification with different biochemical test or lack of sensitivity of cultural method. Twenty samples were positive by two methods and 6 samples were negative by both method. To differentiate *Klebsiella pneumoniae* from *Klebsiella oxytoca* a duplex PCR was performed using species specific primers (16S-23S ITS and *peh*X) respectively. Sixteen out of 27samples could amplify a primer 16S-23S ITS at 130 bp fragment. All the seven samples culturally negative but positive by *gyr*A gene belonged to *Klebsiella pneumoniae* .Meanwhile one sample wrongely identified as *Klebsiella pneumoniae* by biochemical test and could amplify a region of 343bp using *peh*X gene of *Klebsiella oxytoca*. In conclusion, due to difficulties in identifying *Klebsiella* species by biochemical method to overcome the misidentification and wrongly identified problems. A PCR can be used for detection of *Klebsiella* species from clinical samples in poultry farms and identification of the two clinically important *Klebsiella* species *Klebsiella pneumoniae* and *Klebsiella oxytoca*.

Key words: *K. pneumoniae* · *K. oxytoca* · PCR- *peh*X · 16S-23S ITS · *gyr*A

INTRODUCTION

Klebsiella are opportunistic pathogen that cause infections whenever the immune system of affected bird is compromised [1].

In poultry they are found to be associated with different disease as respiratory affections, septicaemia, peritonitis, salpingitis, air sac disease, omphalitis, artheritis, panopthalmitis, intestinal disturbances and drop in egg production [2].

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Klebsiella typically express 2 types of antigens on their cell surface. The first is a lipopolysaccharide (O antigen); the other is a capsular polysaccharide (K antigen). Both of these antigens contribute to pathogenicity [3].

Klebsiella pneumoniae can produce several virulence factors such as smooth LPS, pilli for adhesion to host cells, capsule that are antiphagocytic, siderophores that aid the bacterium in its competition with the host for iron uptake [4].

In clinical microbiology laboratories, identification of *Klebsiella* strains to the species level is often difficult using conventional methods or even by using automated instruments based on classical biochemical tests such as the Vitek and API system. It is often misclassified because some of the species share similar biochemical profile [5, 6].

Phenotypic distinction between *Klebsiella pneumoniae* and *Klebsiella oxytoca* based on existing biochemical test is not reliable and is time consuming and laborious [7].

A number of DNA-based methods for the detection of pathogenic Klebsiella spp. has been developed, including PCR and restriction fragment length polymorphism analysis based on the *gyr*A gene [8].

DNA gyrase, which is composed of two A subunits and two B subunits, is encoded by the *gyr*A and *gyr*B genes. The genes encoding subunit A of DNA gyrase (*gyr*A), a protein that correspond to the main target of fluroquinololnes in Klebsiella is used for detection of genus Klebsiella [9].

A PCR method based on internal transcribed spacer (ITS) gene sequence has been developed for the detection and identification of *K. pneumoniae* subsp. *pneumoniae* [10, 11].

The rRNA genes (16S, 23S and 5S) are ideal candidates for bacterial identification and evolutionary studies, because they are highly conserved within the species [12]

The 16S–23S rRNA gene internal transcribed spacer (ITS) gene sequence, which is not subject to the same selective pressure as the rRNA genes and consequently has a 10-times-greater evolution rate, appears to be able to overcome the apparent limitation of rRNA genes [13, 14].

Kovtunovych[15]reported the development a PCR assay targeting the polygalacturonase (*peh*X) gene that could coding the enzyme polygalacturonase that cleaves a polygalacturonic chain of demethoxylated pectin discriminate *K. oxytoca* from other species of *Klebsiella* and suggested it's use for clinical and ecological monitoring of *Klebsiella oxytoca*.

The purpose of the study reported here was initially to estimate the prevalence of Klebsiella species in chicken suffered from respiratory manifestation, compare between conventional culture method and polymerase chain reaction detection of genus Klebsiella further more differentiate *Klebsiella pneumoniae* and *Klebsiella oxytoca* from clinical samples by duplex PCR.

