Microbes and Infectious Diseases 2021; 2(4): 767-780

Microbes and Infectious Diseases

Journal homepage: https://mid.journals.ekb.eg/

Original article

Detection of virulence genes *(magA* and *rmpA)* and resistance gene *(CTX-M)* in *Klebsiella pneumoniae* isolated from neonates with septicemia

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ARTICLE INFO

Article history:
Received 13 June 2021
Received in revised form 9 August 2021
Accepted 11 August 2021

Keywords:
Neonatal sepsis
K. pneumoniae
Hypermucoviscous
ESβL
Infection control measures

ABSTRACT

Background: Neonatal sepsis (NS) due to *K. pneumoniae* is a major cause of morbidity and mortality in neonates. This study aimed to study the risk factors of NS caused by *K.pneumoniae* in NICU in Menoufia University Hospitals and to detect rmpA and magA virulence genes and CTX-M antibiotic resistance gene. Correlation between compliance of infection control measures and occurrence of NS and its outcome were also evaluated. **Methods:** *Klebsiella pneumoniae* were isolated from blood of neonates with sepsis and studied for hypermucovicosity by string test and detection of *rmpA* and *magA* genes. ESβL production was studied by cephalosporin/clavulanate combination disks and expression of *CTX-M* gene groups. Hand hygiene and other infection control measures compliance were evaluated by observational and practical methods. **Results:** *Klebsiella pneumoniae* was the most frequently isolated organism (31.6%) among neonates with confirmed sepsis. Hypermucoviscous phenotype was detected by string test in 39.6% of isolates while *rmpA* and *magA* genes were found in 47.9% and 8.3% respectively. ESβL production was confirmed in 75% (by cephalosporin/clavulanate combination disk). The *CTX-M* gene was found among 77.8% of ESβL-producing *K. pneumoniae* isolates. There was a negative correlation between hand hygiene and other infection control measures compliance and occurrence of NS. **Conclusion:** Virulence and antimicrobial resistance genes are common among *K. pneumoniae* isolated from neonates with sepsis in our locality. Implementation of infection control measures and proper antimicrobial stewardship programs may be helpful to overcome this problem..

Introduction

Neonates are vulnerable to hospital-acquired bloodstream infections (BSIs) because of immaturity of their immune systems and exposure to multiple risk factors during the perinatal period. This vulnerability is likely to augment the occurrence of neonatal sepsis (NS) which is a clinical syndrome manifested by symptoms, signs of infection and bacterial pathogen isolation from the bloodstream in an infant 28 days of life or younger [1]. Neonatal sepsis is considered a major cause of morbidity and mortality among neonates and is broadly categorized into two categories: Early-onset

DOI: 10.21608/MID.2021.85894.1170
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sepsis (EOS) and late-onset sepsis (LOS) according to the postnatal day of presentation. Early onset sepsis occurs in the first 72 hours of life while LOS occurs after 72 hours [2]. Hand hygiene has been singled out as the most important infection prevention and control (IPC) measure in preventing hospital-acquired NS [3]

Klebsiella pneumoniae is one of the most frequent causes of outbreaks in neonatal intensive care units (NICUs). There are mainly two pathotypes of *K. pneumoniae*: hypervirulent (hvKp) also called hypermucoviscous (hmvKP) and classical (cKp). The hvKp strains exhibit hypermucoviscosity and cause various severe infections [4]. There are phenotypic (string test) and genotypic (virulence genes) methods that can distinguish the hmvKP from ckp strains. Regulator of mucoid phenotype A (*rmpA* and *rmpA2*) and hypermucoviscosity-associated gene A (*magA*) have been found to contribute to the hypervirulent phenotype [5].

Klebsiella pneumoniae utilizes several mechanisms of antibiotic resistance, one of which is production of beta-lactamases [6]. In 2017, the World Health Organization (WHO) included Extended-spectrum beta lactamase (ESβL)-producing *K. pneumoniae* in the list of the most dangerous superbugs [7]. Extended-spectrum beta lactamases enzymes can hydrolyze the extended-spectrum cephalosporin and monobactam antibiotics. These enzymes are divided into several main groups such as sulphydryl variable (SHV), temoneira (TEM) and cefotaximases (CTX-M) [8].

The group of CTX-M type exhibits higher activity against cefotaxime and ceftriaxone than ceftazidime, and becomes the most widely distributed and globally dominant genotypes. It constitutes more than 170 allelic variants, which cluster into five major groups based on similarities in amino acid sequence; CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25. Each group consists of a number of particular variants with dominant variants being restricted in distribution to specific geographic areas, while few others are globally distributed [9].

This study aimed to study the risk factors of NS caused by *K.pneumoniae* in NICU in Menoufia University Hospitals and the antimicrobial resistance profile of these isolates. Detection of *rmpA, magA* and *CTX-M* gene and their relations to virulence and antibiotic resistance were studied. Moreover, correlations between compliance of infection control measures and occurrence of NS and its outcome were analyzed.

Patients and Methods

Study design and population

This cross-sectional analytic study was performed at Medical Microbiology and Immunology, and pediatrics and NICU Departements, Menoufia University Hospitals, during period from April 2019 to June 2020.

