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Prevalence evaluation of *Streptococcus pneumoniae* in neutropenic cancer patients

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Abstract: *Streptococcus pneumoniae* is an important human pathogen that causes both serious invasive infections, such as septicaemia, meningitis and pneumonia, as well as mild upper respiratory infections. It also belongs to the normal nasopharyngeal microbial flora. The majority of cancer patients develop neutropenia, most often due to chemotherapy. The purpose of this study was to identify of pneumococci, among neutropenic cancer patients. A total of 72 nasopharyngeal swabs were collected from cancer patients attending Al-Sader Medical City for chemotherapy who were diagnosed as neutropenic, during 6-month study (2016-2017). All swabs were cultured on blood agar plates, 45 α-hemolytic isolates suspected to be pneumococci were identified by phenotypic methods using and identified using colony morphology, catalase, optochin susceptibility bile solubility and API 20 STREP, and a molecular method using PCR. The results of API 20 STREP test alone showed that out of 45 α-hemolytic isolates only 12 (26.6%) were identified as *S. pneumoniae*. But after combination with the other phenotypic methods which are catalase negative, optochin susceptibility and bile solubility the result was out of 45 α-hemolytic isolates 23 (51.1%) were identified as *S. pneumoniae*. The results of the PCR test showed that from all the 45 α-hemolytic isolates 25(55.5%) were identified as *S. pneumonia*.

Key wards: *S. pneumonia*, among neutropenic cancer, nasopharyngeal, API 20 STREP.

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Introduction:

Neutropenia is identified as the reduction in white blood cells, with a neutrophil count of below $1.5x10^9$/L. This is varies from (16% to 81%) among the patients who receive chemotherapy treatment (1, 2). The occurrence of neutropenia is associated with an increase in mortality, morbidity and treatment costs, in addition to the impossibility of predicting which patients will present it (3).

Because of the type of treatment received and other risk factors, many cancer patients experience a decrease of elements of the immune systems that make them more exposed to various infections (4). The group of neutrophils is one of blood elements whose number is decreases during cancer, which considered the first line of the body defense against diseases. Neutropenia is considered as an oncology emergency and can lead to serious adverse effects such as major infection complications and death (5).

However, Gram-positive bacteria, which became the main microorganisms that can cause life-threatening infections leading to mortality in the immune deficient neutropenic individuals (6). It is hard to prevent the occurrence of neutropenia in people undergoing intensive chemotherapy.

With the evolution of new technologies, pharmacological tools, and drugs, the occurrence of new

microorganisms that used to be nonpathogenic is facilitated and this associated with the increase of antibiotic multi resistances form a serious public health concern at a global level (7).

Infectious complications are a serious cause of morbidity and mortality in cancer patients, especially those with underlying hematological malignancies where autopsy studies demonstrate that approximately (60 %) of deaths are infection related (8,9). Although fewer data exist on infectious mortality in patients with solid organ tumors, approximately (50 %) of these patients are estimated to have an infection as either the primary or an associated cause of death (10). Patients with underlying malignancies are at risk for a wide array of infectious diseases. Bacterial infections predominate, followed by fungal infections. Viral infections occur not infrequently, often as a result of reactivation of latent disease, primarily in patients with hematological malignancies (11-14).

Pneumonia impacts all populations (15, 16). However, the impact of pneumonia on cancer populations is severe, accounting for more morbidity and mortality than other infectious complication (17,18). Lower respiratory tract infections are common among cancer patients. Reports indicate that (13–31%) of leukemia patients receiving chemotherapy and up to 80% of stem cell transplant recipients will experience at least one episode of pneumonia (19,20). The mortality related to pneumonia in these populations is very high with case fatality rates in leukemia patients ranging from (25%–80%) (21, 22).

The purpose of this study was to identify of pneumococci among neutropenic cancer patients infected with pneumonia by phenotypic and molecular methods.

Materials and Methods:

Samples Collection:

A total of 72 nasopharyngeal swabs were collected from neutropenic cancer patients attending Al-Sader Medical City for chemotherapy who were diagnosed as neutropenic, during April 2016, to January 2017.

Culture and identification:

Nasopharyngeal swab of sputum from all patients with suspected pneumonia were cultured. The swabbed samples were held on tubes containing 2 ml of (0.85%) NaCl and vortex for 30 s. They were subsequently cultured on blood agar. The cultures were incubated in (3 to 5%) CO_2 at 35°C.

