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OCCURRENCE OF KLEBSIELLA SPECIES IN RAW MILK MARKET IN ASSIUT CITY AND THE EFFECT OF LOW TEMPERATURE ON ITS VIABILITY

(With 8 Tables)

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(Received at 22/12/2005)

تواجد ميكروبات الكلبسيلا فى اللبن الخام المباع بمدينة أسيوط وتأثير درجات الحرارة المنخفضة على حيويتها

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جمعت مائة وعشرون عينة عشوائية من اللبن الخام للجاموس والأبقار والماعز والأغنام بواقع (٣٠ عينة من كل نوع) من منازل الفلاحين ومحلات بيع الألبان بمحافظة أسيوط وذلك لمعرفة مدى تلوثها بميكروبات الكلبسيلا. كذلك تم دراسة تأثير درجات الحرارة المنخفضة (التبريد والتجميد) على مدى حيوية وبقاء ميكروب الكلبسيلا نيمونى الذى تم عزله والتعرف عليه وقد تم حقنه بـ ١٠×١٧ ^{٨} خلية / جرام فى الزبدة المعقمة. وأيضاً تم القيام بعمل تأثير بعض المضادات الحيوية على ميكروب الكلبسيلا نيمونى المعزولة. وقد أظهرت النتائج أن ميكروب الكلبسيلا نيمونى كان له السيادة على باقى الفصائل المعزولة وذلك بنسبة (١٠ و ١٣,٣٣%) لألبان الجاموس والأبقار على التوالى بينما لم يتم عزله من ألبان الماعز والأغنام وقد كانت النسبة الكلية لميكروبات الكلبسيلا للألبان المختلفة ٢٦,٦٦% و ٢٦,٦٦% لكل من ألبان الجاموس والأبقار أما بالنسبة لفصائل الكلبسيلا المختلفة التى تم عزلها فقد كانت كالتالى: الكلبسيلا أوكسى توكا (٦,٦٦%) فى ألبان الأبقار فقط ، الكلبسيلا أوزونى (٦,٦٦% ، ٣,٣٣% و ٣,٣٣%) لألبان الجاموس والماعز والأغنام على التوالى، الكلبسيلا بلانتيكولا (٦,٦٦% ، ٦,٦٦%) فى ألبان الجاموس والأبقار فقط بينما كانت الكلبسيلا تيراجينا متواجدة فى ألبان الجاموس فقط بنسبة (١,٣٣%). أما بالنسبة لتأثير درجات الحرارة المنخفضة على ميكروب الكلبسيلا نيمونى المعزولة من ألبان الجاموس والأبقار والمحقونة فى الزبد المعقم فقد كانت النتائج كالتالى: عند درجة حرارة التبريد (٥±١°) تزايد العدد من ١٠×١٧ ^{٨} خلية لكل جرام كبداية للحقن إلى ١٠×٩١ ^{٨} ، ١٠×٢٠٦ ^{٨} ، ١٠×٢١٧ ^{٨} و ١٠×٢٢٤ ^{٨} فى اليوم الأول والثانى والثالث والسابع على التوالى بينما تدرج العدد فى التناقص إلى ١٠×١٠ ^{٨} ، ١٠×١١٣ ^{٧} ، ١٠×٢٢٠ ^{٥} و ١٠×٣٧ ^{٣} عند درجة حرارة التجميد (صفر) على التوالى. بينما أختفى الميكروب تماماً بعد الأسبوع الأول عند درجتى التبريد والتجميد

وقد أعزى هذا لإرتفاع نسبة الحموضة حيث تزايدت من ٤,٣% عند البدء إلى ٤,٣ ، ٤,٣ ، ٤,٥ و ٦,٥% فى اليوم الأول والثانى والثالث والسابع على التوالى. وقد تم اختبار حساسية عترات الكلبسيلا نيمونى (٧) المعزولة من ألبان الجاموس (٣ عترة) والأبقار (٤ عترة) لبعض المضادات الحيوية وعددها ثمانية وقد وجد أن العترات حساسة بدرجة عالية للنورفلوكساسين وبدرجة متوسطة للجنتاميسين وبدرجة أقل للسيفوتاكزيم بينما لم يكن لباقى المضادات الحيوية أى تأثير على العترات المعزولة. هذا وقد ناقش البحث الأهمية الاقتصادية للميكروب وتأثيره على الصحة العامة والشروط الواجب اتخاذها لمنع تلوث الألبان ومنتجاتها بهذا الميكروب للحد من خطورته.

