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CHARACTERIZATION OF KLEBSIELLA SPECIES RECOVERED FROM PNEUMOENTERIC BUFFALO CALVES

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SUMMARY

Bacteriological examination was carried out on 95 fecal samples and 95 nasal swabs collected from buffalo calves suffered from diarrhea and respiratory manifestation as well as 50 apparently healthy buffalo calves aged from 1 day up to 2 months old. The obtained results revealed the isolation of 6 strains of *Klebsiella* species from healthy buffalo calves while 14 isolates of Klebsiella sp. were recovered from buffalo calves suffered from respiratory manifestation and 18 isolates from diarrhoeic buffalo calves. All isolates were identified as *K. pneumoniae* subsp pneumoniae , *K. pneumoniae* subsp ozaenae and *K. oxytoca*. The pathogenicity and virulence of different Klebsiella isolates were studied in mice. The study proved that Klebsiella isolates causing mortality rates ranged from 30% up to 80% after intramuscular , subcutaneous and oral routes of infection in mice and death occur usually between day 4 and 21 post infection. This study suggested that the use of ELISA for detection of antibody titre in mice using LPS as coated antigen revealed its efficacy in detection of *klebsiella* infection and cross reactions and it was highly sensitive and specific enough for extensive evaluation in the field.

INTRODUCTION

In Egypt, buffaloes are considered as an important animals among livestock. They posses high efficiency in converting the poor ranges to meat and milk. In addition to their ability to live under a variety of intensive and extensive management conditions.

The genus *Klebsiella* is a member of family Enterobacteriaceae. It is non-motile, rod shaped , gram negative bacteria with a prominent polysaccharides capsule. Serotypes are based on the structural variability of the capsular polysaccharides (*K antigen*) and lipopolysaccharides (O antigen) (Mezyed, 2005). *Klebsiella* species are found widely throughout nature and are often found as part of normal flora of the gastrointestinal tract, respiratory tract and skin of animals (Umeh and Berkowitz, 2004).

Typically, *Klebsiella* are opportunistic pathogen, infections are nosocomial and can give rise to severe diseases such as pneumonia , urinary tract infections, diarrhea and mastitis . The lipopolysaccharides (LPS) are a major structural component of the outer cell membrane. LPS causes macrophage cytotoxicity and intensive cell damage (Farber et al., 1990 and Emery et al., 1991). In addition LPS plays an important role in the virulence of the bacterium based on its antiphagocytic and endotoxin properties (Salman , 2003).

The importance in *Klebsiella* species specially Klebsiella pneumoniae as a health risk affecting animals and human encouraged out the current study to throw lights on the following objects: isolation and identification of *Klebsiella* species from either apparently healthy and diseased buffalo calves ; detection of the pathogenicity and LD_{50} of Klebsiella isolates ; screening of the isolates for the production of endotoxin (LPS) by SDS-PAGE and the antimicrobial susceptibility of the recovered isolates to different antibacterial agents was also done.

MATERIALS AND METHODS.

Bacterial examination:

Bacteriological examination was carried out on 95 fecal samples and 95 nasal swabs collected from buffalo calves suffered from diarrhea and respiratory manifestation in addition to, 50 apparently healthy buffalo calves from Sed`s station (Beni-Seuf Governorate) during the period from August 2006 up to February 2007.

All collected samples were cultured into nutrient broth for 24 hrs at 37°C, then an inoculum was transferred onto MacConkey bile salt agar, Eosin methylene blue agar and Hektoen enteric agar plates.

All inoculated plates were incubated aerobically for 24-48 hrs at 37°C. Smears from these Suspected mucoid colonies were stained with Gramís method and stained by Indian ink to reveal the presence of capsules. Pure cultures of the suspected colonies were identified morphologically, culturally and biochemically according to Collee et al. (1994) and Koneman et al. (1997).

Experimental infection:

Mice were used as experimental animals for studying the pathogenicity of the isolated strains of Klebsiella species. Preparation of the tested

grains was done according to Gradwhol (1956) and Barrow (1992) as follows: twenty four hours of pure culture of each of the tested *Klebsiella* species namely: *Klebsiella pneumoniae* subsp *pneumoniae*, *Klebsiella pneumoniae* subsp *ozaenae* and *Klebsiella* oxytoca were suspended separately in sterile saline solution . Using Mac-Farland opacity tube No " 2 " corresponding to $5x10^9$ viable organisms per ml. was prepared.