Before culturing, direct smears from samples were prepared and microscopically checked after staining with Gram method. The samples were qualified as clinically significant in case the numbers of WBC were 10 cells per field of microscope at high power. Moreover, identification of *S. pneumoniae* was based on phenotypic methods using colony morphology, α-hemolytic on blood agar, catalase negative, optochin susceptibility, bile solublity and API 20 STREP, and a molecular method using PCR (23). Blood agar plates were inoculated with suspected *S. pneumoniae* morphology and an optochin disk (Hi-media, India) was placed in the center of each plate. Optochin susceptibility was defined after incubation for 18 to 24 h at 35°C in 5% CO_2 with $\geq$ 14 mm inhibition zone as positive (24).

DNA isolation:

Several colonies of each *S. pneumoniae* isolate were suspended in 1.5 ml microfuge tube containing 1 ml of phosphate buffer saline (pH 7.5). Genomic DNA was extracted with the Wizard® Genomic DNA Purification Kit (Promega, USA) for isolating Genomic DNA from Gram positive bacteria. According to the kit manual each sample tube were centrifuged at 13,000–16,000 × g for 2 minutes to pellet the cells and the supernatant was removed, then 10mg/ml lysozyme (Sigma) were added to the resuspended cell pellet, and gently pipeted to mix. The purpose of this pretreatment is to weaken the cell wall so that efficient cell lysis can take place, and then proceeding with the kit protocol.

PCR primers:

The oligonucleotide primers for the sequences of highly divergent and species-specific regions between the 16s and 23s rRNA genes were designed as previously described by Saruta (25). The primer sequences were SP I: 5'-AGG ATA AGG AAC TGC G-3', and SP II: 5'-CTT ATT TTC TGA CCT TTC A-3'. The primers synthesized by AccuOligo® Bioneer Corporation USA. A 247bp fragment should be amplified from the genomic rDNA spacer region.

PCR amplification

The detection of species-specific regions between the 16s and 23s rRNA genes in all *S. pneumoniae* isolates was done according to the Experimental Protocol of AccuPower® TLA PCR PreMix tube, the PCR reaction mixture was performed using 5µl of the template DNA, 4µl of each primer (10pmole/µl, 2µl forward and 2µl reverse), were added to each AccuPower® TLA PCR PreMix tube. Distilled water was added to the tubes to a final volume of 20 µl.

The PCR program consisted of initial denaturation at 94 ºC for 5 min, and 30 cycles involving denaturation at 94 ºC for 1 min, and annealing at 55 ºC for 2 min and extension at 72 ºC for 3 min followed by a final extension at 72 ºC for 5 min. All reaction mixtures were held at 4°C (25).

Electrophoresis

The PCR products were electrophoresed through 1% agarose (Sigma Chemical Co., St. Louis, Mo.) dissolved in Tris-borate-EDTA buffer TBE, stained with ethidium bromide. Electrophoresis was conducted at 80 V for 60 min; the bands were visualized with a UV trans-illuminator.

Results:

Bacterial Strains:

The colony morphology and α-hemolytic on blood agar reviled that from all the 72 samples included in this study 45 (62.5%) α-hemolytic isolates suspected to be pneumococci. The results of API 20 STREP test alone showed that out of 45 α-hemolytic isolates only 12 (26.6%) were excellent species identification according to the criteria of the system. But after combination with the other phenotypic methods which are catalase negative, optochin susceptibility and bile solubility the result was out of 45 α-hemolytic isolates 23 (51.1%) were identified as *S. pneumoniae.*

The results of the molecular identification method represented by the

PCR test showed that from all the 45 α-hemolytic isolates 25(55.5%) were identified as *S. pneumoniae* (Table 1), (Figure 1).

The 247bp PCR amplification products of for the sequences of highly divergent and species-specific regions between the 16s and 23s rRNA genes were visualized on agarose gel (Figure 2).

