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Widespread acquisition of antimicrobial resistance of *Klebseilla pneumoniae* isolated from raw milk and the effect of cinnamon oil on such isolates

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Abstract

The incidence of *Klebsiella* spp. (K) in raw milk of different animal species was determined. A total of 240 random raw milk samples were collected from cows, buffaloes, sheep and goats in Assiut and Qena cities, Egypt (30 samples each). Thirty one strains of *Klebsiella* were isolated from raw milk. On the basis of biochemical characterization the strains were divided into 4 species as follow *K. pneumonia, K. ozaenae, K. planticola* and *K. rhinoscleromatis.* The most prevalent isolated species was *K. pneumonia* which was isolated from 13.33% of buffaloe's milk samples collected from Assiut City and from 10% of Goat's milk samples collected from Qena City. *K. pneumonia* show in vitro resistance to almost all tested types of antibiotics except for Ciprofloxacin and Ofloxacin. The antimicrobial effect of cinnamon oil on *K. pneumonia* isolates was tested via performing disk diffusion method and minimum inhibitory concentration. The results revealed that cinnamon oil showed maximum activity with MIC values ranging from 1:25 mg/ml to 1:6.25 mg/ml. The study concluded that cinnamon oil could be used as an effective antimicrobial.

Introduction

Members of the genus *Klebsiella* (K) are frequently incriminated in different infections. Klebsiella pneumonia was isolated from mastitic cows especially from those kept in wood products bedding (Carter, 1995). In humans, *K. pneumoniae* is an important cause of nosocomial infections like pneumonia, septicaemia, urinary tract infection, and life-threatening septic shock (Alves *et al*., 2006; Hentschke *et al*., 2010). *Klebsiella rhinoscleromatis,* that is unable to utilize citrate, induces tissue destructive infections in the nose and pharynx besides its effect on the urinary tract soft tissue as a secondary invader (Joklik *et al*., 1992).

Epidemic and endemic nosocomial infections caused by *Klebsiella* spp. are leading causes of morbidity and mortality (Cryz *et al*., 1985). Recently, World Health Organization also warned the community that multidrug resistant bacteria are emerging worldwide which is a big challenge to healthcare. If we didn't take immediate action then antibiotics may lose their power to cure diseases (Young *et al*., 2011). Multidrug resistant bacteria cause serious nosocomial and community acquired infections that are hard to eradicate by using available antibiotics. Medicinal plants and their essential oils have been shown to possess antibacterial, antifungal, antiviral, insecticidal, and antioxidant properties (Burt, 2004).

Cinnamon *(Cinnamomum verum)* and their essential oil contain both antifungal and antibacterial activity that can be used as antibiotics and to prevent food spoilage due to bacterial contamination (Dragland *et al*., 2003). For these reasons, this study aimed at (i) isolation and identification of *Klebsiellae* from raw milk in Assiut and Qena cities in Egypt, (ii) to quantify the occurrence of antimicrobial resistance, and (iii) to evaluate the ability of cinnamon oil to inhibit the growth of different multi-drug resistant *K. pneumoniae* isolates with different resistance patterns.

Materials and Methods

Samples collection

A total of 240 random raw milk samples were collected from cows, buffaloes, sheep and goats in Assiut and Qena cities, Egypt (30 samples each). Milk sample were mixed and tested for heat treatment using Storch test (Lampert, 1975).

Enrichment procedure

One ml of the prepared milk samples were inoculated into a sterile test tube containing 10 ml of luria enrichment broth (Biolife) (selective enrichment

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broth for *Klebsiella* spp.).The inoculated broth was incubated at 37°C for 24 hours.

Isolation of Klebsiella *spp.*

Isolation was done using surface spreading technique on MacConkey- Inositol- Carbenicillin agar according to Atlas and Parks, (1994). The plates were incubated for 24 hours at 37°C. Typical *Klebsiella* colonies appear pink to red colonies indicating inositol fermentation. Colonies presumed to be *Klebsiella* were transferred to nutrient agar slants, which were incubated at 37°C for 24 hours for morphological and biochemical tests.

Identification of isolates

Klebsiella isolates were identified microscopically according to (Harrigan and MacCance, 1976) and by motility test (Baron *et al*., 1994). The biochemical procedures of *Klebsiella* identification have been standardaized by (Koneman *et al.*, 1992). They involve Methyl red test, voges proskauer test, citrate utilization test, urease test and indol production test. Besides these tests Triple sugar iron (TSI) agar reaction (Baron *et al.*, 1994), Sugar fermentation reaction (A.P.H.A. 1984), Decarboxylation of ornithin and lysine (FAD 1998), Growth at 5 °C and 41°C (Kwan and Skura, 1985) and Gelatin hydrolysis (Cowan and Steel, 1974) were also checked.