For this purpose, 10 mice were used in each group , every mice received 0.25 ml of each tested organisms either *Klebsiella pneumoniae* subsp pneumoniae , *Klebsiella pneumoniae* subsp ozaenae or *Klebsiella* oxytoca by different routes of inoculation (I/M, S/C and orally) and 10 animals were used non infected and kept as control.

The infected animals were observed daily up to 21 days to record their general health condition, clinical signs. Post mortem examinations were performed on dead cases. Bacteriological re-isolation of Klebsiella species was attempted among the internal organs of dead mice.

Extraction and purification of LPS of Klebsiella pneumoniae

Extraction and purification of endotoxin from *Klebsiella pneumoniae* were performed according to Qureshi and Takayama (1982) and Barrow (1992) and the purity was checked up by SDS-PAGE according to Sambrook et al. (1989).

Estimation of the mean lethal dose (LD_{50}) for LPS:

The LD_{50} for the extracted LPS was determined by IV injection in Swiss white mice as described by Lynn and Collahan (1976). 8 groups (10 mice each) were used for each isolates. Various concentrations of LPS were diluted in PBS (ranged from 300-1000 ng /ml). The mean lethal dose LD_{50} was calculated within 3 days.

Antiserum against Klebsiella (Obied et al., 1996):

The cells were grown in brain heart infusion broth for 18 hr and washed three times with phosphate buffered saline pH 7.2 and finally suspended to a concentration of 10^8 cell ml-1 in PBS containing 0.5 % formalin. The suspension was emulsified in an equaled volume of freund's complete adjuvant and 1 ml injected S/C in NewZaeland rabbits . The booster dose was given on day 14 followed by another dose at day 21. The animals were bled out at day 28 and the serum separated.

Protection test:

For animal immunization, groups of ten mice (weighing 20-25 g) were injected I/P with 100 ug of purified LPS in 0.01 M Tris hydrochloride buffer (pH 8.0). The animals were then rested for 14 days before challenge with *Klebsiella* species (Straus, 1987). An over night culture grown in brain heart infusion broth at 28°C was pelleted at 4,500 Xg for 5 min at 4°C and washed 3 times in

sterile normal saline solution. The pellet was re-suspended in 2 or 3 ml of sterile normal saline solution to obtain approximately bacterial count of $5X10^6$/ ml (Berendt et al., 1977) as determined by Miles and Misra (1938) technique and turbidity McFarland standards. Each mouse was given 1 ml I/P.

The control mice were inoculated I/P with 0.01 M Tris hydrochloride buffer (pH 8.0) (Straus, 1987).

ELISA:

The indirect method of ELISA was carried out for detection of *Klebsiella pneumoniae* antibodies. The indirect method was carried out according to Barrow (1992) and Nicholas and Cullen (1991) as follows:

The microplates were coated with LPS. of *Klebsiella pneumoniae* in a concentration of 100 µg / well and incubated over night at 37°C then all plates were washed and blocked of all wells using BSA 1:10 in distilled water (100 µl / well), then incubated for 1 hr. at 37°C and washed. Sera were added in a dose of 100 µl well (1: 100 dilution) and incubated for 1 hr at 37°C and washed. Conjugate of the goat anti ovine IgG was used in a dose of 100 µl / well and incubated at 37°C for 1 hr then washed. A.B.T was added and all tested plate was read by using spectrophotometer at 405 n.m.

Antibiogram for the local isolates of *Klebsiella pneumoniae*

The agar disc diffusion antibiotics technique was conducted according to Finegold and Martin (1982).

RESULTS AND DISCUSSION

Buffaloes are considered as important animals, mainly for meat and milk production. However, limited attention pledged to question the health performance of these profitable animals especially under intensive husbandry conditions. Enteritis and pneumonia in buffalo calves provoked by a multitude of both infections and non infectious cases. As shown in Table (1), bacteriological examination of 50 apparently healthy buffalo calves revealed the isolation of 4 isolates of *K. pneumoniae* subsp *pneumoniae* and 2 isolates of *K. oxytoca* with an incidence of 8.0% and 4.0% respectively. On the other hand bacteriological examination of nasal swabs collected from buffalo calves suffering respiratory manifestation revealed that 14 out of 95 nasal swabs were harboured *Klebsiella* microorganisms (14.74 %). The most predominant isolates were *K. pneumoniae* subsp *pneumoniae* (9 isolates) (9.47%) ; *K. pneumoniae* subsp *ozaenae* (3 isolates) (3.16%) and *K. oxytoca* (2 isolates) (2.1%).