The study involved 152 neonates with confirmed sepsis by positive blood culture. Maternal and neonatal data including complete blood count (CBC) and C- reactive protein (CRP) were obtained.

Specimen collection and isolation of *K. pneumoniae*

One to two blood samples (1-2 ml) within 15 min were aseptically drawn at the peak of the neonatal fever from different peripheral venous sites. The inoculated blood culture media were incubated aerobically at 37°C in the BD BACTEC FX40 blood culture instrument. Blood cultures without any microbial growth after 5-7 days of incubation were considered negative [10]. Positive samples were sub cultured on different bacteriological media.

Klebsiella pneumoniae isolates were identified by the standard microbiological methods [11] and confirmed by Vitek-2 system. String test was used to identify hypermucoviscosity as previously described [12].

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the disk diffusion method against different antimicrobial agents (Oxoid, Basingstoke, UK) and minimal inhibitory concentration (MIC) of colistin was determined by broth micro dilution method according to CLSI guidelines [13].

Screening for ESβLs production:

Klebsiella pneumoniae isolates showing zone of inhibition ≤ 21 mm for ceftazidime, ≤ 27 mm for cefotaxime, and ≤ 25 mm for ceftriaxone were considered potential ESβL-producers [13].

Phenotypic confirmation of ESβLs production:

Cephalosporins/clavulanate combination test: *Klebsiella pneumoniae* isolates were considered ESβL-producers if the inhibition zone around the combined ceftazidime/clavulanic acid (30/10 μg) disk was at least 5 mm larger than that of ceftazidime (30 μg) disk alone [13].

Detection of the studied genes

Bacterial DNA was extracted and purified using genomic DNA purification kit (QIAamp DNA Mini Kit (**Germany**)

The used primers [14] for detection of *CTX-M* gene groups (ESβL gene) by multiplex PCR were:

Group 1: F: AAA AAT CAC TGC GCC AGT TC
R: AGC TTA TTC ATC GCC ACG TT

Group 2: F: CGACGCTACCCCTGCTATT
R: CCAGCGTCAGAT TTT TCA GG

Group 8: F: TCG CGT TAA GCG GAT GAT GC
R: AAC CCA CGA TGT GGG TAG C

Group 9: F: CAA AGA GAG TGC AAC GGA TG
R: ATT GGA AAG CGT TCA TCA CC

Group 25: F: GCA CGA TGA CAT TCG GG
R: AAC CCA CGA TGT GGG TAG C

Amplification was done by: an initial denaturation at (94°C for 5 min), followed by 30 cycles of [(DNA denaturation at 94°C for 25 sec), primer annealing (at 52°C for 40 sec), primer extension (72°C for 50 sec)], and final extension (72°C for 6 min) [14].

The used primers [15] for detection of *magA* and *rmpA* virulence genes by multiplex PCR were:

magA: F:GGTGCTCTTTACATCATTGC
R: GCAATGGCCATTTGCGTTAG

rmpA: F: ACTGGGCTACCTCTGCTTCA
R:CTTGCATGAGCCATCTTTCA

Amplification was done by: an initial denaturation at (95°C for 5 min), followed by 30 cycles of [(DNA denaturation at 94°C for 1 min), primer annealing (58°C for 1 min), primer extension (72°C for 1 min)], and final extension (72°C for 10 min) [16]

Electrophoresis was performed on agarose gel 1.5% (Fermentas, Lithuania) stained with ethidium bromide (Sigma, USA) for 20 minutes.

The products were visualized by UV trans-illuminator and compared with 1 kb DNA ladder for *magA* and *rmpA*: (*rmpA* at 516 bp and *magA* at 1283 bp) and with 50bp DNA ladder for detection of *CTX-M* groups (group 1 at 415 bp, group 2 at 552 bp, group 9 at 205 bp, group 8 at 666 bp and group 25 at 327 bp)

Infection control compliance in NICU

In the present infection control study, we assess the technique, compliance of hand hygiene among HCWs and factors related to the compliance and application of general infection control measures before and after a period of training and education about general infection control measures in NICU which included; on-site educational and training sessions, instruction about proper technique, importance and five moments of hand hygiene, when and how to use personal protective equipment (PPE), how to manage blood and body fluids, the proper technique of sterilization for instruments and devices in NICU and when to apply, safe injection practices (i.e., aseptic technique for parenteral medications), environmental cleaning measures etc**.**

About 40 visits to NICU were done during the study period to assess hand hygiene compliance using observational form documented from WHO [17] and a checklist documented from supreme council of university hospitals, Egypt [18] to assess compliance of infection control measures in NICU. Hospital-acquired septic cases were recorded and followed-up to determine the incidence and outcome of sepsis in our NICU and to correlate them with the infection control measures application before and after the training period of infection control measures.

Statistical analysis

Data were collected, tabulated and analyzed by statistical package for the social sciences (SPSS, version 23). Categorical variables were compared by Chi-square ($\chi 2$) test. Z test was used to compare two proportions in two groups. Pearson correlation was used to show correlation between two continuous normally distributed variables. Kappa test (K) was used to determine the degree of matching between different elements (statistical significance was set at p-value <0.05).