Antimicrobial sensitivity testing of Klebsiella Pneumoniae

The antibiotic sensitivity test and their interpretation were done using the disc diffusion method according to (Baron *et al*., 1994) as follow; *Klebsiella Pneumoniae* strains isolated in this study from milk samples of different animal species were grown at 37°C for 24 h in luria enrichment broth to obtain 1x106 cfu/ml. by comparing its opacity to MacFarland 4 turbidity standard. MacConkey-Inositol- Carbenicillin agar plates without antibiotics were swabbed with broth culture and allowed to solidify. The following antibiotics discs (Oxoid) were used to determine the pattern of resistance; Amoxicillin 20 µg, Amickacin 30 µg, Cefotaxime 30 µg, Chloramphenicol 30 µg, Ciprofloxacin 5 µg, Gentamicin, 10 µg, Ofloxacin 5 µg, Piperacillin 100 µg, Tetracycline 30 µg and Trimethoprim 2.5 µg. The discs of ten antibiotics were placed on the surface of agar plates using sterile forceps. All plates were incubated at 37°C for 24 h. The inhibition growth zone diameter was measured in mm with a ruler. The zone diameter (mm) of resistance was accepted as recommended by NCCLs (2002). A control plate containing no antibiotic was also inoculated with the broth.

Cinnamon oil disk preparation

Cinnamon oil with different concentrations of (1:100, 1:50, 1:25, 1:12.5, and 1:6.25 mg/ml) was kindly obtained by the Department of organic chemistry Faculty of Science, Assiut University and was sterilized by autoclaving at 121oC for 15 min (Mahmoud, 1993). The disks were prepared according to Barry (1976) in which empty sterilized discs (Whatman no. 6 mm diameter) were impregnated with 50 µL per disk with different concentrations of cinnamon oil and kept in a sterile container in the refrigerator to be used during one month. The disks were placed on and swabbed over the surface of MacConkey- Inositol- Carbenicillin agar plates without antibiotics that inoculated with 25 µl of the previously prepared inoculum. All petridishes were sealed with sterile laboratory parafilm to avoid eventual evaporation of the test samples. The plates were left for 30 min at room temperature to allow the diffusion of oil, and then they were incubated at 37°C for 24 h. After the incubation period, the zone of inhibition was measured. Inhibition of bacterial growth in the plates containing tested oil was judged by comparison with growth in blank control plates. The MICs were determined as the lowest concentration of oil inhibiting visible growth of K. pneumoniae on the agar plate according to the method recommended by the NCCLs (2000).

Statistical analysis

The incidence of *Klebsiella* spp. was calculated by dividing the number of positive samples by the total number of examined samples. Data were entered into the Microsof Excel spreadsheet. Comparison between minimal inhibitory concentrations (MIC) of cinnamon oil with antibiotic sensitivity test was done using Pearson's correlation coefficient. The correlation coefficient was 0.90 and was significant at $P \leq 0.001$.

Results and Discussion

Milk and dairy products are essential foods for consumers in all ages. Contamination with virulent pathogens renders them to be a source of public health hazard. The possible contamination sources are either mastitic dairy cows (Carter, 1995) and/or unhygienic production environment circumstances (personnel, utensils and machines, transportation, storage). The gastrointestinal tract and the hands of personnel were reported as principal reservoirs of Klebsiella (Podschun and Ullmann, 1998). Results

Table 1. Incidence of *Klebsiella* spp. in the examined milk samples

Types of examined samples	Sources of samples			
	Assiut city		Qena city	
	No. of positive samples/30	%	No. of positive samples/30	%
Cow's milk	3	10	5	16,66
Buffaloe's milk	6	20	2	6.66
Sheep milk	4	13.33	2	6.66
Goat's milk	2	6.66	7	23.33
Total	15	49.99	16	53.31

Table 2. Incidence of different *Klebsiella* spp. recovered from the examined milk samples collected from Assiut city