Table(1): Number of Bacterial isolates identified by different methods during this study

Identification Method	No. of *S. pneumoniae* Isolates*
API 20 STREP alone	12 (26.6%)
All phenotypic methods	23 (51.1%)
PCR test	25(55.5%)
Total No. of α-hemolytic Isolates	45 (62.5%)

***P <0.05 significant**

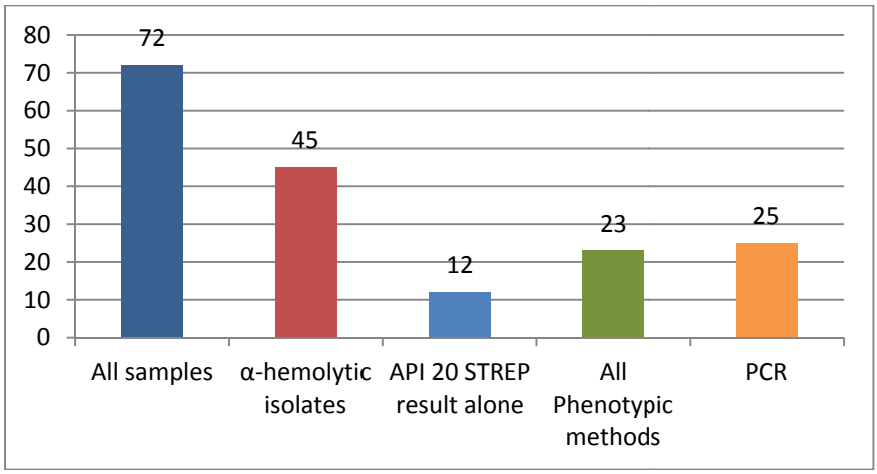

Figure (1): The results of identification of *S. pneumoniae* isolates using different methods.

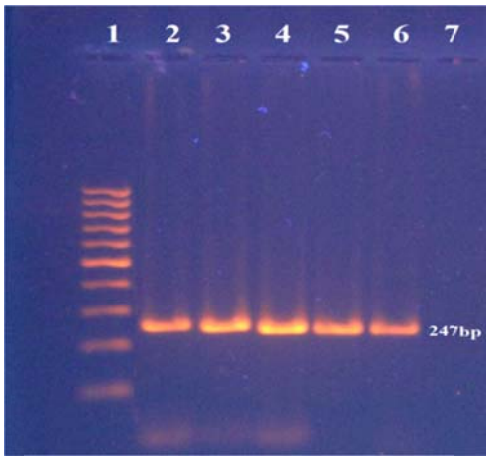

Figure (2): Agarose gel electrophoresis of PCR products after amplification of highly divergent and species-specific regions between the 16s and 23s rRNA genes. Lane 1: molecular weight marker (GeneRuler™ 100 DNA Ladder, SM0243-Fermentas); Lanes 2-6: SP I-SP II primer pair (247bp); Lane 7: Negative result.

Discussion

Neutropenic pneumonia remains a challenge for the clinician and a threat to the patient. The clinical approach requires integration of traditional microbiologic techniques as well as targeted molecular diagnostics (26). Identification of *S. pneumoniae* is necessary for adequate patient care and is a prerequisite for accurate epidemiological surveillance of this bacterium.

Blood cultures are the cornerstone of diagnostic workup of neutropenia, as they provide pathogen identification and susceptibility pattern. Since their sensitivity is significantly lower once antibiotic therapy has been started, they should be performed immediately when infection during neutropenia is suspected. While novel diagnostic methods, mostly based on Polymerase Chain Reaction (PCR) systems, improved the yield of blood cultures, particularly if drawn after the onset of antibiotic therapy, such as in case of persistent fever, and result in shorter time to microbiological diagnosis (27).

Our finding shows that the sequences of SP I and SP II primers employed for *S. pneumoniae* molecular identification are generally different from those required for other *Streptococcus* species. The data showed sensitivity enough to detect *S. pneumoniae* at the level of 3 cfu in a single-step PCR without cross-reaction, and that was agreed with Saruta *et al* . (25).

The API 20 STREP results out of 45 α-hemolytic isolates only 12 (26.6%) were identified as *S. pneumoniae* from this findings we concluded that the API 20 STREP is not an effective system for the identification of *S. pneumoniae* and that is agreed with Bosshard et al (28). While after combination with the other phenotypic methods which are catalase negative, optochin susceptibility and bile solubility the result was out of 45 α-hemolytic isolates 23 (51.1%) were identified as *S. pneumoniae* which is statistically significant.

The results of the molecular identification method showed that from all the 45 α-hemolytic isolates 25(55.5%) were identified as *S. pneumoniae* this result did not differ significantly from the previous result.

Furthermore the combination of nucleic acids amplification with mass spectrometry could permit obtaining the results within 6 hours, but no data are so far available in hematology or oncology settings (29).

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