MATERIALS AND METHODS

A total of 360 samples (lung, liver, spleen and intestine) were collected from 90 chicken suffered from respiratory manifestation. Dakhalia Governorate, Egypt.

Bacteriological Examination: Samples collected internal organs were inoculated directly on nutrient broth and incubated aerobically at 37°C for 18-24 hours [16], then a loopful from incubated broth was streaked on MacConkey's bile salt agar and Xylose Lysine Desoxycholate (XLD) agar and incubated at 37°C for 24-48 hrs. The suspected mucoid lactose fermenting colonies were purified and identified biochemically by catalase, oxidase, indole production, methyl red, Voges-Proskauer, citrte utilization, lysine decarboxylase, urea hydrolysis and sugar fermentation according to Barbara *et al*. [17].

A total of 33 clinical samples, 20 *Klebsiella* culture positive (10 *Klebsiella pneumoniae* and 10 *Klebsiella oxytoca*) and 13 suspected organs that yield mucoid lactose fermenting colony by the cultural method but differ in one or more of biochemical tests (urea and citrate) so considered negative by cultural method. Then the organs pre-enriched on buffered pepton water for DNA extraction.

Extraction of Bacterial DNA: according to QIAamp DNA mini kit instructions Catalogue no. 51304.

Molecular detection of genus *Klebsiella* by *gyr*A gene and *K. pneumoniae*, *K. oxytoca* using species specific primer (16S-23S ITS and *peh*X) respectively: Oligonucleotide primers used in PCR from Metabion (Germany), primers sequence and product shown in Table (1). Preparation of PCR Master Mix according to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit:

Table 1: Oligonucleotide primers sequences used for amplification of genus *Klebsiella* and species specific primers genes:

Target gene	Primers sequences	Amplified segment (bp)	Reference
*Klebsiella gyr*A	CGC GTA CTA TAC GCC ATG AAC GTA ACC GTT GAT CAC TTC GGT CAG G	441	Brisse and Verhoef, 2001
K.pneumoniae 16S-23S ITS	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTC	130	Turton *et al*., 2010
*K. oxytoca peh*X	GATACGGAGTATGCCTTTACGGTG TAGCCTTTATCAAGCGGATACTGG	343	Chander *et al*., 2011

Table 2: Cycling conditions of the different primers during PCR:

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	Final extension
*gyr*A	94°C 5 min.	94°C 30 sec.	55°C 45 sec.	72°C 45 sec.	72°C 10 min.
K.pneumonia and K. oxytoca	94°C 5 min.	94°C 30 sec.	55°C 30 sec.	72°C 40 sec.	72°C 10 min.

Preparation of *gyr*A Uniplex PCR Master Mix: PCR was carried out with template DNA (6 µl), forward and reverse primers (1µl), 12.5 µl of Emerald Amp GT PCR master mix (2 x premix) and 4.5 µl of PCR grade water in a total volume of 25 µl [18].

Preparation of *K. pneumonia* and *K. oxytoca* Duplex PCR Master Mix: PCR was carried out with template DNA (8 µl), forward and reverse primers (1 µl for each), 25 µl of Emerald Amp GT PCR master mix (2 x premix) and 13 µl of PCR grade water in a total volume of 50 µl.

Temperature and time conditions of the primers during PCR are demonstrated in Table 2 according to specific author and Emerald Amp GT PCR mastermix (Takara) kit. Electrophoresis of amplified products was carried out according to Sambrook and Russell [19]. Using 1.5% agarose gel stained with ethidium bromide and detected by UV transillunination. Amplified genes were identified on the basis of fragment size. The ladder was mixed gently by pipetting up and down. 6 µl of the required ladder were directly loaded.

RESULTS

Bacteriological examination of internal organs from 90 diseased chicken revealed isolation of Klebsiella species from 33 birds with an incidence 36.67% out of 360 internal organs. Klebsiella species was recovered from 82 organs with an overall incidence 22.78%.

