Polymerase chain reaction (PCR) as a rapid tool for identification of virulent bacteria, infecting neonates in a neonatal intensive care units (NICUs).

By

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Abstract:

This study evaluated the nature to bacterial infections in a neonatal intensive care unit (NICU) at Zagazig University Hospital, Zagazig , Egypt . Rapid diagnosis and identification of the infectious bacterial pathogens by polymerase chain reaction (PCR) is recommended herein. Out of 70 microbial cultures ordered by physicians from many clinical samples, 50 cultures showed bacterial growth. The 50 bacterial cultures obtained were identified by biochemical methods. They were categorized into 6 groups ; were arranged in the following descending order according to number the identified strains : *Escherichia* (E.) *coli* (group 1 , 22 isolates) > *Staphylococcus* (S.) *aureus* (group 2 , 10 isolates > *Streptococcus* (St.) *pneumonia* (group 3 , 6 isolates) > *St* . *agalactiae* (group 4 , 5 isolates) > *St. pyogenes* (group 4 , 5 isolates) > *Klebsiella* (Kb.) *pneumonia* (group 6 ,3 isolates) . Antibiotic sensitivity profiles

showed variability in susceptibility of isolates to the 10 studied antibiotics. Certain virulence genes for selected bacterial strains from each bacterial group were rapidly detected by multiplex PCR ; this indicated that PCR technique could be used as a rapid for identification of infectious bacteria and in turn a diagnostic tool for infectious diseases .It reduces time and personal efforts faced microbiologists throughout biochemical identification.

Introduction:

Newborn infections are normally detected in newborns throughout two months after birth, their prevalence in neonates within the first month after birth is more higher than older ages; such infections are either acquired from the external environments especially neonatal intensive care units (NICUs) or transmitted via placental transfer from mothers **(1)** . Neonates are more susceptible to infections much more than older infants; because their immune systems are not completely developed and, therefore , can not fight many infections **(2)** . This clearly shows that there is a need to develop rapid diagnosis protocols for infectious microbes.

Neonatal infections is the important cause of neonatal death in NICUs due to many infectious organisms such as *E. coli*, *St. pyogenes* , *methicillin* or vancomycin resistant *S. aureus* (MRSA or VRSA respectively), *Kb. pneumonia*, *Pseudomonas aeruginosa* organisms *(P. aeruginosa)* and other infectious organisms **(3).**Those infections were showed to be associated with decrease in health care in NICUs . Also the use of many medical devices and lack of a child's maturation with non-developed immune system of neonates increase the chance of infection **(4)**. This clearly shows that there is a need to continue research to find out rapid diagnosis and treatment protocols for such infections of neonates in NICUs.

Diagnosis of infectious organisms by their culturing on agar media lasts long time (24 h) to record culture characteristics such as Gram stain and cell morphology and lasts other 24 h to study their antibiotic sensitivity; the culturing of infectious organisms on agar media could gave elusive results in many cases since contamination may be occurred from handling processes, decrease in sterilization or other reasons **(5,6).** There is a high incidence of infectious organisms in air , food , soil , and even hospitals **(7,8,9).** To avoid elusive results of bacterial culturing on agar media and possible contamination by

such highly frequent pathogenic organisms, rapid diagnosis by PCR technique within 2 h only is necessary and to concur with recent work in this respect.

The present work was undertaken to **(1)** know the causal pathogens of infections in NICUs at Zagazig University Hospitals, Zagazig, Egypt and to (**2)** calculate their prevalence and to (**3**) develop rapid identification processes for the isolated bacteria.

Material and Methods

Ethical approval: This study was approved by the institutional board member of Zagazig University ,Egypt. Parents of neonates admitted to NICU were informed about the nature and purpose of medicinal analysis needed. The study subjects were not exposed to any harms or risk.

Collection of clinical samples:

Seventy clinical samples including urine, stool, blood, eye and ear swabs, central line tubes, aspiration of endotracheal tubes, umbilical cords were collected from NICUs at Zagazig University Hospitals in the period from October/2013 till December/2016 and transferred immediately to Laboratory of Microbiology in the same hospitals for their microbiological analysis. In addition, the necessary analysis prescribed by physicians such as complete blood count (CBC), liver functions, kidney functions, erythroside sedimentation rate (ESR), carbon reactive protein (CRP) were made and will be considered in another study .

Isolation and identification of the infectious bacteria:

The collected samples were streaked by either sterile loops or swabs (ear, eye & throat swabs); endotracheal tubes, central lines and umbilical cords were also streaked on both MacConkey agar and blood agar plates. After incubation at 37 °C for 24-48 h, growing colonies were purified on another agar plates and slope cultures of pure isolates were made and kept throughout the experimental work **(10, 11, and 12)**. The pure bacterial isolates were subjected to biochemical identification using the biochemical tests reported

previously such as Gram staining, catalase test, urease test, indole production, Voges Proskauer test, production of enzymes and carbohydrate fermentations **(13,14).**

Antibiotic sensitivity test:

Ten antibiotics assimilating most antibiotic groups such as sulphonamide , gentamycin , tobramycin , ampicillin , azitreonam , erythromycin, cefaclor, ampicillin clavulonic acid, ceftazidime, ampicillin/sulbactam; were selected for carrying out the antimicrobial susceptibility test by disc diffusion assay **(15,16).** The bacterial isolates were swabbed onto the surface of Muller-Hinton agar plates and, then, antibiotic impregnated discs were placed aseptically onto this Muller-Hinton agar at appropriate distances separating them from each other, After incubation for 24h at 37°C, diameter of inhibition zones were measured by a millimeter ruler after subtracting diameter of an antibiotic disc. Results were calculated according to CLSI **(17).**

Preparation of bacterial DNA:

Bacterial cultures were grown in their suitable broths until the mid of the exponential phases; then cells were subjected to DNA

isolation protocol by DNA purification Kit **(Wizard Genomic DNA purification kit, Promega corporation , USA)** according to the manufacture's instructions.