Results

Out of the 152 neonates with confirmed sepsis, 73 (48%) presented as EOS and 79 (52%) as LOS. About 73.6%, 76.3% and 78.9% of neonates with confirmed sepsis were CRP positive, had leukocytosis and had immature/total leucocytes (I/T) ratio ≥0.2 respectively. The most frequent clinical presentations were pneumonia (26.3%) followed by respiratory distress (23%), temperature instability (19.7%) and lethargy (9.8%).

The most commonly isolated bacteria were Gram-negative bacilli (53.3%) followed by Gram-positive cocci (43.4%) and fungi (3.3%). *Klebsiella pneumoniae* was the most frequently isolated organism (31.6%), followed by coagulase-negative staphylococci (CoNS) (23.1%), *Staph aureus* (20.4%), *Acinetobacte*r *spp.* (9.9%), *E. coli*, (3.9%) *Klebsiella oxytoca* (3.3%), *Pseudomonas aeruginosa* (2.6%), *Citrobacter spp*. (1.9%) and

Candida spp. (3.3%). Gram-negative bacilli were more abundant in LOS neonates (62.1%). The most frequently isolated organisms in EOS were CoNS (27.4%), followed by *Klebsiella pneumoniae* (24.7%) and *Staph aureus* (23.3%), while, in LOS *Klebsiella pneumoniae* was the most frequent (37.9%) followed by CoNS (18.9 %) and *Staph aureus* (17.7%).

Out of the 48 *K. pneumoniae* isolates, 19/48 (39.6%) displayed hypermucoviscous phenotype (hmvKP) as proved by positive string test while the remaining isolates (29/48) (60.4%) were string test-negative (cKP). Regarding ESβL production, 36/48 (75%) of *K. pneumoniae* isolates were ESβL producers by double disc diffusion method, relation between Hypermucoviscosity and ESβL production showed that 47.3% (9/19) and 93.1% (27/29) of hmvKP and cKP isolates respectively were ESβL producers with a highly significant (*p* value <0.001**) difference between both phenotypes (hmvKP and cKP) regarding ESβL production **(Table 1)**.

There was a significant difference between neonates infected with hmvKP and cKP regarding neonatal birth weight, mechanical ventilation use, while there was no significant difference regarding other studied parameters between the two phenotypes. In addition, there was significant difference between neonates infected with ESβL and non-ESβL producing *Klebseilla pneumoniae* regarding submission to mechanical ventilation (91.6% in ESβL producers and 41.6% in non-ESβL producers) **(Table 2)**.

Antibiograms of the isolated *Klebsiella pneumoniae* showed that cKP isolates exhibited higher antibiotic resistance rates than hmvKP for most the tested antibiotics except for Monobactam (Aztreonam), Carbapenems (Ertapenem, Imipenem, Meropenem) and Quinolones (Ciprofloxacin, Levofloxacin) groups with a significant difference regarding susceptibility to Amoxicillin-clavulanic acid, Doxycycline, Cefixime, Levofloxacin and Piperacillin/tazobactam.

ESβL-producing *K. pneumoniae* exhibited higher antibiotic resistance rates than non-ESβL producers for all the tested antibiotics except for Tigecycline and Colistin. There was a significant difference regarding susceptibility to Amoxicillin/clavulanic acid, Piperacillin/tazobactam, Ceftriaxone, Amikacin, Levofloxacin, Piperacillin, Cefotaxime and Gentamycin.

Polymerase chain reaction results showed that, 23/48 (47.9%), 4/48 (8.3%) and 28/48 (58.3%) of *Klebsiella pneumoniae* isolates were positive for *rmpA, magA* and *CTX-M* genes respectively **(Figure 1)**. The most prevalent single group of *CTX-M* gene was group 1 (42.8%), followed by group 9 (32.1%), group 25 (7.2%) and group 8 (3.7%), while the most prevalent combined groups were group (1 and 9) (7.2%) followed by group (1 and 8) and group (8 and 9) (3.7% for each) **(Table 3)**.

Frequency of *rmpA, magA* and *CTX-M* genes among *K. pneumoniae* isolates in relation to onset of sepsis, showed that 8/18 (44.4%), 3/18 (16.7%) and 10/18 (55.6%) of isolated *Klebsiella pneumoniae* from EOS were positive for *rmpA, magA* and *CTX-M* genes respectively. On the other hand, 15/30(50%), 1/30 (3.3%) and 18/30 (60%) of isolated *Klebsiella pneumoniae* from LOS cases were positive for *rmpA, magA* and *CTX-M* genes respectively. There was no significant difference between *K. pneumoniae* isolated from EOS and LOS cases regarding carriage of *rmpA, magA* and *CTX-M* genes.