The prevalence rate of *K. oxytoca* was 10.83% which was higher than that of *K. pneumoniae* and *K.rhinoscleromatous* 7.78% and 4.17 respectively. The highest percentage of isolation of *K. pneumoniae* was in lung 10% followed by liver 8.89% then intestine 6.67% and finally in spleen 5.56%. Meanwhile he highest percentage of isolation of *K.oxytoca* was in spleen followed by liver then intestine and finally lung by (15.56%, 12.22%, 10% and 5.56%) respectively. While *K. rhinoscleromatous* was recovered from liver then intestine and was equal in lung and spleen with percentage rate (5.56%, 4.44 %, 3.33% and 3.33%) respectively as shown in Table 3.

Detection of Genus Klebsiella by Genus Specific Gene (*gyr*A): A total of 33 clinical samples were screened by PCR to amplify the (*gyr*A) gene. Twenty-seven samples harbor *gyr*A gene and could amplified at 441bp fragment. As shown in Fig. (1, 2 and 3).

It was noticed that 7 samples in lane (4, 8, 12, 14, 15, 17 and 23) out of 13 Klebsiella culture negative could amplified the 441bp with incidence 53.85% as shown in Fig. (1, 2).

Identification and Differentiation of Klebsiella by Using Duplex Polymerase Chain Reaction: 27 *Klebsiella* positive samples detected by *gyr*A gene were differentiated by duplex polymerase chain reaction either to *Klebsiella pneumoniae* or *Klebsiella oxytoca* according to species specific gene *peh*X for *Klebsiella oxytoca* which was amplified at 343 bp fragment and 16S-23S ITS for *Klebsiella pneumoniae* which was amplified at 130 bp fragment, There were 59.26% (16/27) samples positive for *Klebsiella pneumoniae* and 40.74%(11/27) samples was *Klebsiella oxytoca* by species specific genes. As shown in Fig (4 and 5)

It was noticed that all 7 samples in lane (4, 8, 12, 14, 15, 17 and 23) detected by *gyr*A and negative by cultural method belong to *Klebsiella pneumoniae*. Meanwhile sample in lane 22 improbably identified as *K. pneumoniae* by biochemical test but could amplify 343bp by *peh*X gene of *K.oxytoca*. As shown in Fig. (5).

Table 3: Prevalence of different *Klebsiella* spp. in examined samples.

		K. pneumoniae		*K. oxytoca*		*K. rhinoscleromatous*		Total *Klebsiella*	
Samples	No of examined samples	No	%*	No	%*	No	%*	No	%*
Lung	90	9	10	5	5.56	3	3.33	17	18.89
Liver	90	8	8.89	11	12.22	5	5.56	24	26.67
Spleen	90	5	5.56	14	15.56	3	3.33	22	24.44
Intestine	90	6	6.67	9	10	4	4.44	19	21.11
Total	360	28	7.78**	39	10.83**	15	4.17**	82	22.78**

*The percentage was calculated according to the total number of each organ

**The percentage was calculated according to the total no. of organs

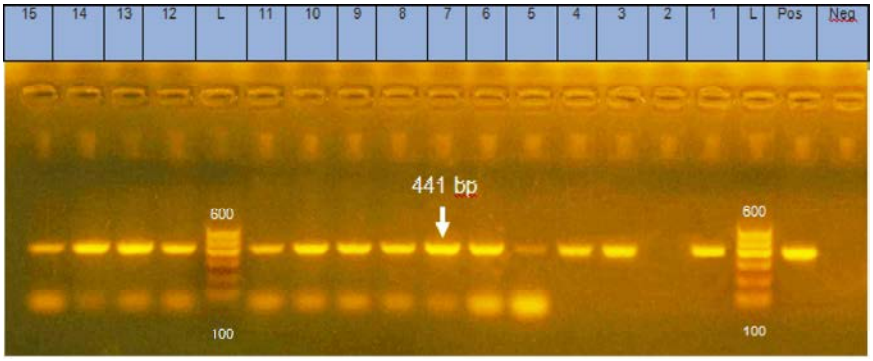

Fig. 1: Agar gel electrophoresis showing PCR amplification at 441 bp fragment for *gyr*A gene
M) 100 bp DNA ladder; control positive Klebsiella species, control negative *E.coli*, lane (1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15) positive samples; lane (2) negative sample