Polymerase chain reaction (PCR) for amplification of virulence genes:-

Multiplex PCR was monitored to detect some virulence genes such as hia ; icuD ; fim H ; yia A ; Tsb E4C2 ; mag A; kan R ; scp A ; sda B ; Sdc ; spy CEP which encode hemolysin within *S. aureus* , aerobactin ; fimbria adhesion; enteric toxin and siderophore receptor within *E. coli;* outer membrane protein, promoter region of kanamycin resistance cassette within *St. pneumonia*; superantigen of *St. pyogenes* (scpA , sda B and Sdc) ; protease activity within *St. pyogenes* genomes respectively. The specific primers used are given in Table 1 (Promega, USA) . The PCR mixture consisted of 30 picomoles of each primer, 10 mg of chromosomal DNA , 200 μm dNTPs and 2.5 units of Taq polymerase in 50 μl polymerase buffer. The PCR was carried out for 30 cycles in 94 °C for 2 min. , 55 °C for 1 min. and 72 °C for 2 min . After completion , a fraction of the electrophoresis and the remnant was purified using QIA quick PCR purification reagents

(Qiagen).DNA bands were photographed as described previously **(18,19) .**

Table 1- Primers used for the Multiplex PCR:

Gene	The expressed material	Organism	Primer used	Reference
Hia	Hemolysin	*S. aureus*	5`- TAATGAATCCTGTCGCTAATGCC-3` F 5`-CACCTGTTTTTACTGTAGTATTGCTTCC-3` R	(Biotek corporation, china)
icuD	Aerobactin receptor	*E. coli*	5-ATGGCATCACTGCCGATTCTTT -3` F 5-AGTGAGTTAAAGCAGCAGCCTC- 3` - R	(20)
fimH	fimbrial adhesion	*E. coli*	5`- ATTCCTCACAATCAGCGCACTT-3` F 5`-ATCAGCAGTACAGCAAACAGGG-3` R	(20)
yiaA	Enteric toxin	*E. coli*	5`-TGAAGTGTTCAGGAGACCGCTG -3` F 5` -ATGGAGAATGCGTTCCTCAAC -3` R	(20)
TsbE4c2	Siderophore receptor	*E . coli*	5` -GAGTAATGTCGGGGCATTCA-3` F 5` -CGCGCCAACAAAGTATTACG -3` R	(20)
magA	Outer membrane protein	*Kb. pneumonia*	5`-CGCCGCAAATACGAGAAGTG -3` F 5`-GCAATCGAAGTGAAGAGTGC -3` R	(21)
KanR	Promoter region of Kanamycin resistance cassette	*St. pneumonia*	5`-GACGAACTCCAATTCACTGTT -3` F 5`-AGATTTAGATGTCTAAAAAGC -3` R	(22)
scpA	Superantigen	*St. pyogenes*	5`-GCTCGGTTACCTCACTTGTCC -3` F 5` - CAATAGCAGCAAACAAGTCACC -3` R	(23)
sdaB	Superantigen	*St. pyogenes*	5`-TATAGCGCATGCCGCCTTTT -3` F 5` -TGATGGCGCAAGCAAGTACC- 3` R	(23)
Sdc	Superantigen	*St. pyogenes*	5`-AAGCTTAGAAACTCTCTCGCCA-3` F 5`-AGTTCCAGTAATAGCGTTTTTCCGT -3` R	(23)

spyCEP	**Protease activity**	***St. pyogenes***	**5`-GATCCGGCCCATCAAAGCAT -3` F** **5` -AGCTGCCACTGATGTTGGTG 3 `-R**	**(23)**

Results

Out of 70 microbiological cultures, only 50 of them showed bacterial growth and were numbered from 1-50.The diagnosis of neonates infections and their medicinal analysis such as CBC, ESR and CRP were made by physicians (data not shown).They will be considered for publication in another article .Pure bacterial colonies were purified and slope cultures were made and kept in refrigerator throughout the study period ,and sub cultured every 2 months on brain heart infusion broth (BHI,Oxoid).

From the 50 isolates,16 bacterial isolates were obtained from urine (codes UR1- UR16),15 isolates from stool (codes ST1 – ST15),10 isolates from blood (codes BL1- BL10),3 isolates from eye swabs (codes EY1 – EY3),3 isolates from throat swabs (codes THR1 – THR3) and 3 isolates from ear swabs(codes ER1 – ER3).

Urine samples were taken from neonates suffering from urogenital system infections such as cystitis and pyelonephritis. Stool samples were taken from patients of fever and diarrhea. The 10 blood

cultures requested were taken from patients suffering from pneumonia, meningitis and bacteremia. The 3 swabs from eyes were taken from neonates suffering from conjunctivitis. The 2 swabs from ears were taken from neonates suffering from otitis. Finally, the 3 throat swabs were withdrawn from neonates suffering from inflammation of throat.

Based on cultural, morphological and biochemical characteristics, the 50 bacterial isolates were classified into 6 groups as follows:

Group 1: This group includes 22 isolates; all of them were Gram negative bacteria, rod shaped motile cells. They showed positive results regarding catalase, indole production, methyl red, gelatin liquification reactions and blood hemolysis. They were able to utilize glucose, lactose, maltose, maninitol, L- arabinose and D-sorbitol. Other biochemical testes showed negative results. On MacConkey agar, those 22 isolates grew well and produced rose pink to red colonies. By surveying bacterial characteristics given in **Berge's Manual of Systematic Bacteriology (24),** the 23 isolates of this group could be identified as bacterial strains belonging to *E. coli.*

Group 2: The obtained results indicated that the 10 isolates of group 2 were Gram positive coccoid bacteria and non-motile cells. They showed positive results with regard to catalase, coagulase, Voges Proskauer test, urease reaction, blood hemolysis, and gelatin liquification, fermentations of glucose lactose, maltose, mannitol, sucrose, and salicin. Other biochemical testes showed negative results. They grew on Mannitol salt agar and gave golden yellow colonies and on Baird – Parker agar media they gave blue colonies. Following the diagnostic key of **Berge's Manual of Systematic Bacteriology (20),** the eight isolates of this group were identified as belonging to *S. aureus*.

Group 3: The 6 isolates of this group were Gram positive coccoid bacteria and non-motile cells. They showed positive results with regard to lactase fermentation, blood hemolysis, catalase and mannitol utilization but showed negative results regarding other tests. According to the previous literatures **(24),** those bacterial isolates of this group were identified as belonging of *St. Pneumonia*.

Group 4 : The five isolates of this group showed identical cultural and morphological results like that obtained by bacteria of

group 3.They also gave similar results regarding biochemical results like bacterial isolates of group 3 except for positive results for sucrose utilization , Voges Proskauer test and fermentation of maltose ;they also gave positive CAMP test (Collection of Anti-Microbial Peptides) test (Fig.1). According to the previous literature **(24),** those bacterial isolates of this group were identified as belonging to *St. agalactiae*.