There was a significant (*p* value<0.001) difference between hmvKP and cKP isolates regarding *rmpA* and *magA* distribution but with no significant difference regarding *CTX-M* genes. Results showed that 17/19 (89.5%), 4/19 (21.1%) and 10/19 (52.6%) of hmv *Klebsiella pneumoniae* strains were positive for *rmpA, magA* and *CTX-M* genes respectively. In contrast 6/29 (20.5 %), none (0%) and 18/29 (62.1%) of ckp strains were positive for *rmpA, magA* and *CTX-M* genes respectively**.**

There was a significant (*p* value<0.001) difference between ESβL-producing and non-producing *Klebsiella pneumoniae* regarding *CTX-M* gene distribution but no difference regarding *rmpA* or *magA* genes. About 50%, 11.1% and 77.8% of ESβL-producing *Klebsiella pneumoniae* were positive for *rmpA, magA* and *CTX-M* genes respectively. On the other hand, 41.7 % of non-ESβL were positive for *rmpA* and none of them (0 %) were positive for *magA* or *CTX-M* genes**.**

There was a moderate agreement (Kappa test = 0.498) (*p* value<0.001) between *rmpA* gene and string test. On the other hand, there was fair agreement (Kappa test = 0.244) between *magA* gene and string test for detection of hypermucoviscosity among *Klebseilla pneumoniae* isolates **(Table 4)**

After the training period of infection control measures, the compliance to hand hygiene has increased and the infection rates have decreased

as the overall HCWs hand hygiene compliance rate increased from 61.5% and 70.2% in the period before education and training to 90.4% and 95.3% after this period, also general infection control measures compliance in NICU rates increased significantly (65.3% and 69.4 vs. 92.5% and 94.4), in addition sepsis recovery rate increased from 58.5% to 93.8% and as an overall result, there was a strong negative correlation between compliance to hand hygiene and general infection control measures in NICU, and occurrence of NS, and a strong positive correlation between compliance to hand hygiene and general infection control measures in NICU, and recovery rate of neonates with sepsis **(Figure 3)**

Other infection control measures in NICU included during this infection control study are:

- Availability of adequate and proper PPE (gloves and surgical masks) and indication of use.
- Formula preparation and bottle feeding: ensure that all feeding and preparation equipment (bottles, teats, lids) are washed thoroughly in hot soapy water using brushes with scrubbing the inside and outside of the equipment and that any feed that has not been consumed within two hours is thrown away
- Staff exclusion: staff with infected wounds or skin infections on exposed parts of their body or respiratory infections are excluded until recovery
- Linen management: proper collection of used linen, transport and changing
- Environmental safety: proper environmental surfaces cleaning and disinfection (daily and when contaminated)
- Waste management: proper waste segregation using colored bags in covered containers.

Table 1. Relation between hypermucoviscosity and ESβL production among *Klebsiella pneumoniae* isolates.

ESβL production	**Hypermucoviscosity**				X^2	***p* value**
	hmv*KP* (No=19)		c*KP* (No=29)			
	No	%	**No**	**%**		
ESβL (36)	9	47.3	27	93.1	12.8	**<0.001****
Non- ESβL (12)	10	52.6	2	6.8		

- Hypermucoviscosity was detected by string test.

- ESβL production was detected by cephalosporin/clavulanate combination disk method.

- hmvKP :hypermucoviscous Klebsiella pneumoniae .

- cKP: classic Klebsiella pneumoniae.

Table 2. Neonatal demographic and clinical data and maternal risk factors in relation to ESβL production (ESβL, Non-ESβL) and hypermucoviscosity (hmvKP, Ckp) of *K. pneumoniae* isolates.

Neonatal demographic and clinical data	***Klebseilla pneumonia*e isolates (No=48)**					
	Hypermucoviscosity			**ESβL production**		
	hmv*KP* **(**No =**19)**	**c*KP*** **(**No =**29)**	**χ2 (*p*-value)**	**ESβL** **(**No =**36)**	**Non-ESβL** **(**No =**12)**	**χ2 (*p*-value)**
	No (%)	**No (%)**		**No (%)**	**No (%)**	
Gender						
Male	10(52.6)	19(65.5)	0.7	13(36.1)	7(58.3)	1.8
Female	9(47.4)	10(34.5)	(0.4)	23(63.9)	5 (41.7)	(0.2)
Gestational age						
<37 weeks	15(78.9)	16(55.2)	2.8	25(69.4)	6(50)	1.5
37-40 weeks	4(21.1)	13(44.8)	(0.09)	11(30.6)	6(50)	(0.3)
Birth weight						
low	17(89.5)	18(62.1)	4.4	27(75)	8(66.7)	0.3
Normal	2(10.5)	11(37.9)	**(0.04*)**	9(25)	4(33.3)	(0.7)
Intrauterine distress						
Yes	2(10.5)	2(6.8)	0.2	2(5.5)	2(16.6)	1.4
No	17(89.4)	27(93.1)	(0.7)	34(94.4)	10(83.3)	(0.2)
Mechanical vent.						
Yes	17(89.4)	21(72.4)	5.9	33(91.6)	5(41.6)	17.3
No	2(10.5)	8(27.6)	**(0.04*)**	3(8.4)	7(58.4)	**(<0.001**)**
I.V line						
central	13(68.4)	15(51.7)	1.3	21(58.3)	7(58.3)	-
peripheral	6(31.5)	14(48.2)	(0.3)	15(41.7)	5(41.7)	-
Out come						
Discharged	11(57.8)	16(55.1)	0.03	18(50)	9(75)	3.1
Referred	3(15.8)	5(17.2)	(0.9)	6(16.7)	2(16.7)	(0.2)
Died	5(26.3)	8(27.5)		12(33.3)	1(8.3)	
Mode of delivery						
Vaginal	10(52.6)	10(34.5)	1.6	14(38.9)	6(50)	0.4
C.S	9(47.4)	19(65.5)	(0.2)	22(61.1)	6(50)	(0.4)
PROM						
Yes	9(47.3)	13(44.8)	0.02	17(47.2)	5(41.6)	0.1
No	10(52.6)	16(55.1)	(0.9)	19(52.8)	7(58.3)	(0.7)
maternal fever						
Yes	4(21.1)	6(20.7)	0.001	7(19.4)	3(25)	0.2
No	15(78.9)	23(79.3)	(0.9)	29(80.6)	9(75)	(0.7)
Chorioamnionitis						
Yes	2(10.5)	6(20.7)	0.8	5(13.9)	3(25)	0.8
No	17(89.5)	23(79.3)	(0.4)	31(86.1)	9(75)	(0.4)
Maternal UTI						
Yes	2(10.5)	2(6.9)	0.2	3(8.3)	1(8.3)	-
No	17(89.5)	27(93.1)	(0.7)	33(91.7)	11(91.7)	-