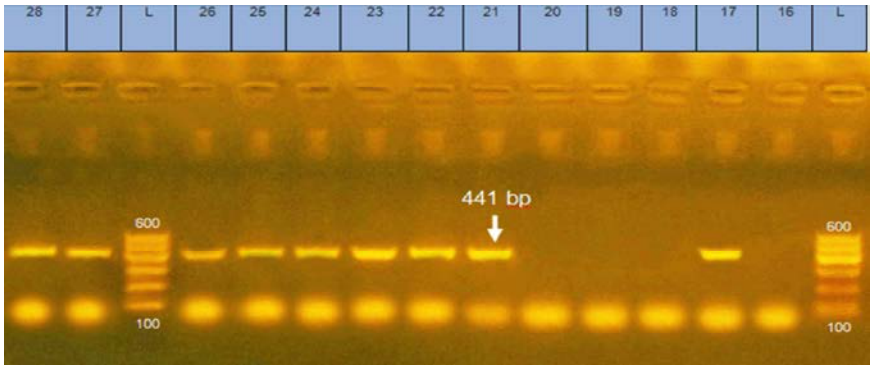

Fig. 2: Agar gel electrophoresis showing PCR amplification at 441 bp fragment for *gyr*A gene
M) 100 bp DNA ladder, lane (17, 21, 22, 23, 24, 25, 26, 27 and 28) positive; lane (16, 18, 19 and 20) negative

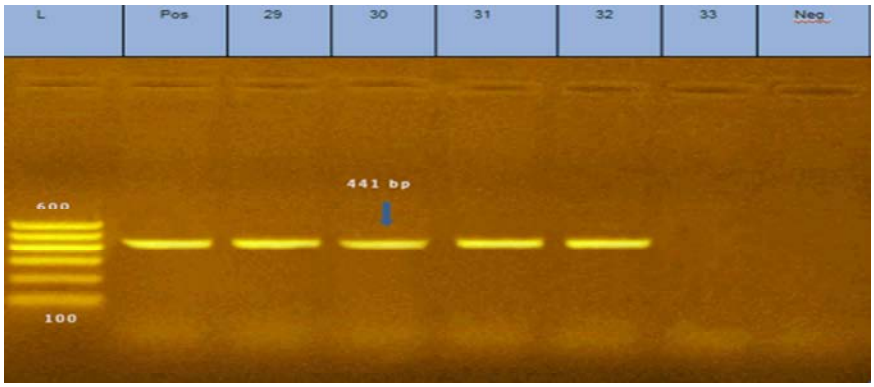

Fig. 3: Agar gel electrophoresis showing PCR amplification at 441 bp fragment for *gyr*A gene. M) 100 bp DNA ladder, lane (29, 30, 31and 32) positive; lane (33) negative

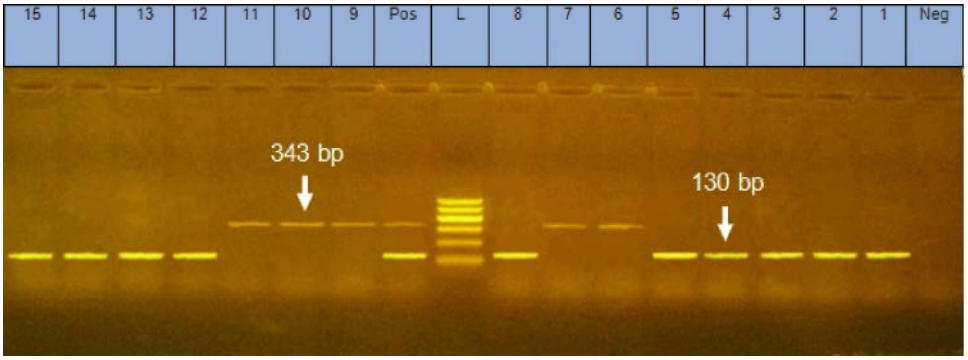

Fig. 4: Agar gel electrophoresis showing PCR amplification at 130 bp and 343bp fragment for 16S-23S ITS and *Peh*X gene respectively
Lane (1, 2, 3, 4, 5, 8, 12, 13, 14 and 15) +ve for 16S-23S ITS gene at 130 bp for *K.pneumoniae*; Lane (6, 7, 9, 10 and 11) +ve for *Peh*X gene at 343 bp for *K.oxytoca*.