Group 5: The 4 isolates of this group were Gram positive, coccoid bacteria and non-motile cells. They showed positive results with regard to lactose fermentation, sucrose utilization and blood hemolysis but showed negative results regarding other tests. According to the diagnostic key reported previously **(24),** those bacterial isolates of this group were identified as belonging to *St. pyogenes.*

Group 6 : The three isolates of this group were Gram negative, rod shaped, non-motile bacterial cells. They produced positive results with regard to catalase, Voges Proskauer, urease, lysine decarboxylation, gelatin liquification testes, and fermentation of glucose, lactose, maltose, mannitol sucrose, L- arabinose and D-sorbitol but showed negative results regarding other biochemical tests.

According to the previously reported diagnostic key **(24),** the bacterial isolates of this group were identified as strains of *Kb. pneumonia.*

At the end of this step, the 50 bacterial isolates obtained from neonates at NICUs in Zagazig University Hospital, Zagazig, Egypt were classified and identified to the bacteria namely : *E. coli ,S. aureus, St. pneumoniae, St. agalactiae, St. pyogens, Kb. pneumonia. E. coli* was the most frequent strains isolated from NICUs. This was followed by *S. aureus* strains, and then *streptococci*. *Kb. pneumoniae* strains were the lower frequent ones.

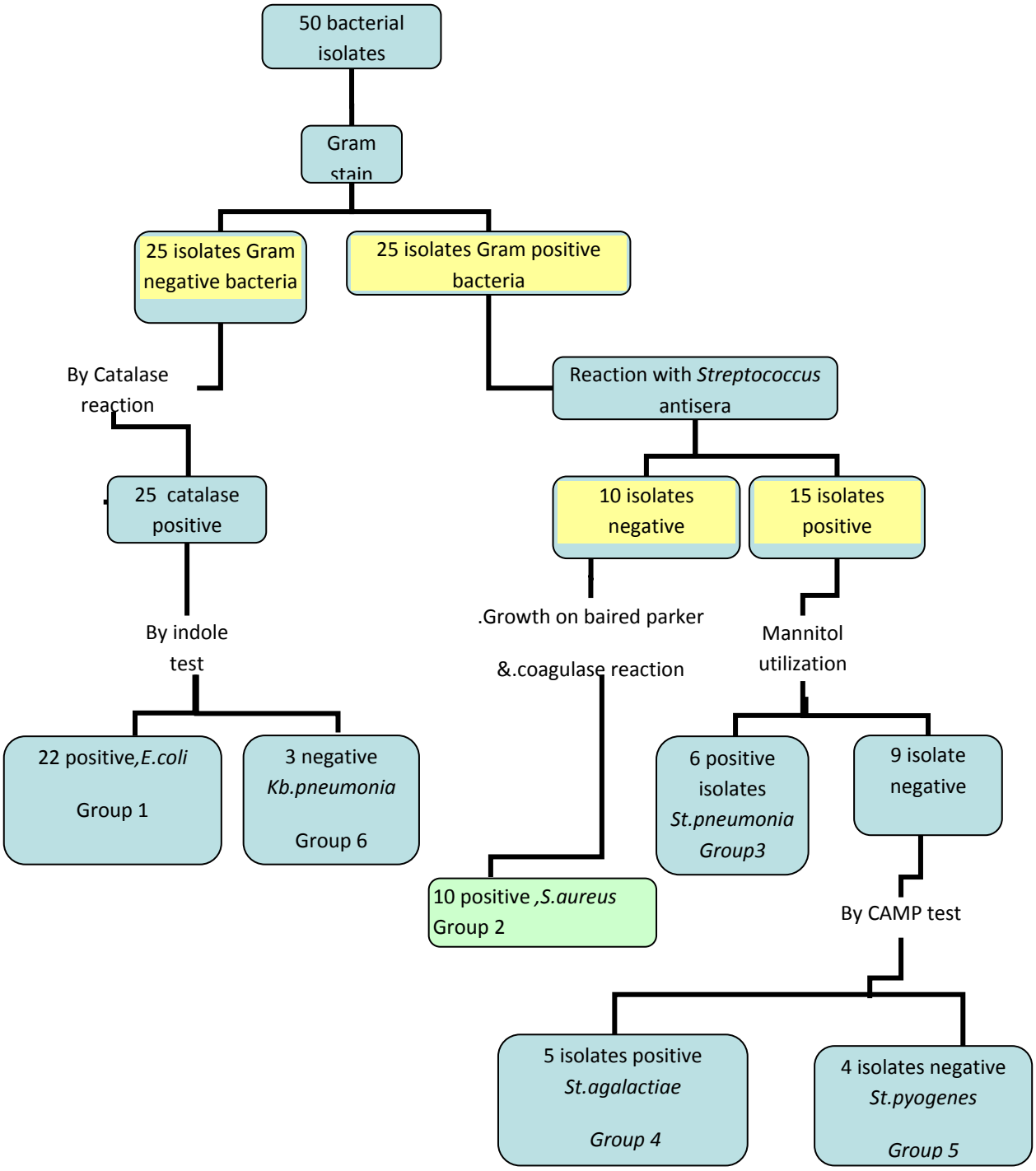


Figure 1:Key for differentiation of the infectious bacteria isolated from neonates admitted to NICUs in Zagazig

University Hospitals,Zagazig, Egypt.

The 50 identified bacterial strains were studied for their sensitivities to antibiotics .Results are given in Table 2 .*E. coli* strains were showed to be multi-drug resistant as all *E. coli* strains resist the action of 3 antibiotics tested. Other *E. coli* strains showed variability in antibiotic resistance; no strain was either completely resistant or sensitive to certain antibiotic. *S. aureus* strains were more resistant to ampicillin/sulbactam, ampicillin clavulanic acid, cefaclor, erythromycin, ampicillin, ceftazidime and sulphonamide and sensitive to gentamycin and tobramycin. Except for aztreonam ,*S. aureus* ST3 resist the action of the all antibiotics used. The antibiotic resistance profiles of *St. pneumonia*, *St. agalactiae*, *St. pyogenes*, *Kb. pneumonia* are given in Table 2.Almost all of these bacterial strains were multi-drug resistant .They showed variability in their sensivities or resistant profiles. No one strain was either resistant or sensitive to the all antibiotics used. In view of *Klebsiella* strains, they were of higher resistance to the antibiotics used than other bacteria.

Table (2): Antibiotic susceptibility profiles of identified bacteria.