As regards to diarrheic cases, 18 out of 95 cases were harboured *Klebsiella* species (18.95%), *K. pneumoniae subsp pneumoniae* (9 isolates) (9.47

K. pneumoniae subsp ozaenae (5 isolates) (8 %) and *K. oxytoca* (2 isolates) (4.21 %) these findings are similar to that mentioned Mezyed (2005) who recorded that *K. pneumoniae* and *K. oxytoca* were the common isolates from buffalo calves suffered from respiratory manifestation and diarrheic cows and from buffalo calves.

Table (1) : Prevalence rate of *Klebsiella* species recovered from buffalo calves suffered from diarrhea and respiratory manifestation.

Type of isolates	General health condition					
	Apparently healthy (50)		buffalo calves with respiratory manifestation (95)		Diarrheic buffalo calves(95)	
	No	%	No	%	No	%
K. pneumoniae subsp . *pneumoniae*	4	8.0	9	9.47	9	9.47
K. pneumoniae subsp *ozaenae*	0	0.0	3	3.16	5	5.26
K.oxytoca	2	4.0	2	2.1	4	4.21
Total	6	12	14	14.74	18	18.95

% was calculated according to the No. of examined animals in each status. ( ) = No.

The obtained results proved that all isolates of *Klebsiella oxytoca* were negative for oxidase, H_2S, motility, gelatin liquefaction at 22°C. They were positive for indole production, citrate utilization and urease activity, methyl red test and Voges Proskauer test. Isolates proved to be *Klebsiella pneumoniae* subsp ozaenae as they were negative to oxidase , indole production , Voges Proskauer test , H_2S , urease , motility and gelatin liquefaction at 22°C. Isolates proved to be *Klebsiella pneumoniae* subsp *pneumoniae* because they were negative for oxidase , indole production , H_2S , motility and gelatin liquefaction at 22°C. The use of biochemical tests was of vital worth in *Klebsiella* species judgment and this observation was analogous to that mentioned by Simmons-Smith et al. (1985) who recorded that each biotype has distinct characters for its biochemical activity which finally considered as one of the most important method for *Klebsiella* identification.

Table (2) :Results of experimental infection of local isolates of *Klebsiella* biotypes using various routes of injection in mice

No. of groups	*Klebsiella* species	Subgroup	Rout of injection	No. of dead mice at different interval day						No. of dead / no. of infected	Mortality rate
				0	4	7	10	15	21		
I.	*K. pneumoniae* subsp . *pneumoniae*	A	Oral	0	0	1	1	1	1	4/10	40
		B	S / C	0	0	1	1	2	1	5/0	50
		C	I /M	0	1	2	1	2	1	7/10	70
II.	*K. pneumoniae* subsp *ozaenae*	A	Oral	0	0	1	1	1	2	510	50
		B	S / C	0	1	1	2	2	1	7/10	70
		C	I /M	0	1	2	2	2	1	8/10	80
III.	*K. oxytoca*	A	Oral	0	0	0	1	1	1	3/10	30
		B	S / C	0	1	1	2	1	1	6/10	60
		C	I /M	0	2	0	1	2	1	6/10	60
IV.	Control		Non	0	0	0	0	0	0	0/10	0.0

The results of experimental infection of the three Klebsiella biotypes in mice by different routes were investigated. The mortality rates reached to 70%, 80% and 60% with *K. pneumoniae* subsp . *pneumoniae; K. pneumoniae* subsp *ozaenae* and *K. Oxytoca* respectively by I/M routes as shown in Table (2). As regards to subcutaneous rout, the mortality rate reached to 50%, 70% and 60% among the tested isolates respectively. The mortality usually occurred between 4-21 days. These findings are in agreement with the results recorded by Riad et al. (2002).

[illegible] of LPS extracted from Meiseila species

[illegible]	Mortality in mice after injection								LD50 µg/ml
	30	40	50	60	70	80	90	100	
[illegible]	4/10	6/10	6/10	7/10	7/10	8/10	9/10	10/10	58
[illegible]	1/10	3/10	3/10	5/10	6/10	8/10	8/10	9/10	80
[illegible]	0/10	2/10	3/10	4/10	6/10	7/10	8/10	9/10	88

[illegible] of extracted LPS in age mi

(3) shows the LD50 values of LPS extracted from Meiseila species. All these isolates as LPS with LD50 values of 58,80 and [illegible] M. [illegible] and M. [illegible] respectively. The LD50 of the isolates was used for the determination of virulence of [illegible] in mice by intraperitoneal injection of 24 hr. broth culture of each isolate. It is clear that all of the tested isolates from diseased buffalo calves showed high virulence with low LD50. Similar results were reported by Mohamed (2005) who reported that LD50 of endotoxin purified from Meiseila isolates of diseased buffaloes varied from 1.5 to 2.5 µg/ml, the corresponding LD50 of isolates obtained from apparently healthy animals (buffalo calves) were ranged from 8.5 to 15.5 µg/ml.