χ2: Chi-square test, *hmvKP :hypermucoviscous Klebsiella pneumoniae, cKP: classic Klebsiella pneumoniae, <37 Weeks: preterm, 37-40 Weeks: full-term, Low birth weight : <2500g, Normal birth weight: 2500-4200g, *significant, **highly significant, PROM: premature rupture of membrane, C.S: caesarian section, I.V: intra venous line, UTI: urinary tract infection*.

Table 3. Frequency of *CTX-M* gene groups among *CTX-M*-positive *Klebsiella pneumoniae* isolates.

***CTX-M* gene groups**	**Base pair**	**Positive**	
		No	%
Single groups			
Group 1	415	12	42.8
Group 2	552	0	0
Group 9	205	9	32.1
Group 8	666	1	3.7
Group 25	327	2	7.2
Combined groups			
Group 1 and 9	415 and 205	2	7.2
Group 1 and 8	415 and 666	1	3.7
Group 8 and 9	205 and 666	1	3.7

Table 4. Degree of agreement between *rmpA*, magA and string test for detection of hypermucoviscosity among *Klebseilla pneumoniae* isolates.

The studied genes		**String test**				**Symmetrical measurement**
		Positive (No =19)		**Negative (No =29)**		
		No	**%**	**No**	**%**	
***rmpA* gene**	**Positive (No =23)**	17	89.5	6	20.5	**Kappa test = 0.498 (*p*-value <0.001)**
	Negative (No =25)	2	10.5	23	79.3	
***magA* gene**	**Positive (No =4)**	4	21.1	0	0	**Kappa test = 0.244 (*p*-value: 0.01)**
	Negative (No =44)	15	78.9	29	100	

Kappa agreement

- *< 0 Less than chance agreement*
- *0.01–0.20 Slight agreement*
- *0.21– 0.40 Fair agreement*
- *0.41–0.60 Moderate agreement*
- *0.61–0.80 Substantial agreement*
- *0.81–0.99 Almost perfect agreement*

Figure 1. Distribution of *rmpA*, *magA* and *CTX-M* genes among isolated *Klebsiella pneumoniae*.

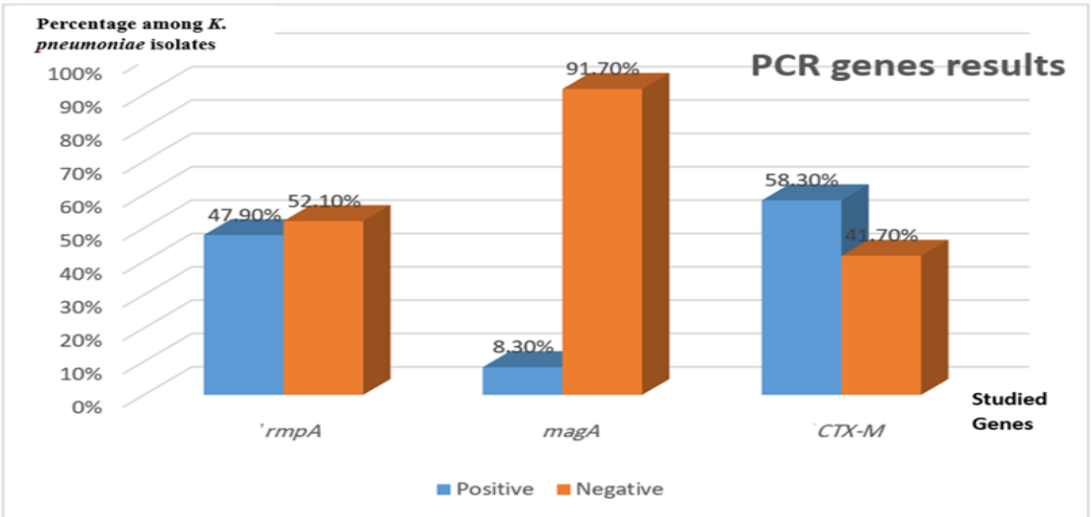
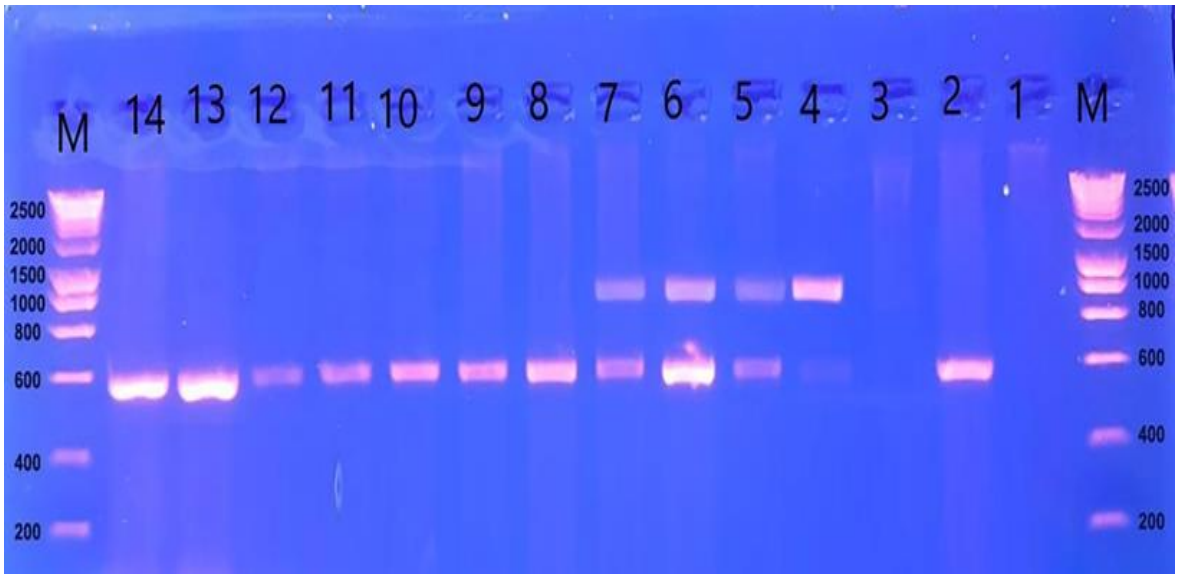