Isolate no&code	Sulphonamide	gentamycin	tobramycin	ampicillin	aztreonam	Erythromycin	cefaclor	Ampicillin clavulanic acid	ceftazidime	Ampicillin sulbactam
***E .coli* UR1**	R	S	R	R	R	R	R	S	R	S
***S. aureus* UR2**	S	S	S	S	S	S	S	I	S	R
***E. coli* UR3**	R	S	S	R	R	R	R	S	R	S
***E. coli*UR4**	S	S	S	S	S	S	S	S	R	S
***Kb. pneumonia* UR5**	R	S	R	R	R	S	R	R	R	R
***E. coli* UR6**	R	S	R	R	S	R	R	S	R	S
***E. coli* UR7**	S	S	I	R	R	R	S	S	I	R
***E. coli* UR8**	R	S	S	R	I	R	R	S	R	S
***E. coli* UR9**	R	R	R	R	R	R	R	S	R	S
***S. aureus* UR10**	S	R	R	S	R	R	R	S	R	R
***Kb. pneumonia* UR 11**	R	R	R	R	R	S	R	R	S	R
***E. coli* UR12**	R	S	R	R	R	R	R	R	R	R
***E. coli* UR13**	R	S	R	R	S	R	R	R	R	R
***E. coli* UR14**	R	R	S	R	S	R	R	I	R	R
***Kb. pneumonia* UR 15**	R	R	R	R	S	R	S	R	R	R
***E. coli* UR16**	R	R	R	R	R	R	R	S	R	S
***S. aureus* EY1**	R	S	S	R	R	R	I	S	R	R
***S. aureus* EY2**	R	S	S	R	S	R	R	R	R	R

Isolate no&code	Sulphonamide	gentamycin	tobramycin	ampicillin	aztreonam	Erythromycin	cefaclor	Ampicillin clavulanic acid	ceftazidime	Ampicillin sulbactam
***St. pneumonia* EY3**	S	R	R	S	R	S	R	S	R	S
***S. aureus* EY4**	R	S	S	R	R	R	R	S	S	R
***E. coli* ST1**	R	S	R	R	R	I	R	I	R	I
***E. coli* ST2**	R	R	S	R	R	S	R	S	R	S
***S. aureus* ST3**	R	R	S	R	S	R	R	S	R	R
***E. coli* ST4**	R	R	S	R	R	R	R	R	R	I
***E. coli* ST5**	R	S	R	R	R	R	R	S	R	S
***E. coli* ST6**	R	S	S	R	S	R	R	R	R	R
***S. aureus* ST7**	S	S	S	R	S	R	I	R	R	R
***S. aureus* ST8**	R	S	S	I	S	R	I	R	R	R
***E. coli* ST9**	R	R	S	R	S	R	R	S	R	R
***E. coli* ST10**	S	R	S	R	S	R	S	R	R	R
***S. aureus* ST11**	S	S	S	S	S	S	S	I	S	R
***E. coli* ST12**	R	R	R	S	S	R	R	R	R	R
***E. coli* ST13**	R	R	S	R	R	S	R	I	R	R
***E. coli* ST14**	S	S	S	S	R	R	R	I	R	R
***S. aureus* ST15**	R	R	S	R	R	R	R	S	R	R
***St .pyogens* THR1**	R	S	S	R	S	R	R	R	R	R
***St. pyogens* THR2**	R	R	R	R	S	R	R	S	R	S
***St .pyogens* THR3**	R	S	S	I	R	R	I	R	R	R
***St. pneumoniae* EA1**	R	S	S	R	R	R	R	R	R	R
***St. pneumoniae* EA2**	R	K	R	S	R	R	R	S	R	R
***St. pneumoniae* BL1**	R	S	R	S	R	R	R	S	I	S
***St. pneumoniae* BL2**	R	R	S	R	R	S	R	S	S	R
***St. agalactiae* BL3**	R	R	R	S	R	R	R	R	R	S

Isolate no&code	Sulphonamide	gentamycin	tobramycin	ampicillin	aztreonam	Erythromycin	cefaclor	Ampicillin clavulanic acid	ceftazidime	Ampicillin sulbactam
***St .agalactiae* BL4**	R	R	R	R	S	R	R	R	R	S
***St. agalactiae* BL5**	R	S	R	S	R	R	I	R	R	R
***St. pneumoniae* BL6**	R	R	S	S	R	R	S	S	R	S
***St. agalactiae* BL7**	R	R	R	S	R	S	R	S	R	S
***St. agalactiae* BL8**	R	S	R	R	R	R	R	R	R	S
***E. coli* BL9**	S	R	R	S	S	R	S	S	R	R
St. pyogens BL10	S	R	R	S	R	R	R	S	R	S

Due to elusive biochemical identification of pathogenic bacteria obtained from neonates and from the environments of NICUs which lasts long time and many handling efforts, the rapid molecular identification of pathogenic bacteria by detection of their marker virulence gene(s) is currently necessary. This gives sound and rapid evidence for existence of bacterial pathogen and indicates on virulence of such bacteria.

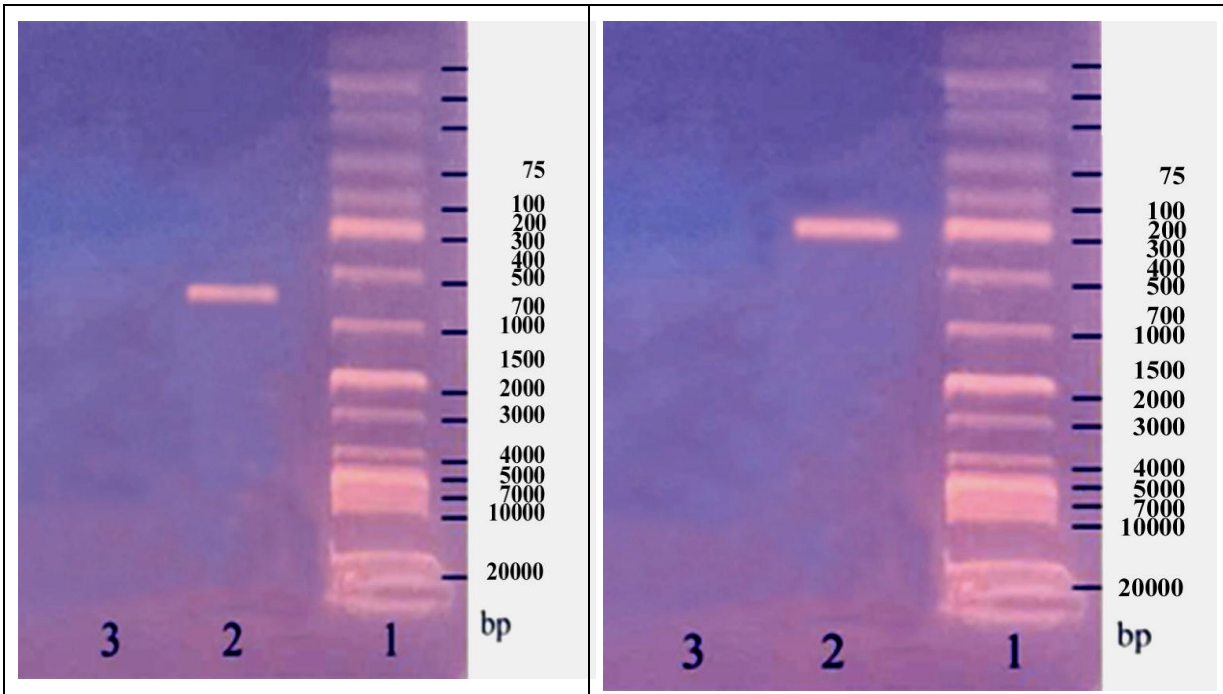
Primers used for detection and their specific virulence genes are given in Table 1. DNA values were extracted from bacteria as given in Material and Methods. PCR technique was carried out for detection of each virulence gene within the specific bacterium. After