SUMMARY

One hundred and twenty random samples of raw buffalo's, cow's, goat's and sheep's milk (30 of each) were collected from different farmer's houses and dairy shops in Assiut City to be examined for the presence of Klebsiella organisms on MacConkey Inositol Carbenicillin agar. The results revealed that K. pneumoniae was the most prevalent species among the Klebsiella organisms isolated (10 and 13.33%) for buffalo's and cow's milks. K. oxytoca (66.6%) in cow's milk only, K. ozaenae (2.66, 3.33 and 3.33%) for buffalo's, goat's and sheep's milk respectively, K. planticola (6.66 and 6.66%) in buffalo's and cow's milk respectively and K. terrigena revealed 1.33% in buffalo's milk. Klebsiella organisms isolated from raw milk of different animals were (18) 15%, and they were 26.66% for buffalo's and cow's milks. Concerning the second part dealing with the effect of cold temperature (5±1°C) on the viability of K. pneumoniae in sterile butter samples revealed that, there is a gradual increase in the number of K. pneumoniae from $17x10^8$ cells/g as an initial count to $91x10^8$, $206x10^8$, $217x10^8$ and $224x10^8$ cells/g in the first, second, third and seventh day respectively. While a remarkable decrease in case of freezing temperature (0°C) from $17x10^8$ cells/g as an initial count to $10x10^8$, $113x10^7$, $220x10^5$ and $37x10^3$ in the first, second, third and seventh day respectively. K. pneumoniae inoculated in sterile butter completely disappeared and could not be detected after the first week in both chilling and freezing temperatures due to the high acidity percentages which reach from 4.3% at the zero time to 4.3, 4.3, 4.5 and 6.5%. Most of the isolated K. pneumoniae strains were highly sensitive to Norfloxacin, moderately to Gentamicin and weakly to cefotaxime but were resistant to other antibiotics used. The public health hazard and suggestive measures were discussed to prevent milk and milk products from contamination with Klebsiella organisms.

Key words: *Klebsiella, pneumoniae, ozaenae, oxytoca, planticola, terrigena, Buffalo's, cow's, goat's and sheep's milk.*

INTRODUCTION

Klebsiella is a Gram negative fecal coliform bacteria in the family Enterobacteriaceae. It could be isolated by Berry's (1933) and was named after Edin Klebs, a late 19 Century (Burnet *et al.,* 1978). It was responsible for several fatal pneumonia (Obiamiwe, 2002) and a variety of clinical syndromes in human due to consumption of milk (Koneman *et al.,* 1992).

In recent years a gradual awareness of the occurrence of Klebsiella organisms in both human being and animal species spread throughout the world while, a considerable interest in Klebsiella pneumoniae as an opportunistic pathogen responsible for nosocomial infection and trend tward multiple antibiotic resistance (Dechamps *et al.* 2004). From the side point of view K. pneumoniae is the most medically important species in the genus Klebsiella. As it is also responsible for acute renal failure to acute pylonephritis with extensive necrosis of the kidney (Creyghtgon *et al.* 2001), wound infections, meningitis, endocarditis, pleuritis, enteritis and acute or chronic diarrhoeal disease (Anderson and Janoff, 1998). In addition, the organism was incriminated in causing lung infection (Lim *et al.* 1995) and mastitis in animals (Shoshani *et al.* 2000) resulting in economic losses. Thirteen epidemic outbreaks of nosocomial bacteremias attributed to Klebsiella organisms in USA during 1983-1991 (Goto *et al.,* 2003). Moreover, serious mortality rates from Klebsiella reaches 5% even with antimicrobial therapy and approaches 100% for immunocompromised patients (Al-Rabea *et al.,* 1998).