Figure 2. Agarose gel electrophoresis for the PCR-amplified products of *KP rmpA*, *magA* and *CTX-M* genes.

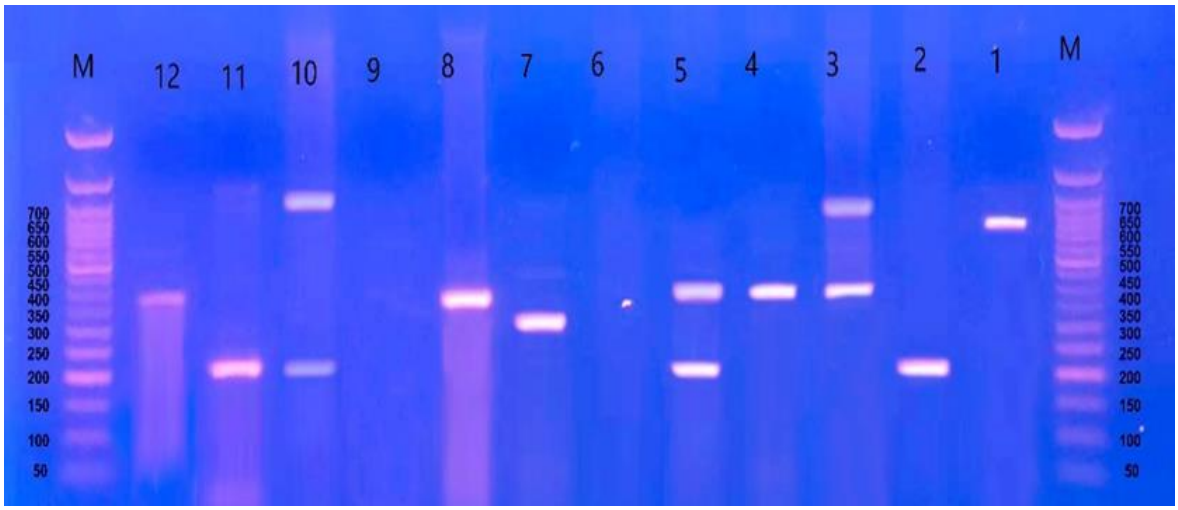


a) Multiplex PCR for *rmpA* and *magA* genes:

Lane M: DNA molecular size marker (1 kb DNA ladder).

-Lanes 2, 5, 6, 7, 8,9,10,11,12,13 and14 were positive for *rmpA* gene (516 bp).

-Lanes 4, 5, 6and 7 were positive for *magA* gene (1283 bp).



b) Multiplex PCR for *CTX-M* groups:

Lane M: DNA molecular size marker (50 bp DNA ladder).

