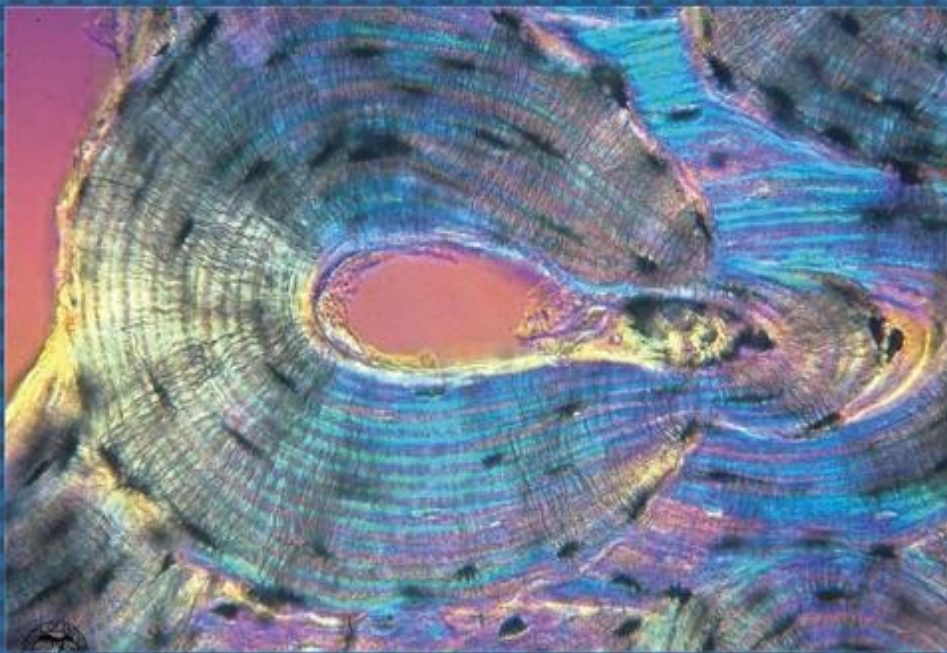




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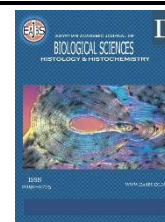
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Phenotypic Detection of Some Virulence Factors among Hypervirulent *Klebsiella pneumoniae* and Classic *Klebsiella pneumoniae* Associated with Urinary Tract Infections

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ABSTRACT

The present study aimed to investigate prevalence and some virulence factors among hyper virulent *Klebsiella pneumoniae* (HVKP) and classic *Klebsiella pneumoniae* (CKP) isolated from urinary tract infections. Three hundred urine samples collected from patients with urinary tract infections were cultured on MacConkey agar for primary isolation of *K. pneumoniae* isolates. Vitek 2 Compact System was used for identification of *K. pneumoniae*. HVKP and CKP were detected by string test. Some virulence factors (biofilm formation, haemolysin, and urease production) possessed by the bacterial isolates were investigated phenotypically.

Sixty bacterial isolates belonged to *K. Pneumoniae* where 15 (25%) isolates belonged to HVKP as viscous string measuring >5 mm in length and 45 (75%) isolates belonged to CKP where viscous string measuring <5 mm in length. The results of haemolysis test showed that 60% of HVKP isolates gave β -haemolysis, while 51.1% of CKP isolates gave β -haemolysis. All bacterial isolates (100%) of HVKP and CKP showed the ability to produce urease. Phenotypically, all HVKP and CKP isolates showed the ability to form biofilm while only 33.3% and 11.1% isolates showed high ability to form biofilm respectively by Tissue Culture Plates methods (TCP).

INTRODUCTION

Klebsiella pneumoniae, a Gram-negative bacterium, is one of the major pathogens associated with nosocomial infections (Chen *et al.*, 2018). There are two distinct species of *K. pneumoniae*, Classical *K. pneumoniae* (CKP) and Hypervirulence *K. Pneumoniae* (HVKP) (Bialek-Davenet *et al.*, 2014). CKP is characterized by being an opportunistic pathogen that causes infections in hospitalized patients, while HVKP is characterized by being hyper mucosal and causing life-threatening diseases including liver abscesses and eye inflammation in healthy people (Shon *et al.*, 2013; Navon-Venezia *et al.*, 2017). HVKP was first described in Taiwan in the mid-1980s in apparently healthy adults, with liver abscesses developing and arising in the brain and eyes (with or without bacteremia), clinical cases that rarely occur in CKP infection (Lee *et al.*, 2006; Shon *et al.*, 2013). CKP strains can be differentiated from HvKP strains by their mucosal viscosity and iron carrier arrangement (Russo and Marr., 2019). Strains of HVKP showed the ability to produce iron carriers in larger quantities, in order to obtain iron more efficiently, and showed a hyper mucosal phenotype, as the capsular

polysaccharide is synthesized by the *rmpA* and *rmpA2* genes, which is screened for by a string assay (Tang *et al.*, 2021).