Five subspecies are related to K. pneumoniae: K. ozaenae and K. rhinoscleromatis (Rennie and Duncan, 1974) which are responsible for nasal cavity infections were identified as ozena and rhinoscleroma (Gamea and Tatawi, 1990 and Zohar *et al.,* 1990). Also, K. oxytoca which produces infections similar to those caused by K. pneumoniae (Mahon and Manuselis, 1995) and usually occur in newborn populations in the hospital (Monnet and Freney, 1994). K. planticola, K. terrigena and K. ornithinolytica (Izard *et al.,* 1981 and Al-Tarazi, 2001). Klebsiella speeies are more related to 100% of immunosupressed individuals (Al-Rabea *et al.,* 1998) causing respiratory, intestinal, meningial infections due to heat labile and heat stable enterotoxins responsible for the virulence of Klebsiella species (Dhand *et al.,* 2001).

The organisms have been found in the environmental conditions surrounding the dairy animals, soil, dust, water, grass, bedding materials and fecal matter of animals and humans as the organisms are considered as normal flora of 30 to 40% of the intestinal tract of both animals and humans (Buttiaux, 1959). Also, they are found in oropharyngeal and biliary tracts (Quinn *et al.*, 1994).

Milk and milk products are liable to be contaminated by Klebsiella species during milking, handling, transportation, processing and storing as the organism can multiply at 5°C in refrigerator (Patterson and Gibbs, 1977).

Klebsiella organisms are implicated in many cases of food poisoning outbreaks (Hörvath *et al.*, 1964). And several investigators could isolate K. organisms from raw mastitic milks of different animals (Newman and Kowalski, 1973, Barkema *et al.*, 1998 and Peng *et al.*, 2003).

Because of the involvement of milk and milk products in Klebsiella infections this work was planned to study the following items:

1- Isolation of Klebsiella organisms from raw buffalo's, cow's, goat's and sheep's milk.
2- Identification of the isolated Klebsiella.
3- Studying the effect of cold storage (chilling and freezing) on the viability of isolated K. pneumoniae in sterile butter.
4- Antimicrobial susceptibility of the isolated K. penumoniae.

MATERIALS and METHODS

Collection of samples:

One hundred and twenty random samples of buffalo's, cow's, goat's and sheep's milk (30 of each) were collected from different farmer's houses and dairy shops in Assiut City in clean sterile containers in an ice box and transferred to the laboratory without delay and kept in refrigerator. The collected samples were subjected to Storch's test (Lampert, 1975) to exclude the heat treated milk samples.

Isolation of Klebsiella organisms (Bagley and Seidler, 1978)

One milliliter of each prepared raw milk sample was inoculated into Klebsiella enrichment broth (Atlas and Parks, 1994) and incubated at 37°C for 24 hs. Then loopfuls from enrichment broth were streaked on MacConkey-Inositol-Carbenicillin agar and incubated at 37°C for 24 hrs. The large mucoid and red colony with red pigment was picked up on nutrient slope tubes for further identification. The isolated Klebsiella

organisms were identified according to Edward's and Ewing, 1972; Niazi *et al.*, 1977 and Cruickshank *et al.*, 1978;

Experimental part:

A strain of K. pneumoniae previously isolated and identified from milk samples was grown in 10 ml of Klebsiella enrichment broth and incubated at 37°C for 24 hs. The culture was decimally diluted and plated to enumerate the organism. The dilution continues to achieve an inoculum level of $17x10^8$ cells/ml as an initial count.

The effect of the cold storage of butter on the viability of K. pneumoniae isolated:

220 gr. of sterile butter were used to be injected with the previously isolated and identified K. pneumoniae ($17x10^8$ cells/g as an initial count). Butter was divided into two portions. One portion was stored at chilling temperature (5±1°C) while the other was stored at freezing temperature (0°C). Growth rate and viability of K. pneumoniae was detected by applying the surface plating technique for the first three days then weekly on MacConkey Inositol Carbenicillin agar for 24 hs at 37°C.