agarose gel electrophoresis of the obtained PCR products, four virulence gene(s) were detected within *E. coli*. It was shown that the *E. coli* UR9 strain contain icuD; yiaA; TspE4C2; fim H gene(s) as distinctive bands characterizing them of about 534 bp; 11bp; 152 bp; 170 bp were showed respectively(Figure 2).

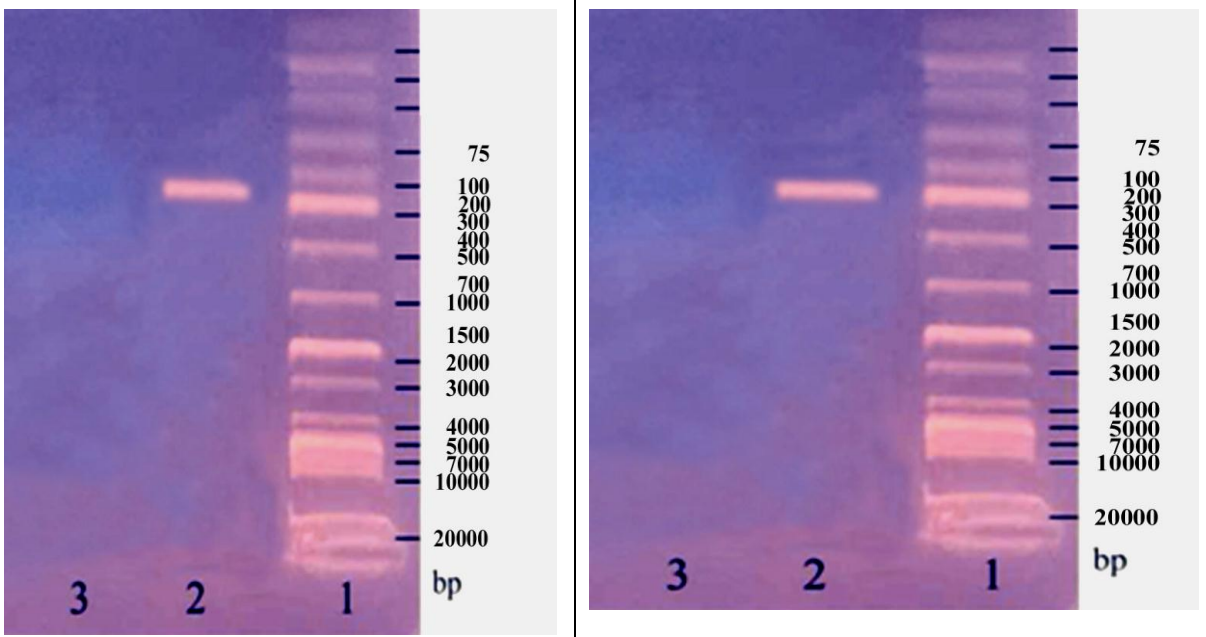
Concerning the *S. aureus* ST15 strain assimilating group 2, it was the second dominant bacterium isolated from patients admitted to NICU in Zagazig University Hospital and, therefore, its detection by PCR technique throughout 2h is necessary. The hia gene encoding α-hemolysin toxin of *S. aureus* was detected Figure 3.A DNA band indicating this gene of molecular mass of about 680 bp was appeared. The *St. pneumonia* EA1 bacterium was fingerprinted by Kan R gene which encodes for promotor region of kanamycin resistance cassette. Results are given in Figure 4. This promoter cut of kan R gene was detected successfully as DNA band of a molecular mass of about 550 bp was detected. *St. pyogenes* appeared herein (THR1,THR2,THR3,BL10) was showed to be virulent pathogens and cause many diseases for both neonates and adults. Hence, the complete identification of this pathogen is important. Virulence genes of this pathogen are certain markers that give rapid detection of this

pathogen and facilitate rapid treatment of its pathogenic cases. Some of their virulence genes were detected within genome of this pathogen. Results are given in Figure 5. It was shown that different virulence gene(s) were detected within *St. pyogenes* THR1, genome. The scpA; sdaB; sdc; CED gene(s) were showed as DNA bands of a molecular masses of about 700 bp; 600 bp; 500 bp; 300 bp were demonstrated respectively.

Finally *Kb. pneumonia* was tested by a specific marker gene namely: magA that encodes outer membrane protein responsible for invasive behavior. Results are given in Figure 6. This marker gene was detected distinctively as DNA band of about 1200 bp was showed. At the end of the results employed herein, it was shown that PCR technique is a rapid test for identification of bacterial cultures and could reduce the time and handling efforts exerted by microbiologists' .It also identify the virulence of the infectious bacterium.



(a) E. coli UR9 *(b) E. coli* UR16



(c) E. coli ST9 *(d) E. coli* BL9

Figure 2. .Agarose gel electrophoresis of PCR products of the amplified genes :icuD (a);yia (b) ;TspE4 C2 (c); fim H (d) from *E. coli* UR9 genome with a molecular masses of

about 534bp ;211bp ;152bp ; 170 bp that encode aerobactin ; enteric toxin;siderophore receptor ; fimbrial adhesion respectively .Lane 1,lane 2 are DNA markers,PCR product the UR9 stain respectively.