K. pneumoniae possess several virulence factors that increase their pathogenicity, including capsule formation, hemolysin, urease production, and biofilm formation (Dadi *et al.*, 2020). capsule consists of a capsular polysaccharide is the most important virulence factor for *K. pneumoniae*, as it prevents phagocytosis by the host cells (Mantin and Bachman, 2018). More than 70 serotypes of capsules were recorded. K1 and K2 serotypes were associated with HVKP are responsible for human respiratory tract infections, while K8, K9, K10, and K24 serotypes are responsible for urinary tract infections (Shon *et al.*, 2013; Paczosa and Mecsas, 2016). Hemolysin is a type of toxin secreted by some bacterial species that destroys the cell membrane of red blood cells, leading to their lysis (Mare *et al.*, 2020). *K. pneumoniae* have the ability to produce urease, which breaks down urea into two parts of ammonia and one part of carbon dioxide (Meknonnen *et al.*, 2020). Ammonia combines with magnesium and phosphate ions, forming a precipitate of triglycerides, causing infection of the urinary tract and stones (Duran *et al.*, 2022). Biofilm is an assembly of microorganisms with their secretions, as the bacteria show new characteristics after the formation of the biofilm that they did not show during free growth because their growth within the biofilms leads to a change in their gene expression on surface markers and changes in nutrient metabolism (Siddique *et al.*, 2020). Biofilm provides bacterial cells with a way to hide from the body's immune system and protection from the immune response as well as protection from toxins, antibiotics, and inappropriate environmental conditions such as drought and ultraviolet radiation (Crabbé *et al.*, 2019).

It is important to detect HVKP due to its high mortality in comparison to multidrug-resistant and CKP infections, where coexisting hepatobiliary disease is

a potential risk factor for these infections (Parrott *et al.*, 2021). Also, most of the *K. pneumoniae* infections were caused by CKP strains that cause infections in immunocompromised patients, and occurrence of multiple antibiotic resistance *K. pneumoniae* strains was the reason why urinary tract infections became difficult to treat and these bacteria became life-threatening (Kuehn., 2013) so that this study aimed to investigate the prevalence of HVKP and CKP associated with UTI and detect some of their virulence factors.

MATERIALS AND METHODS

Isolation and Identification of *K. pneumoniae*:

Vitek 2 Compact System (ID/GN) was used to identify *K. pneumoniae* that isolated from 300 urine specimens collected from patients with UTI.

Detection of HVKP and CKP:

String test described previously (Chang and Ong, 2022) was followed up to differentiate HVKP from CKP. The test is considered positive if a viscous string measuring >5 mm in length was obtained by stretching bacterial colonies on blood agar base and MacConkey agar plates with a bacteriology inoculation loop or needle which referred to HVKP, while it was considered negative if a viscous string measuring <5 mm in length which referred to CKP.

Detection of Haemolysin and Urease Production:

Blood agar base plates (supplemented with 5% human blood) were inoculated with young bacterial colonies by streaking methods. Hemolysis pattern was detected by appearance of either complete blood hemolysis known as β -hemolysis or partial hemolysis (greenish zone around colonies) known as α -hemolysis or non-hemolysis pattern. Tubes of urea agar base supplement with 40% sterile urea were inoculated with fresh bacterial colonies and incubated for 6-48 hrs. at

37°C. Changing the colour of media from yellow to red referred to the ability of isolates to produce urease (MacFaddin, 2000).

Detection of Biofilm Formation:

Congo Red Method (Vuotto *et al.*, 2017) and Tissue Culture Plats (Hassan *et al.*, 2011) used to detect the

ability of bacterial isolates to form biofilm. Changing the colour of congo red media from red to black indicate positive results while the quantity of biofilm was detected by measuring the optical density of biofilm layer as mentioned in Table 1.

Table 1: Optical density (OD) of biofilm-producing isolates (Hassan *et al.*, 2011).