Measurement of pH:

The pH of butter was determined using a pH meter (an Orion Model 701) equipped with standard electrode.

Antimicrobial susceptibility of K. pneumoniae:

Antibiotic sensitivity test of K. pneumoniae strains isolated from raw buffalo's and cow's milk was carried out according to (Baron et al., 1994) using eight antibiotic sensitivity discs. Ampicillin (AM_{10}), Cefadroxil (FR_{30}), Cafotaxime (CTx_{30}), Gentamicin (CN_{10}), Norfloxacin (NoR_{10}), Rifampin (RA_5), Streptomycin (S_{10}) and Tetracyclin (TE_{30}).

RESULTS

The obtained results were tabulated in Tables 1-8.

Table 1: Incidence of Klebsiella species in the examined raw milk samples.

Type of milk	No.	Klebsiella species	
		No.	%
Bufaloe's	30	8	26.66
Cow's	30	8	26.66
Goat's	30	1	3.33
Sheep's	30	1	3.33
Total	**120**	**18**	**15**

Table 2: Incidence of Klebsiella species isolated from raw buffaloe's milk.

Kelbsiella species	Positive samples	
	No/30	%
K. pneumoniae	3	10
K. oxytoca	-	-
K. planticola	2	6.66
K. ozaenae	2	6.66
K. terrigena	1	3.33
Total	**8**	**26.66**

Table 3: Incidence of Klebsiella species isolated from raw cow's milk.

Kelbsiella species	Positive samples	
	No.	%
K. pneumoniae	4	13.33
K. oxytoca	2	6.66
K. planticola	2	6.66
K. ozaenae	-	-
K. terrigena	-	-
Total	**8**	**26.66**

Table 4: Incidence of Klebsiella species isolated from raw goat's milk.

Kelbsiella species	Positive samples	
	No/30	%
K. ozaenae	1	3.33
Total	**1**	**3.33**

Table 5: Incidence of Klebsiella species isolated from raw sheep's milk.

Kelbsiella species	Positive samples	
	No./30	%
K. ozaenae	1	3.33
Total	**1**	**3.33**

Table 6: Effect of cold temperature on K. pneumoniae isolated from raw buffalo's and cow's milk in sterile butter.

Time	Count/g	
	Freezing	Chilling
0	$17x10^8$	$17x10^8$
First day	$10.0x10^8$	$91x10^8$
Second day	$113x10^7$	$206x10^8$
Third day	$220x10^5$	$217x10^8$
First week	$37x10^3$	$224x10^8$
Second week	0	0

Table 7: pH in butter samples during period of storage.

Time	Acidity %	
	Freezing	Chilling
0	4.3	4.3
First day	4.3	4.3
Second day	4.3	4.3
Third day	4.5	4.5
First week	6.5	6.5
Second week	Not examined	Not examined

Table 8: Antibiotic sensitivity of Klebsiella pneumoniae isolated from raw buffalo's and cow's milk.

Antibiotic disc	No. of samples	Reaction
Ampicillin (AM_{10})	7	-
Cefadroxill (CFR_{30})		-
Cefotaxime (CTx_{30})		+
Chloramphinicol (C_{30})		-
Gentamycin (CN_{10})		++
Norfloxacin (NoR_{10})		+++
Rifampin (RA_5)		-
Streptomycin (S_{10})		-
Tetracyclin (TE_{30})		-

(-) Resistant ++ Moderately sensitive
+ Weak sensitive +++ Highly sensitive

DISCUSSION

Data obtained and recorded in Table 1 showed that the incidence of Klebsiella species from the examined raw milk samples of different animals was 15%. These results were completely in agreement with Vijayalakshmi *et al.*, 2001 (15%), near to Rahman *et al.*, 1992 (12.2%), higher than Kumari and Gupta, 2002 (2.46%) and Al-Ashmay, 2004 (2%). On the other hand, lower than Newman and Kowalski, 1973 (54%), while, these results were in disagreement with Grhon et al., (2004) who failed to detect Klebsiella species from milk samples examined.