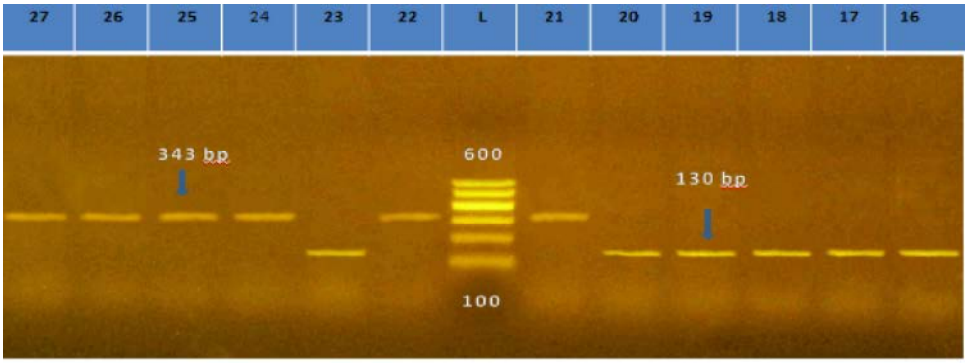

Fig. 5: Agar gel electrophoresis showing PCR amplification at 130 bp and 343bp fragment for 16S-23S ITS and *Peh*X gene respectively
Lane (16, 17, 18, 19, 20 and 23) +ve for 16S-23S ITS gene at 130 bp for *K.pneumoniae*
Lane (21, 22, 24, 25, 26 and 27) +ve for *Peh*X gene at 343 bp for *K.oxytoca*
Sample (30, 31 and 32) in lane (16, 18 and 19) respectively
Sample (21, 22, 23, 24, 25, 26, 27 and 28) in lane (20, 21, 22, 23, 24, 25, 26 and 27) respectively

DISCUSSION

The diseases of bacterial etiology represent important factors in poultry production, therefore the sources of spreading the infection in poultry flocks and possible economic losses, which they induce, need to be investigated especially bacteria which could cause infection with high rate of morbidity and mortality [20]. The control measures of klebsiellosis mainly depend on the isolation, identification and treatment of the infected birds.

Klebsiella species were recovered from 33 out of 90 diseased chicken with an incidence 36.67%.

This finding is lower than that reported by Türkyilmaz [21] who recovered Klebsiella species with a prevalence rate 47.1%. While it was higher than that reported by Hossain [22] and Khalda [23] and Aly [2] and Younis [1] with a percentage rate (6%, 8.69%, 10.2%, 10% and 15%) respectively.

The variable results of *Klebsiella* incidence could be attributed to differences of hygiene and sanitary measures in examined farms.

In the present study 82 isolates of Klebsiella were recovered from 360 organs (lung, liver, spleen and intestine) with an overall incidence 22.78% as shown in Table 3.

The isolation rate of Klebsiella from liver was higher than other organs. These results disagree with Younis [1] who said that of Klebsiella species recovered from lungs is higher percentage than the other organs. The wide distribution of *K. pneumoniae* in the lungs, spleen and liver of birds affected could probably indicate concurrent extra–intestinal infections Türkyilmaz [21].

One of the most important tests to differentiate between Klebsiella species is the indole test which is negative in *K.pneumoniae, K. rhinoscleromatous* and *K.ozonae*. While it was positive in *K.oxytoca* and *K.ornitholytica* Koneman [24] reported that the production of tryptophan could be used to separate the two principle species as *K. pneumoniae* and *K. oxytoca*.