The OD at a wavelength of 570 nm	The ability to form a biofilm	Adhesion ability
Less than 0.120	Non	Non
From 0.120 - 0.240	Medium	Medium
More 0.240	High	High

RESULTS

The results of string test showed that out of 60 *K. pneumoniae* isolates only 15 (25%) isolates gave positive results, as a viscous string measuring >5 mm in length of colonies grew on blood agar base (BAB) and a viscous string measuring >20 mm in length of colonies grew on MacConkey agar, indicating that these isolates belong to HVKP. The results also showed that 25 (41.66%) isolates showed a medium mucoid pattern, as a viscous string measuring 5 mm in length of colonies grew on BAB, and 20 (33.33%) isolates did not show any viscous or mucoid pattern on the

culture medium which indicated that these isolates were belong to CKP (Fig. 1).

The results of haemolysis test showed that 9 (60%) HVKP isolates gave β -haemolysis, while 6 (40%) HVKP isolates showed no haemolysis of blood agar base. Among CKP isolates only 23 (51.1%) isolates gave β -haemolysis, while 22 (48.9%) CKP isolates showed no haemolysis of blood agar base (Fig. 2). In addition, all isolates of HVKP and CKP showed the ability to produce urease where the colour of the urea agar base culture was change to pink colour after 24hr of incubation (Fig. 3).

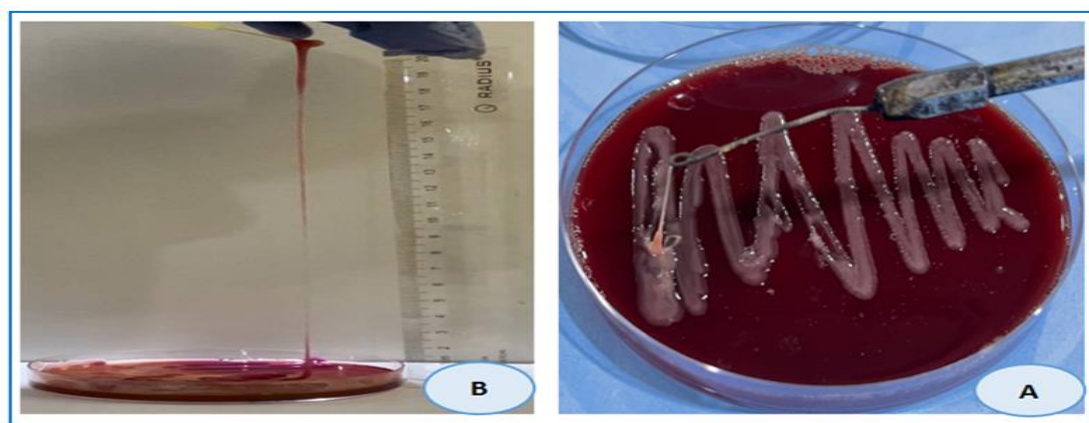


Fig.1: String test for detection of hyper-virulent *Klebsiella pneumoniae* (HVKP), Isolates grown on. (A): Blood Agar Base, (B): MacConkey Agar.

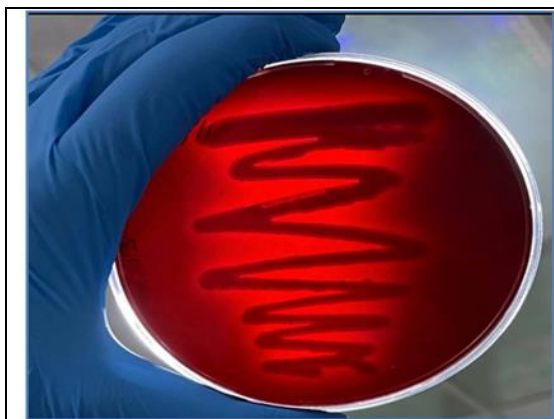


Fig.2: B-Haemolysis test of *K. pneumoniae*.

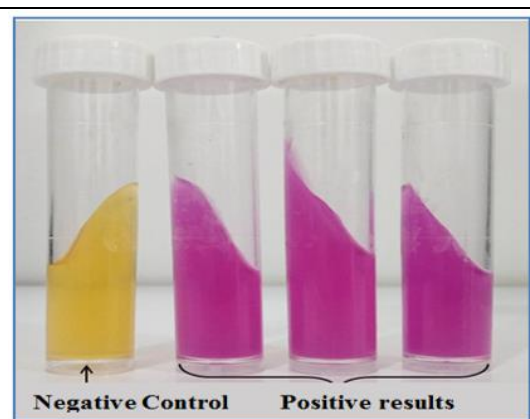


Fig. 3: Urease test of *K. pneumoniae*.