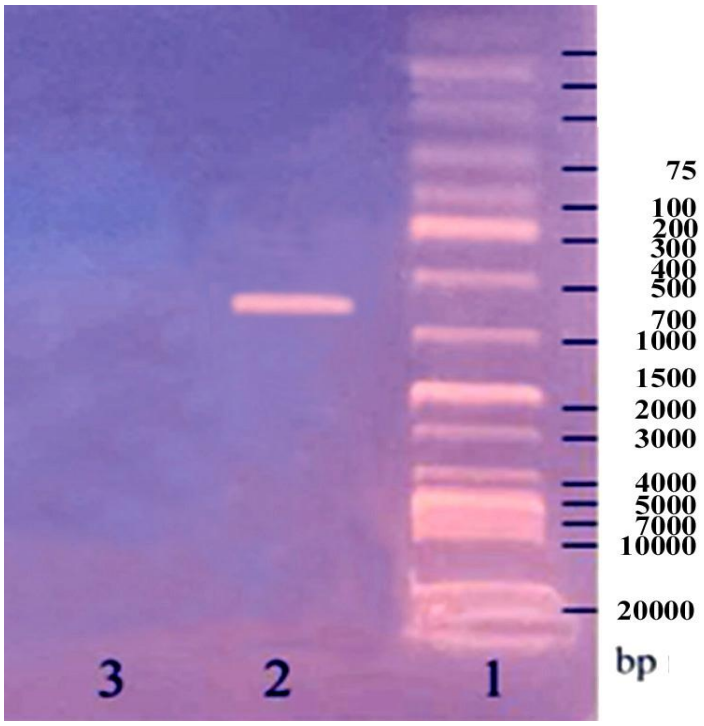


Figure 3. Agarose gel electrophoresis of hia gene (680 bp) of *S. aureus* ST15 genome which encodes α homolysin toxin .Lanes 1,2 are DNA marker,PCR product of *S. aureus* ST15 respectively.

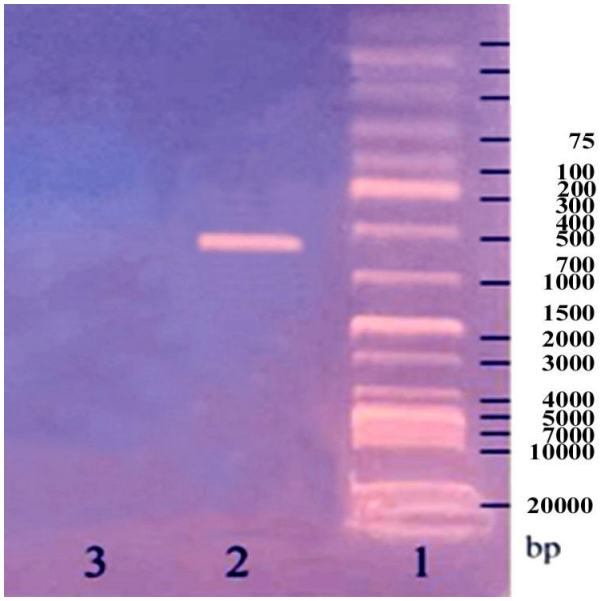
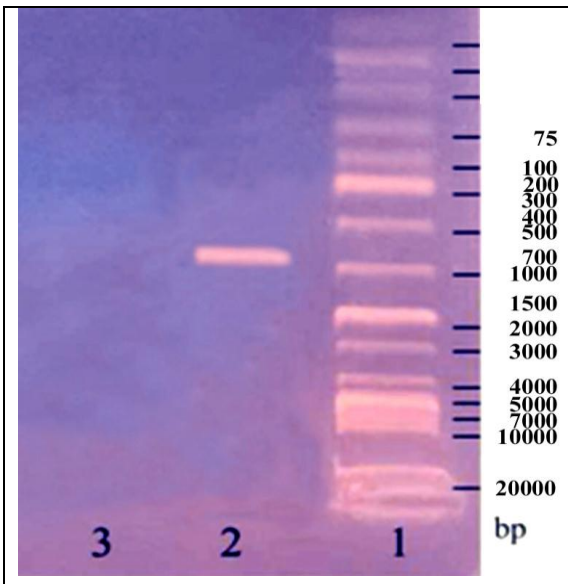
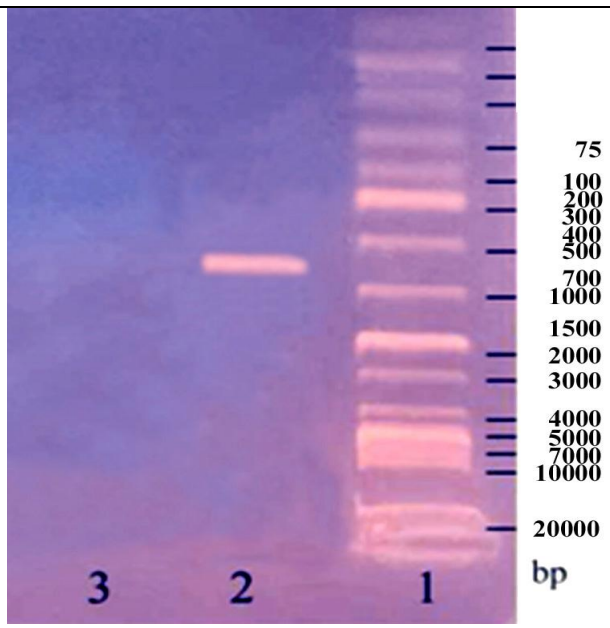


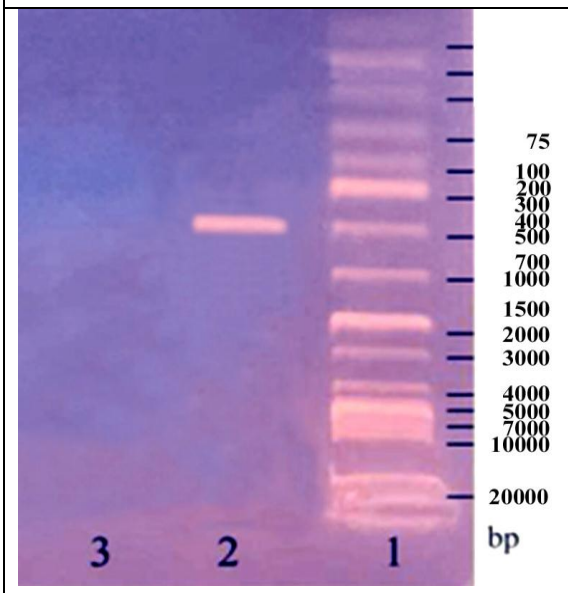
Figure 4. Agarose gel electrophoresis of PCR product of *St. pneumoniae* EA1 genome ;indicating on the promoter region of Kanamycin resistance cassette (KanR gene ,500 – 800 bp).Lane 1 is a marker DNA of known molecular mass. .