Indole negative Klebsiella isolates could be differentiated by urease production test as *K.pneumoniae* gave positive result while *K. rhinoscleromatous* and *K. ozonae* gave negative result also methyl red test could differentiate between indole negative Klebsiella species as *K. pneumoniae* gave negative result while *K. rhinoscleromatous* and *K. ozonae* gave positive result. At the same time *K. pneumoniae* citrate positive while *K. rhinoscleromatous* gave negative result and *K. ozonae* mostly negative for citrate.

Indole positive Klebsiella species could be differentiated by methyl red and ornithine decarboxylase test as *K.oxytoca* gave negative result while *K.ornitholytica* gave positive result.

K. pneumoniae could be isolated with a percentage rate (7.78%) while higher percentage rate (64% and 73.33% and 18%) was reported by Ajayi [25], Younis [1] and Kumbish [26] respectively. A lower incidence was recorded by Dashe [27] and Yehia and Riyadh [28] with percentage rate (1.5% and 3.33%) respectively. *K. oxytoca* was recovered with an incidence (10.83%) while reported that it was isolated with a percentage rate (1.66% and 26.67%) detected by Yehia and Riyadh[28] and Younis [1] respectively.

Current biochemical methods of identification are time consuming and often are inconclusive because related species e.g, *K.pneumoniae* and *K.oxytoca* or even *Enterobacter aerogenes*, often present similar biochemical patterns lopes [11].

Distinctive identification of the species *K. oxytoca*, *K. pneumoniae*, *K. planticola*, *K. ornithinolytica* and *K. terrigena* is difficult based on phenotypic tests and misidentifications are frequent in routine clinical microbiology Kovtunovych [15].

*Gyr*A primers was designed based on the alignment of the *gyr*A and *par*C *E.coli* sequences with those of other bacteria available in public sequence database. These primers were amplify a portion of the genes broader than those generally amplifed to determine solely mutations in the quinolones resistance determining region that were used for all Klebsiella species Brisse and Verhoef [9].

Twenty- seven samples were positive by *gyr*A and could amplify a region of 441bp as shown in Fig. (1, 2 and 3). It was noticed that 7 samples in lane (4, 8, 12, 14, 15, 17 and 23) were positive by PCR more than cultural method. The negative result by culture might be unexplained by loss of viability of bacteria with specimen handling or lack of sensitivity of cultural method. It was most probaply due to misidentified with different biochemical tests. All twenty positive *Klebsiella* by cultural method could amplifying a region of 441 bp fragment. This result in accordance with Chander [7] and Younis [1].

To discriminate of *K.pneumoniae* and *K.oxytoca*, the two *Klebsiella*species of major clinical importance.

Duplex PCR was performed with 27 samples that yielded positive result with *gyr*A. Sixteen samples could amplify a region of 130 bp 16S-23S ITS primer (10 culturally identified as *K.pneumoniae* and 7 samples in lane (4, 8, 12, 14, 15, 17 and 23) untyped biochemically as they differ in one or more of biochemical test (urea and citrate).

It was noticed that one sample in lane 22 wrongly identified biochemically as *K.pneumoniae* but it was *K.oxytoca* by PCR and this result in accordance with Kovtunovych [15].reported that 5 (3%) *K.oxytoca* straines had been identified incorrectly by biochemical test and detected by PCR.

Monnet[29] said that up to 13% of Klebsiella isolates were misidentified using biochemical method.

Further biochemical tests were recommended by Kovtunovych [15] to differentiate *K. oxytoca* from *K.pneumoniae* by biochemical test as (growth at 10°C, D-Melezitose, gas production from lactose at 44.5°C and pectate degradation).

CONCLUSION

Due to difficulties in identifying Klebsiella species by biochemical method to overcome the misidentification and wrongly identified problems. PCR can be used for detection of Klebsiella species from clinical samples in poultry farms and identification of the two clinically important *Klebsiella* species *Klebsiella pneumoniae* and *Klebsiella oxytoca* by duplex PCR

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