The results of biofilm formation by Congo red test showed that all isolates of HVKP and CKP have the ability to form biofilm by changing the colour of the medium to black. The results of the quantitative assessment of the biofilm formation showed that only 5(33.3%) HVKP isolates and 5(11.1%) CKP isolates have high ability to produce a biofilm and showed a high ability to adherence where the optical density values of the biofilm produced by these

isolates ranged between 0.243 - 0.395. The percentage of HVKP and CKP isolates that showed moderate ability to produce biofilm and adherence were 60% (9 isolates) and 62.2 % (28 isolates) respectively, where the optical density values of the biofilm produced by these isolates ranged between 0.124 - 0.230. Only 1(6.7%) HVKP isolates and 12(26.7%) CKP isolates showed weak or no biofilm formation (range of optical density 0.056 - 0.118) (Table 2).

Table 2: The Percentage of Hypervirulent *K. pneumoniae* and Classic *K. pneumoniae* produce biofilm

Biofilm Pattern	NO (%) of Hypervirulent <i>K. pneumoniae</i>	NO (%) of Classic <i>K. pneumoniae</i>
Non	1 (6.7)	12 (26.7)
Medium	9 (60%)	28 (62.2)
High	5 (33.3)	5 (11.1)
Total	15	45

DISCUSSION

K. pneumoniae represented the second cause of respiratory tract infections after viruses, as it causes infections that affect the upper and lower respiratory tracts, and it also causes respiratory tract infections for hospitalized patients, and it comes second after *E. coli* in causing digestive system infections (Predic *et al.*, 2020). The polysaccharide capsule is the main virulence factor responsible for producing the hypermuco-viscous phenotype characteristic of *K. Pneumoniae* (Lee *et al.*, 2010). It acts as a shield to protect the bacterial cells from the process of phagocytosis by the

immune cells and protects the bacterial cells from the effect of antibiotics by preventing their deposition on the surface of the bacterial cells and it also works to activate the innate immunity of the cells (Lawlor *et al.*, 2005; Paczosa and Mecsas, 2016). Despite the virulence of these strains, some studies indicated that some strains of *K. pneumoniae* do not have this phenotype and that some HVKP strains may not cause purulent liver abscesses (Yu *et al.*, 2007).

Hemolysin is considered one of virulence factors that contribute to increasing the pathogenicity of bacterial cells. It helps in providing nutrients to bacterial cells, as the lysis of blood cells

leads to the provision of iron to bacterial cells, and also helps to secrete histamine and destroy white blood cells (Liu *et al.*, 2020). The results of the current study showed the ability of some *K. Pneumoniae* isolates to produce hemolysin, this may be due to the occurrence of a genetic mutation in *ns-h* in *K. Pneumoniae* isolates, which leads to showing their ability to produce hemolysin. Some studies indicated that in the absence of the aerobactin system, bacterial cells resort to hemolysin production as an alternative pathway for obtaining iron (Sukhan *et al.*, 2010).

Urease is one of the important virulence factors possessed by isolates of *K. Pneumoniae* bacteria. It breaks down urea into ammonia NH₄ and carbonic acid H₂CO₃ and transforms the medium into an alkaline one (Boer and Hausinger, 2012) as the liberation of ammonia leads to an increase in the pH of the medium and thus a change in the color of the medium due to the sensitivity of the reagent in the culture medium to a change in the pH (Mishra and Agrawal, 2012).

Biofilm is an important virulence factor that bacteria possess. It contributes to enhancing the bacteria's ability to overcome host defences and play role in many bacterial infections (Clegg and Murphy, 2016). It also promotes transmission of resistance genes, increases expression of efflux pumps genes, and reduces antibiotic permeability into bacterial cells (Uruèn *et al.*, 2021). The results of the current study showed a high percentage of *K. pneumoniae* isolates that produce a biofilm, which plays an important role in increasing the virulence of these isolates. The ability of the bacterial isolates to produce a biofilm is attributed to their possession of fimbria type 1 and type 3, in addition to the presence of LPS and capsule, as these are important virulence factors that help biofilm formation (Clegg and Murphy, 2016). *K. pneumoniae* biofilm contributes to infection and the development of severe HVKP infections not only in

immunocompromised patients but also in healthy adults (Khaertynov *et al.*, 2017).

CONCLUSION

K. pneumoniae represented one of the most causes of UTI and prevalence of CKP followed by HVKP. Distribution of virulent factors that increase the pathogenicity of HVKP and CKP, particularly biofilm formation plays an important role in spreading of antibiotic resistance among isolates.

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