-Lane 1 positive for group 8 (666bp)

-Lane 2, 11 positive for group 9 (205bp)

-Lane 3 positive for group 1 and 8 (415 bp, 666 bp)

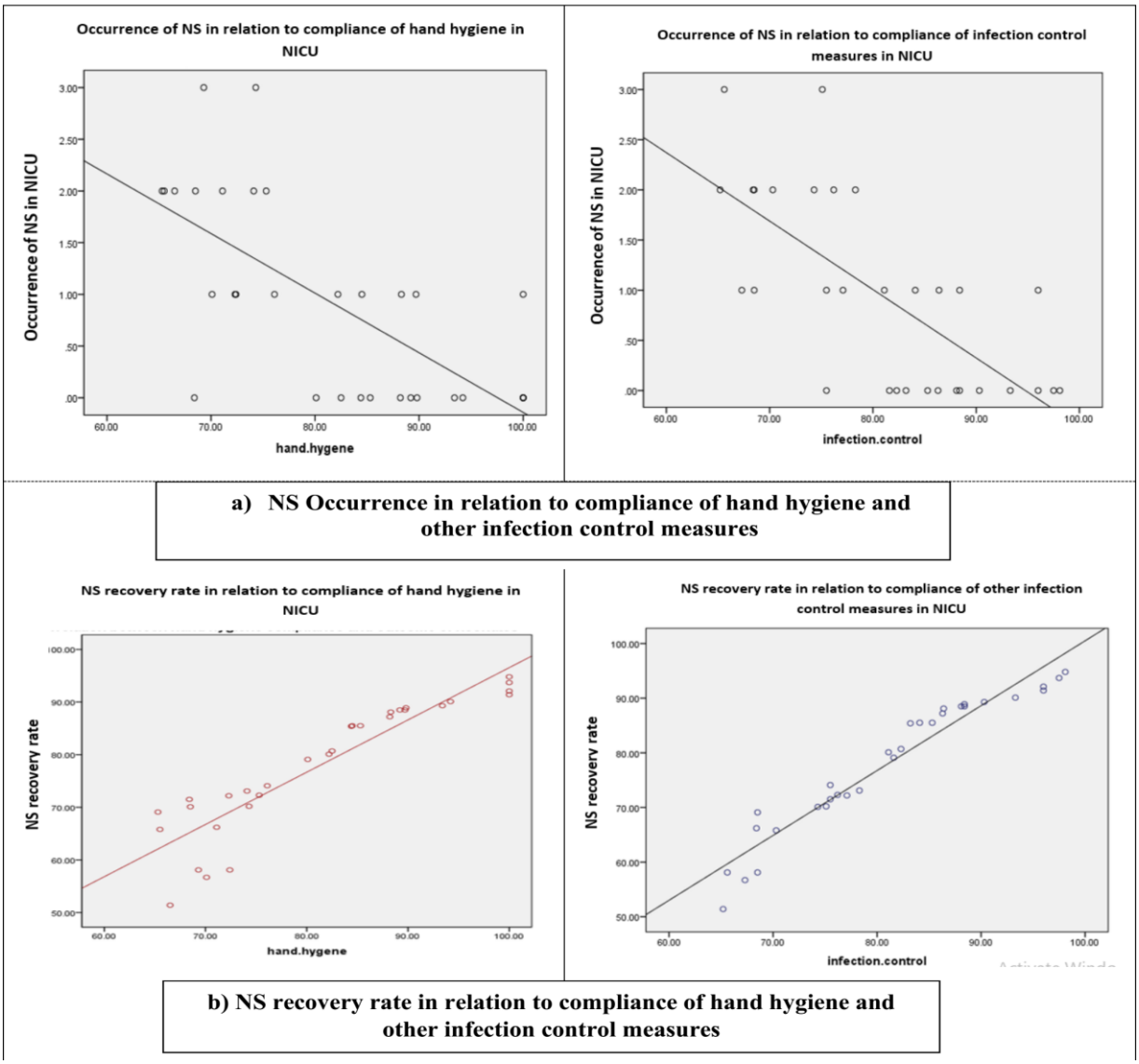
-Lane 4, 8, 12 positive for group 1(415bp)

-Lane 5 positive for group 1 and 9 (415 bp, 205 bp)

-Lane 7 positive for group 25 (327 bp)

- Lane 10 positive for group 9 and 8 (205 bp , 666 bp).

Figure 3. Correlation between compliance of infection control measures and NS occurrence and recovery rate in NICU.



Discussion

Blood culture remains the gold standard method for diagnosis of NS [19]. In our study, 48% and 52% of neonates with confirmed sepsis had EOS and LOS respectively. Of the confirmed NS cases, 73.6% were CRP positive, 76.3% had leukocytosis and 78.9% had I/T ratio ≥0.2. Similar results were reported [20-23].

This study showed that the most prevalent isolated bacteria were Gram-negative bacilli (53.3%) followed by Gram-positive cocci (43.4%). *Klebsiella pneumoniae* was the most frequently isolated organism, followed by CONS, Similar results were reported [24,25] who found that the majority of NS cases were caused by Gram-negative isolates. [26] showed that the most frequently isolated organism was *Klebsiella spp* followed by CONS, on the other hand, [27] found that the predominant isolates were *S. aureus*. Our results showed that Gram-negative isolates were significantly more abundant in LOS neonates than EOS neonates. However, [28] reported that Gram-negative organisms were the predominant agents in both EOS and LOS. This difference may be related to changes in place and time [29].

In the current study, 39.6% of *K. pneumoniae* isolates were hmvKP (as proved by positive string test) and 75% of isolates were ESβL producers (by double disc diffusion method) and that 47.3% of hmvKP were ESβL producers. In agreement with our results, [30] showed that 37.4% of *K. pneumoniae* were hmv and [7] reported that 60% were ESBL-producing *K. pneumoniae* and [31]

who reported that 47.5% of hmvKP were ESβL producers.