It is worth to mention that high incidence of Klebsiella species may be attributed to the free living of Klebsiellae in the external environmental conditions surrounding the dairy animals (soil, sewage, water and bedding) and faecal matter of both animals and humans that contaminate teat or udder surface (Richter *et al.*, 1992) and

consequently, contaminate milk which creats a good medium for multiplications of many microorganisms including Klebsiella. Also, the lack of hygiene and sanitation during handling, processing distribution as stated by Montgomerie (1979) that hands of the personal are the main factors for transmission of infection by Klebsiella. It is evident from Tables 2 and 3, that the incidence of Klebsiella species from raw buffalo's and cow's milk was 26.66% for each. That was higher giving than Horya (2005) 14.7 and 5.3% respectively. This may due to the differentiation in the culture media used, number of samples and hygienic conditions under which they are collected, also the seasons of collection. Klebsiella species obtained from raw buffalo's milk as shown in Table 2 were identified as K. pneumoniae (3 isolates) 10%, K. ozaenae (2 isolates) 6.66%, K. planticola (2 isolates) 6.66% and K. terrigena (1 isolate) 3.33%, while 4 isolates (13.33%) K pneumoniae, 2 isolates (6.66%) K. oxytoca and 2 isolates (6.66%) K. planticola in cow's milk (Table 3). It is evident from the tabulated data that K. pneumoniae was the most prevalent species in the examined raw buffalo's and cow's milk samples. This came in line with El-Essawy and Riad, 1989 (10%), close to Silva and Costa, 2001 (13.5%), Alonso *et al.,* 2002 (13.8%) and Seleim *et al.,* 2002 (7.3%), higher than Singh and Sharma, 1999 (3.4%) and Horya, 2005 (1.3%) and the results were lower to El-Essawy and Riad, 1989 (18%). At the same time the results were in disagreement with several investigators who failed to detect K. pneumoniae from raw milk samples El-Masry (1996) and Ahmed and Sotohy (2003). The high prevalence of K. pneumoniae in the examined raw buffalo's and cow's milk samples may be explained by the numerous outbreaks of mastitis in cattle as the organism was incriminated in causing acute and chronic mastitis and variety of infections in other domestic animals. Also, K. pneumoniae implicated as an etiological pathogen in human patients suffering from, gastro, respiratory and urinary infections. Also, from Tables 2 and 3 it is clear that K. ozaenae could be isolated from raw buffalo's milk only and it revealed 6.66%. This result was lower than El-Essawy and Riad, 1989 (14%) and higher than Horya, 2005 (3.3%). Al-Tarazi (2001) stated that K. ozaenae was the most frequent species among Klebsiella. It is responsible for an infection of the nasal cavity called Ozena which occurs in eldery persons and manifested by nasal congestion of bad smell and sinusitis (Tondo *et al.,* 2004). With regarding to Tables 2 and 3 K. oxytoca isolated from raw cow's milk only and could score also (6.66%). The obtained result was lower than Podshun *et al.,* 1998 (72%), near to Singh and Sharma, 1999 (5.3%) and

similar to Opsomer *et al.* (2001). It is worth to mention that K. oxytoca produces infections similar to those caused by K. pneumoniae (Mahon and Manuselis, 1995). It usually occur in new born populations in the hospital (Monnet and Freney, 1994). Also, K. oxytoca could be isolated from faeces and blood (Farmer, 1985). K. planticola among Klebsiella species which was obtained from raw buffalo's and cow's milk with the same percentages 6.66%. The giving result was in accordance to Podshun *et al.*, 1998 (8.7%) and Singh and Sharma, 1999 (5.3%) while, it was higher than Horya, 2005 (0.7%). K. planticola could be isolated from urin, blood and respiratory tract of humans causing diseases not less than that caused by K. penumoniae (Mahon and Manuselis, 1995). K. terrigena another species of K. pneumoniae obtained only from raw buffalo's milk 1 (3.33%). The organism has been found in soil and water surrounding the dairy animal (Mahon and Manuselis, 1995). As shown in Tables 2 and 3 Buffalo's and Cow's milk achieved the same percentages of isolation 26.66%. This was in contrary to Sprong *et al.* (2001) and Horya (2005).