Isolated strain	Cow's milk		Buffaloe's milk		Sheep milk		Goat's milk	
	No./30	%	No./30	%	No./30	%	No./30	%
K. pneumoniae	3	10	4	13.33	2	6.66	2	6.66
K. ozaenae	–	–	1	3.33	–	–	–	–
K. rhinoscleromatis	–	–	1	3.33	2	6.66	–	–

illustrated in Table 1 revealed that the incidence of *Klebsiella* spp. was nearly similar in examined milk samples collected from both Assiut (49.99%) and Qena (53.31%) cities. Higher incidence (82.3%) and (59.7%) were detected by El-Sukhon (2003) and Gundogan and Yakar (2007), respectively. The obtained results indicate that dairy cattle might represent the most possible contamination sources. Presence of *Klebsiella* in milk is not surprising in view of the fact that they are widely distributed in nature and contaminate milk. The problem was complicated by the absence of cool system that may enhance the multiplication of pathogenic microorganisms.

From the results recorded in Table 2 it is clear that the most prevalent isolated species was *K. pneumoniae* which was isolated from 13.33% of buffaloe's milk samples collected from Assiut City. Lower prevalence 9.7% was recorded by El-Sukhon (2003). Higher prevalence of 58% was detected by Gundogan and Yakar (2007). Data recorded in Table 3 showed that *K. pneumoniae,* was the most prevalent species in the examined milk samples collected from Qena City. *K. ozaenae, K. planticola* and *K. rhinoscleromatis* could be isolated in variable percentage from the examined cows, buffaloes, sheep and goats milk samples collected from Qena City.

Concerning antimicrobial sensitivity of 18 *Klebsiella Pneumoniae* isolates against 10 chosen antimicrobial agents it was found that high prevalence of amoxicillin and tetracycline resistance was detected for *Klebsiella pneumonia* (100%). Variable resistance was found also against cefotaxim (77.8%), Chloramphenicol (61.1%), Gentamicin (88.9%), Piperacillin (94.5%) and Trimethoprim (83.3%). Multiple resistances to antimicrobial agents were very common in the present isolates. The emergence of resistance to antimicrobial agents is a global public health concern not only because it kills but because it increases health costs and threatens patient care (Young, 2011). Moreover, uses of broad spectrum antibiotics, insufficient aseptic condition and technique with inadequate control of infections spread had aggravated this problem.

Disc diffusion method was used to evaluate the zone of microbial growth inhibition at various concentrations of cinnamon oil and the results showed that inhibition zones varied from 2-22 mm in diameter. *K. pneumonia* proved to be sensitive to the effect of cinnamon oil in which the growth was inhibited with a zone of 13 mm in diameter, this zone considers a good result if it is compared with the results of antibiotic sensitivity test in which it formed 100% resistance for the tested antibiotics. Depending on these results, it can be concluded that antibacterial effect of cinnamon oil is almost close to the effect of amoxicillin and tetracycline. Similar results were recorded by Prabuseenivasan *et al.,* (2006), who found that cinnamon oil (in concentration of 1:5 mg/ml) was significantly able to inhibit the growth of *K. pneumonia*. In another study Simic *et al.*, (2004), suggested that cinnamon oil was very effective against pathogens even in moderate concentrations.

Minimal inhibitory concentration (MIC) of cinnamon oil was determined and compared with

Table 3. Incidence of different *Klebsiella* spp. recovered from the examined milk samples collected from Qena City

Isolated strain	Cow's milk		Buffaloe's milk		Sheep milk		Goat's milk	
	No./30	%	No./30	%	No./30	%	No./30	%
K. pneumoniae	2	6.66	–	–	2	6.66	3	10
K. ozaenae	1	3.33	1	3.33	–	–	1	3.33
K. planticola	1	3.33	–	–	–	–	1	3.33
K. rhinoscleromatis	1	3.33	1	3.33	–	–	2	6.66

the results of antibiotic sensitivity test and the results revealed that cinnamon oil showed maximum activity with MIC values ranging from 1:25 mg/ml to 1:6.25 mg/ml. It seems that the oil was found to be strongly bactericidal especially the concentration 1:6.25 proved to be a cidal concentration and its action was similar to the action of amoxicillin and tetracycline. Similar results were reported by Prabuseenivasan (2006), and Mau *et al.*, (2001), who stated that cinnamon oil showed promising inhibitory activity even at low concentration and the antibacterial activity of cinnamon oil was probably due to their major component, cinnamaldehyde and their properties could be multiple.

Conclusion

In conclusion, cinnamon oil showed antibacterial activity against *K. pneumoniae* isolates so it could be used as antibacterial supplement in the developing countries. In addition to in vivo studies would be needed to evaluate the potential of this oil as an antibacterial agent.

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