In this study, there was significant differences between neonates infected with hmvKP and cKP regarding birth weight and mechanical ventilation. However, no significant difference was detected regarding gender, gestational age, birth weight, intrauterine distress, type of IV line or mortality rate. There was a significant difference between neonates infected with ESβL and non-ESβL producing strains regarding submitting to mechanical ventilation. However, no significant difference was found regarding other studied parameters. These results agree with other investigators [32] who showed that invasive procedures as (CVC insertion, umbilical catheter), continuous positive airway pressure, and birth weight were significantly associated with infection by ESβL-producing *K. pneumoniae*. Invasive operations such as peripherally inserted central catheter (PICC) and mechanical ventilation can damage the body's natural barrier, decrease immunity and increase the probability of bacterial colonization or infection. Moreover, the long-term use of antibiotics in NICU may increase the number of drug-resistant strains [33].

In this study, cKP isolates exhibited higher antibiotic resistance rates for most of the tested antibiotics than hmvKP except for Monobactam, Carbapenems and Quinolones groups. In addition, ESβL-producing *K. pneumoniae* exhibited higher antibiotic resistance rates than non-ESβL producers for all the tested antibiotics except for tigecycline and colistin, with significant difference regarding amoxicillin/clavulanic acid, piperacillin/tazobactam, ceftriaxone, amikacin and levofloxacin. These results agree with that reported by [4] who showed that the resistance rates to almost all antibiotic agents among cKP were significantly higher than that of the hvKP group, with the exception of ampicillin, imipenem, and meropenem, some investigators [7,34,35] reported that, the rates of resistance were much higher among ESβL positive isolates than ESβL negative isolates.

Emergence of antibiotic-resistant hmvKP in NICUs is of great concern because this may offer a new threat for critically ill neonates. Co-occurrence of virulence and antimicrobial resistance is becoming a global and widespread problem that must require adequate surveillance and treatment [7]. Our results showed that 47.9% of the studied *Klebsiella pneumoniae* isolates (89.5% of hmvKP and 20.5% of cKP) harbored the *rmpA* gene, with a highly significant difference (p<0.001) between the two groups. Moreover, the percentage of *rmpA* (50%) and *magA* (11.1%) positive isolates were non-significantly higher among ESβL-producing *K. pneumoniae* compared to non ESβL-producing strains. However, different results were reported by [36,37] who detected *rmpA* gene in 72.9% of cKP and 96.4% of hmvKP isolates. Also, [7] found that 28.6% of *rmpA* positive isolates were ESβL-producers. To our knowledge, the relation between the studied genes and onset of sepsis was not previously studied. Our results showed that there was no significant difference between *K. pneumoniae* isolated from EOS and LOS cases regarding carriage of *rmpA*, *magA* and *CTX-M* genes.

In this study, about 58.3% of *K. pneumoniae* isolates harbored *CTX-M* gene, with a highly significant difference (p<0.001) between ESβL- and non-ESβL-producing *K. pneumoniae.* Presence of the gene in 77.8% in ESβL-producing *K. pneumoniae* isolates indicates its dissemination in our locality. Others reported 70% and 92.3% carriage rates [38,39]. The most prevalent group of *CTX-M* gene among our isolates was group1 (42.8%), followed by group 9 (32.1%), group 25 (7.2%) and group 8 (3.7%), in agreement with other studies [40,41] who showed that the *CTXM-1* group was the most predominant ESβL type among ESβL-producing *K. pneumoniae*.

Our results showed that there was a strong negative correlation between compliance to hand hygiene and other infection control measures in NICU, and occurrence of NS. On the other hand, there was a positive correlation with recovery rate of neonates with sepsis; similarly, there was a significant improvement in the adherence to infection control measures among healthcare personnel after a period of on-site educational and training sessions [42-44] reported similar results after a period of education and training on infection control measures in Egypt and Saudi Arabia respectively.

Limitation

Limitations in this study, included lack of financial facilities that disabled the use of anaerobic cultivation of the samples.

Conclusion and recommendations

Klebsiella pneumoniae is one of the most common causes of NS in NICU in our hospital.

Spread of ESβL-producing *K. pneumoniae* in NICU was strongly associated with abuse and misuse of antibiotics, exposure to invasive procedures, presence of associated comorbidities and prolonged hospital stay. Co-occurrence of virulence patterns and antimicrobial resistance among *K. pneumoniae* isolates is becoming a widespread problem and a major challenge. Proper identification of the causative pathogens, the patterns and rates of antibiotic resistance along with implementation of infection control measures, antimicrobial stewardship programs and constant surveillance, can help in survival of the septic neonates.

Acknowledgment

The authors express thanks and gratitude to the staff members of NICU, Menoufia University Hospitals, Egypt for their valuable help.

Authorship

Each author listed in the manuscript had approved the submission of this version of the manuscript and takes full responsibility for it.

Ethical approval

The study protocol was approved by local ethics committee of the Faculty of Medicine, Menoufia University (No.28419MLCR46). Informed written or signed consent from each neonate's parents or legal guardian was obtained before involvement in this study.

Conflict of interest: The authors have no conflicts of interest to disclose.

Financial disclosure: None.

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