Table (4) : Characterization of LPS of *klebsiella* species by SDS-PAGE

Lanes bands	Molecular weights of *klebsiella* species													
	Marker lane (7)		*K. pneumoniae*				*K. ozaenae*				*K. oxytoca*			
			Lane (1)		Lane (2)		Lane (3)		Lane (4)		Lane (5)		Lane (6)	
	Mol. Weight	Amount	Mol. Weight	Amount	Mol. Weight	Amount	Mol. Weight	Amount	Mol. Weight	Amount	Mol. Weight	Amount	Mol. Weight	Amount
1	175	20.34	18.32	31	17.98	58	17.7	67.70	19.5	36.5	18.99	69.5	19.00	57.4
2	83	17.32	16.91	15.5	15.95	25.3	16.82	8.78	16.99	11.4	17.10	13.9	16.82	15.2
3	62	10.21	15.42	53.4	14.1	15	15.99	13.1	15.67	18.8	15.90	3.54	15.55	9.01
4	47	29.91					13.73	10.3	15.22	33	14.10	13.20	14.40	18.39
5	32.5	12.73												
6	16.5	9.51												

The LPS analysis of *Klebsiella* species was recorded in Table (4). SDS-PAGE analysis showed that *Klebsiella* LPS contain a wide variety of different molecular weight which ranged from 13.7 kDa to 19.5 kDa .

In *Klebsiella pneumoniae* , LPS molecular weight ranged from 14.1 to 18.32 kDa. It was obvious that LPS molecular weight of *Klebsiella ozaenae* ranged from 13.73 kDa to 19.5 kDa. LPS extracted from *Klebsiella oxytoca* molecular weight ranged from 14.10 kDa to 19.00 kDa. Nearly similar finding was recorded by Gennarland (1992) who mentioned that the SDS - PAGE revealed that the major bands of endotoxin have low molecular weight that ranging from 11.7 to 14.0 kDa.

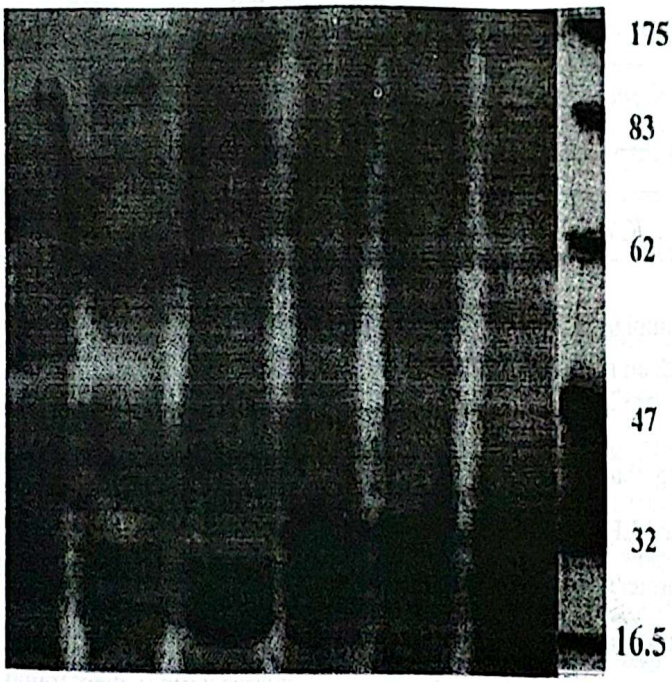

Fig. (1): SDS-PAGE of LPS purified from *Klebsiella* species

Table (5) : Protection of mice by rabbit antiserum antibodies against homologous & heterologous *Klebsiella* LPS.