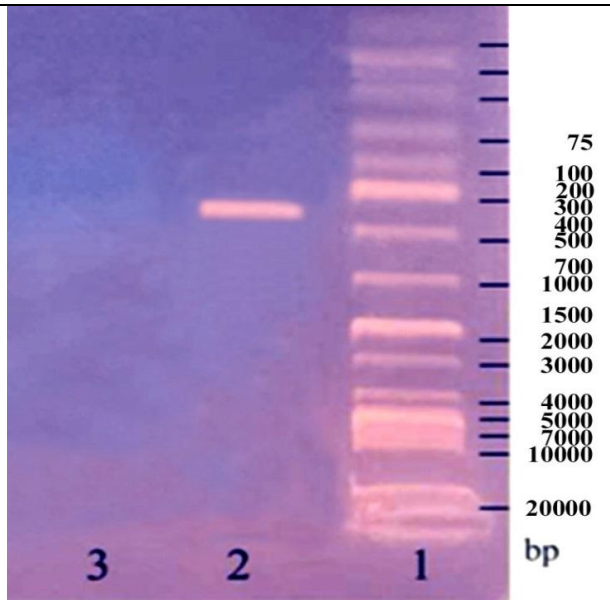
(a) St. pyogenes THR1.



(b) St. pyogenes THR 2



(c)St. pyogenes THR3



(d) St. pyogenes BL10

Figure 5. Agarose gel electrophoresis of the PCR products : scpA(700 bp,a) ;sda B(600bp,b);sdc (500bp,c);CED(300bp,d) genes(Lanes 2) of *St.pyogenes* THR1 genome

;that express on superantigen ;superantigen ; superantigen ;protease activity which digest IL-8 protien to phagocytosis respectively.Lines 1 are DNA markers of known molecular masses.

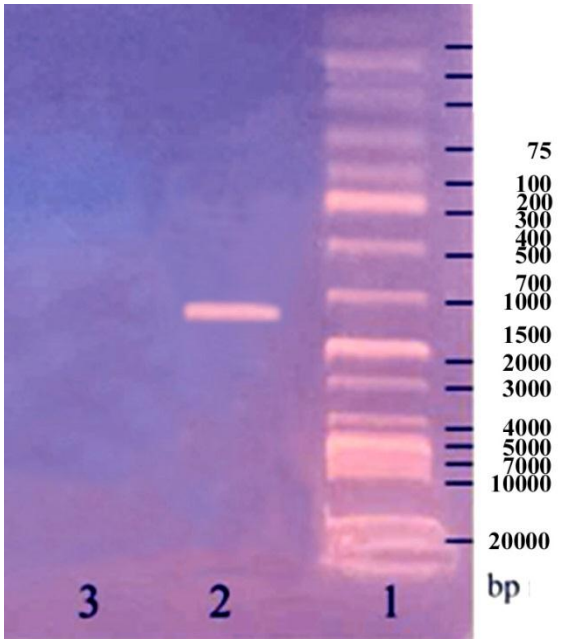


Figure 6. Agarose gel electrophoresis of PCR product (Line 2) magA gene (1200 bp) of *Kb.pneumoniae* UR5 genome that encodes a 43-KD outer membrane protein responsible for invasive behavior of this organism. Line 1 is a DNA marker of known molecular mass.

Discussion

The healthcare-associated infection (HAI) is a serious problem in neonates who are admitted to the NICUs.. It is associated with increases in mortality, morbidity and length of hospital stay. This HAI increases with the degree of both prematurity and low birth weight of neonates; it is also a blood stream infection and is associated with urogenital, respiratory, digestive and neurological infections **(25)**. Due to the high incidence of HAI, there is a need to continue research on infections of neonates admitted to NICUs, to understand more knowledge about infectious pathogens and their resistance to antibiotics. In this regard the present work focused on neonates infections that admitted to NICU in Zagazig University Hospitals, Zagazig, Egypt.

The HAI were detected in many hospitals of Egypt due to the lack of health services and hygiene practices. Maternal and infant mortality rate in Egypt are high, with an infant mortality rate of 17 deaths per 1000 live birth and maternal mortality rate of 55 deaths per 1000 live births **(26)**.

About 70 neonate patients were admitted to NICU in Zagazig University Hospitals; clinical samples were withdrawn from them (26 urine, 25 stool, 10 blood, 3 eye swabs, 3 throat swabs, 3 swabs from ears) and were subjected to microbiological analysis. This was to know the nature of the infective microbe and its resistance or sensitivity to different antibiotics. Those microbial cultures were necessary for diagnosis of neonate pathogenic cases; this is in agreement with recent guidelines **(27).**Out of 26 urine samples;25stool samples ,only 16 urine samples;15 stool samples were of positive microbial growth. This showed that the symptoms appeared were due to something else such as metabolic, nutritional or inherited diseases. The study employed herein focused on cultures showed microbial growth.

Diagnosis of pathogenic cases was preliminary done by physicians. The 16; 15; 10; 4, 3, 2 patients, from which urine; stool; blood; eye swabs; throat swabs; ear swabs were taken were diagnosed as urogenital infections ;diarrhea with or without fever ;septicemia with several reasons; conjunctivitis; pharyngitis; otitis respectively. These results are in conform with previous results who studied the surveillance of infection in NICUs(**28**).