Data reported in Tables 4 and 5 varify that raw goat's and sheep's milk samples were free from Klebsiella species except K. ozaenae which revealed 1 (3.33%) for each. The result was near to Kozacinski *et al.* (2002) 0.4% from goat's milk. Rareness of goat's and sheep's milk from Klebsiella species may be in agreement with Hutchinson *et al.* (1985) who stated the lower of risk from unpasteurized goat's and ewe's milk due to the low evidence of contamination. At the same time the giving result was in disagreement with Muchlherr *et al.* (2003) who could isolate different Klebsiella species from raw goat's and sheep's milk 61.6 and 71.4% respectively, El-Battrawy *et al.*, and El-Ganzory, 2002 could isolate K. pneumoniae (10%) and K. oxytoca (16%) from ewe's milk respectively.

The aforementioned results in Table 6, showed that the effect of low temperature (chilling and freezing) on the growth of K. pneumoniae when inoculated into sterile butter with $17x10^8$ cells/g was: in case of chilling temperature, a significant increase in the cell counts of K. pneumoniae from $17x10^8$ as initial count to $91x10^8$, $206x10^8$, $217x10^8$ and $224x10^8$ cells in the first, second, third and seventh day respectively. The obtained results were in agreement with Hechelmann *et al.* (1974), Johnson *et al.* (1975) and Patterson and Gibbs (1977) and this finding represents the ability of Klebsiella organisms as one of psychrotrophic bacteria to survive at the temperature of refrigeration and could contribute substantially of refrigerated materials. While, in case of

freezing temperature a gradual decline in count was noticed in the first and second day from $17x10^8$ cells as initial count to $10x10^8$ and $113x10^7$ cells respectively. On the other side, in the third and seventh day a remarkable decrease in the count of K. pneumoniae, reached to $220x10^5$ and $37x10^3$ cells, respectively. These results were in agreement with Calcott (1976). Following up to Table 6 it is worth mentioning that K. pneumoniae was completely disappeared after the first week at chilling and freezing temperatures and that may be due to the acidity percentages which revealed significant increase affecting the inoculated organism. From 4.3% at the zero time and reached to 6.5% at the seventh day. Similar findings were reported by several investigators on different microorganisms due to increasing of acidity percentages Huang *et al.* (1993), Rola *et al.* (1994), Abou-Eleinin (1999) and El-Gazzar (1997). It is worth to mention that the freezing temperature has been shown to cause reduction in the growth rate, inactivation and destruction of microorganisms (ICMSF, 1980).

The results in Table 7 which concern antimicrobial susceptibility pointed out that all K. pneumoniae strains isolated from raw buffalo's and cow's milk were found to be resistant to the most antibiotic discs used as reported by Carroll (1971), Braman *et al.* (1973) and Malinowski and Klossowska (2003), while they were highly sensitive to Norfloxaxin as obtained by Grewal *et al.* (2001), Longoni *et al.* (2001) and Ozgur *et al.* (2003), moderately sensitive to Gentamycin and this is pointed out by Dhand *et al.* (2001) and Silva and Costa (2001) and weakly sensitive to Cefotaxime.

In general the excessive use of antibiotics in the treatment of Klebsiella infections in hospitalized patients and animals are of limited value and may lead to prolong the illness and increase the carriage of Klebsielleae in addition to the economic loss due to the misuse of antibiotics in veterinary practice.

In conclusion: milk represents a suitable medium for growth and multiplication of several human pathogens, including Klebsiella. Therefore, strict hygienic measures must be followed during all steps of milk production and its manufacture to dairy products. In addition to particular attention to the management of these dairy goat and sheep flocks in order to avoid the development of cases of clinical mastitis with different microorganisms.

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