Challenge of with LPS *	Preimmune serum	mice protection with rabbit antiserum against LPS		
		K. pneumoniae subsp . *pneumoniae*	*K. pneumoniae* subsp *ozaenae*	*K. oxytoca*
K. pneumoniae subsp . *pneumoniae*	0/8	8/8	8/8	7/8
K. pneumoniae subsp *ozaenae*	1/8	6/8	8/8	5/8
K. oxytoca	2/8	5/8	6/8	8/8

* All immunized mice received 100 µg of purified LPS

** No. of dead mice / no. of inoculated.

Table (5) showing that mice immunized with 10 µg of the purified LPS of homologous strains showed more protection than heterologous strains, this result is nearly similar to Mezyed (2005) who recorded that when mice were immunized with 100µg of the purified LPS of *Klebsiella* species, they were protected against large dose of challenge of the homologous organism more than heterologous strains.

Table (6) illustrated that mice immunized with 100 µg LPS of *Klebsiella* species showed the highest antibody titer of 2 weeks post immunization and this agree, with Cryz et al. (1981) and Straus (1987) who found that non of the mice died when infected with 100 µg purified LPS.

Studying the effect of LPS by small quantities as a mean of increased protection against *Klebsiella*

Table (6): Antibody titre in mice injected with LPS extracted from different *Klebsiella* species (ELISA).

Sera form	Antibody titre at 2 weeks interval	
	1st week post inoculation	2nd week post inoculation
Preimmunization	0	0
Immunization: using LPS of		
K. pneumoniae subsp . *pneumoniae*	320	640
K. pneumoniae subsp *ozaenae*	160	640
K. oxytoca	160	320

Rabbits immunized with unheated LPS preparartion showed titre 1/640 and the antibody level was similar to that obtained by Fohnson and Greisman (1988) who observed that the immunogeicity of LPS (the capacity to induce formation of antibodies). The prepared LPS was used to determine their protective capacity by means of passive mice protection test against homologous bacteria , the obtained result showed that a titre of 1/640 provided 100% protection against challenge by the homologous strain (*K. pneumoniae* subsp . *pneumoniae; K. pneumoniae* subsp ozaenae and *K. oxytoca*).These results agree with that reported by Fohnson and Greisman (1988) who concluded that specific antisera uniformally provide significant protection against lethality induced of enterotoxins from homologous smooth bacterial strains. It is suggested that antibody to core polysaccharides in LPS which is structurally similar in most LPS will neutralize a wide variety of enterotoxins, the idea that postulated a broad spectrum protective antibody against such core antigen in all Gram negative bacteria Fohnson and Greisman (1988) added that immunization with shared cross reactive antigens can protect against challenge with heterologous Gram

Table (7): Antimicrobial susceptibility patterns of *klebsiella* species isolated from the examined animals

Antimicrobial agents	susceptibility patterns																	
	K. pneumoniae (22)						*K. ozaenae* (8)						*K. oxytoca* (8)					
	Susceptible		Intermediate		Resistance		Susceptible		Intermediate		Resistance		Susceptible		Intermediate		Resistance	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Carbenicillin	22	100	0	0	0	0	8	100	0	0	0	0	8	100	0	0	0	0
Cephotaxim	22	100	0	0	0	0	8	100	0	0	0	0	8	100	0	0	0	0
Chloramphenicol	0	0	4	18.18	18	81.82	0	0	2	25	6	75	0	0	2	25	6	75
Flumequine	22	100	0	0	0	0	8	100	0	0	0	0	8	100	0	0	0	0
Nitrofurantoin	20	90.90	2	9.09	0	0	8	100	0	0	0	0	8	100	0	0	0	0
Streptomycin	0	0	4	18.18	18	81.82	0	0	5	62.5	3	37.5	0	0	2	25	6	75
Sulphamethoxazole t rimethoprim	13	59.09	5	22.72	4	18.18	0	0	8	100	0	0	3	37.5	5	62.5	0	0
Ampicillin	0	0	0	0	22	100	0	0	1	12.5	7	87.5	0	0	0	0	8	100

negative bacterium. so in this study foremost aim of this work was mainly to estimate the protective capacity of anti LPS of *K. pneumoniae* subsp . *pneumoniae; K. pneumoniae* subsp *ozaenae* and *K. oxytoca* in mice and the result showed that the used antisera was capable to neutralize the toxic effect of LPS and such antisera were capable of binding heterologous endotoxins (Table 6).

Antimicrobial susceptibility patterns of *Klebsiella* species isolated from examined animals was made and results obtained showed that most of the strains were sensitive to *Carbenicillin ; Cephotaxim* followed by flumequine and nitrofurantoin meanwhile isolates were resistant to chloramphenicol ; streptomycin and ampicillin (Table 7) and this results are in agreement with that mentioned by Bernable et al. (1998) and Mohamed (2003) .

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