To show the relation of infection with the causative organism, it was necessary to identify all the 50 bacterial isolates obtained. The identification processes were carried out according to **Berge's Manual of Systematic Bacteriology (24)**. The identified bacteria were classified and categorized in 6 groups according to their possible characteristics. Bacterial isolates of group 1; group 2; group 3; group 4; group 5; group 6 were identified as belonging to *E. coli; S. aureus; St. pneumonia; St. agalactiae; S. pyogenes; Kb. pneumonia* respectively. Group 1 included *E. coli* and contained the dominant number (22 isolates) (44%). Other published results showed that *E. coli* was the more frequent organism among NICUs **(29).** In previous study, about 418 bacterial isolates from NICU at Zagazig University Hospitals, Egypt were developed neonates infections **(30).** The main neonatal infections were caused by *E. coli* (41%) followed by *Kb. pneumonia* (34.2%) followed by *S. aureus* (26.1%); low birth weight and prematurity were reported to be an important risk factors of neonatal infections. The identified 50 bacterial strains were categorized in a simple identification key. This key could give certain understanding on the bacterial isolates causing infections of neonates admitted to NICU in Zagazig University Hospitals,Egypt and could

simplify further work in this respect. Many identification keys were assigned in this topic; all of them gave an understanding on the incidences of infection and their causative organisms **(31,32)**.

It is of interest to recall herein that certain scientific relationship was obtained between the identified causative organisms and the diagnosed case. Thus *E. coli* was shown to be the main causative organism of urogenital infections such as cystitis, urethritis, prostatitis, and pyelonephritis. This is in conforming to latter published study **(33)**.

E. coli was shown to be also the causative agent of most cases suffering from diarrhea. Acute diarrhea in many cases is conjugated with sepsis of patients **(27)**. Four groups of *E. coli* were involved in infections. Enteropathogenic *E. coli* which cause an outbreaks of diarrheal diseases among both neonates and infants; all of them were from "O" serotypes which cause damage to intestinal villi. Group 2 of *E. coli* is the enterotoxigenic which is correlated with both neonates and infant infections; isolates of this group produce enterotoxins. Group 3 is the verotoxigenic *E.coli*; isolates of this group are hemorrhagic and hemolytic and act locally on gut mucosa and cause also hemolysis and renal failure and could be isolated from

polluted food. Last group is the enteroinvasive *E. coli* that produce a shiga-like toxin with invasion of the intestinal mucosa, resulting in diarrhea containing blood, pus and mucus **(27,34)**.

S. aureus strains were showed to be the causative organisms, of cystitis, conjunctivitis, fever, chronic diarrhea. This was also reported previously **(35)** *S. aureus* was recorded to cause cellulitis, impetigo, abscess; wound infections. It was reported to cause complications conjugated with both bacteremia and septicemia; wherein could be isolated from many cases such as pyelonephritis, osteomyelitis and upper respiratory tract infections **(29)**.

Other infective pathogens such as *Kb. pneumonia; St. pneumonia; St. pyogenes; St. agatactae* were showed to be conjugated with urogenital infection with fever, conjunctivitis, meningitis and pneumonia; pharyngitis and sepsis; meningitis, respectively. This support latter published results in this respect **(35)**.

The existence of drug resistant bacterial strains makes difficulty faced physicians in treatment protocols of neonates' infection. Therefore, antibiotic resistance ability of the 50 isolated and identified strains were carried out **(29)**. The antibiotic susceptibility

was carried out according as described previously **(17)**. Unfortunately, 10 (20%) bacterial isolates were resistant to most (>90%) of antibiotics studied. This was also reported previously **(36).** This phenomenon could be due to defects in technique used or defects in the antibiotic discs used; this was also reported previously **(37)**. About 40 (80%)of bacterial strains were resistant to only ≥3 antibiotics used and this was also reported previously **(17)**. The resistance of a bacterial strains to antibiotics could be due to secretion of enzymes that can degrade the antibiotic used, thickness of bacterial cell wall, modifications of specific site(s) receptors and genetic reasons **(5,7,9)**. It was necessary to study the antibiotic resistance ability of the identified strains to give a protocol guide for physicians at NICU in Zagazig University Hospitals and to give some understanding for further work in this respect to find out other treatment protocols in the future.

Based on the requests of physicians at NICU in Zagazig University Hospitals,Zagazig,Egypt, clinical samples to be cultured were arranged in the following descending manner: urine (32%), stool (30%), blood (20%), eye swabs (8%), throat swabs (6%), ear swabs (4%). This is in agreement with latter published results **(38)**.The

prevalence of the identified bacteria within the withdrawn clinical samples can be arranged in the following descending manner: *E. coli* (44%), *S. aureus* (20%), *St. pneumonia* (12%), *St. agalactae* (10%), *St. pyogenes* (8%), *Kb. pneumonia* (6%). This was also reported previously **(30, 39)**. *E. coli* and *S. aureus* were the more dominant strains as they were isolated from different environments such as air, door knobs, walls, instruments and also due to HAI **(40)**.

It was shown that biological and biochemical identification of bacteria can give elusive identification and last long time and many efforts of microbiologists. Consequently molecular detection of bacteria using PCR techniques is currently requested **(41)**. PCR techniques are used for bacterial identification throughout detection of virulence genes which are markers for each bacterial species **(42)**.The detection of virulent genes give certain understanding on the virulence of each infectious pathogen **(43)**. In this work , *E. coli* UR9 strain was successfully used in such study; in such isolate iucD gene; yia gene; TspE4C2 gene; fim H gene encoding aerobactin; enteric toxin; siderophore receptor as a virulence factor; fimbria adhesion respectively were successfully characterized. This could facilitate identification of *E. coli* strains rapidly within 2h and

this is a promising result wherein physicians can use such data in a successful treatment protocols. This is in agreement with latter published results in this respect **(44)**.

Since *St. pyogenes* was reported to be of dominant prevalence in NICU and is the causative agent of many neonate diseases such as urogenital infections, endocarditis, upper and lower respiratory infections sepsis, osteomyelitis **(45)**, certain virulence genes of this pathogen were of interest to be characterized to give a rapid identification step and successfully treatment protocol. Hence, the scpA; sdaB, sdc spy CED genes that encode super antigens of the first three genes and protease activity which digest IL-8 protein to phagocytosis of *St. pyogenes* THR1 respectively were successfully detected as identification markers. This supported latter published results in this field **(5)**.

Finally the hia gene; mag A gene; kan R gene encoding hemolysin; outer membrane protein responsible for invasive behavior; promoter region of kan R cassette were successfully used as an identification markers of *S. aureus; Kb. pneumonia; St. pneumoniae* respectively. This corroborated other published work in this field.

Further work will be necessary to develop a treatment protocols to inhibit antibiotic resistant strains identified and characterized in this study.

Conclusion:

Fifty bacterial isolates obtained from neonates admitted to NICU in Zagazig Univerisity Hospital, Zagazig , Egypt. They were identified ;*E. coli* strains were the dominant ones.PCR technique gave rapid identification step of these infectious bacteria and reduce long time needed for biochemical identification.

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