Prevalence and Molecular Characterization of Extensively Drug Resistant Hypervirulent (Hypermucoviscous) K. pneumoniae Isolates from Al-Najaf province.

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Abstract

Background: Extensively drug-resistant (XDR) is resistant to all but one or two categories of antibiotics were sensitive, Klebsiella pneumoniae mainly has two path types that pose a danger to our health: classical (cKp) and hypervirulent (hvKp). hvKp is known to cause life-threatening and community-acquired pyogenic infections such as pyogenic liver abscesses, osteomyelitis, endophthalmitis, and metastatic meningitis in healthy individuals. The current study aimed to investigate the prevalence of XDR-hvKp and the genes responsible for resistance in hvKp isolates obtained from Najaf province clinical samples. Methods: about 121 K. pneumoniae clinical isolates were collection from Private Laboratories and Public Health Laboratory in Najaf province during the period of study, determine the antibiotic susceptibility of K. pneumoniae isolates by using Kirby-Bauer disk diffusion method to 25 antimicrobial agents according to the CLSI (2022). Hypervirulent isolates were diagnosed using the test called string test. Molecular detection for main ESBL, AmpC, carbapenemases, AMEs, 16S-RMTase, PMQR, capsular serotypes, and class I integrons genes by PCR. Results: from 121 K. pneumoniae clinical isolates XDR isolates had represented in 23 (19%). Out of 23 XDR isolates, 5 (21.7%) had a positive string test and were identified as hypervirulent K. pneumoniae. The isolates showed significantly higher resistance to most antibiotics and the PCR analyses demonstrated that four of these isolates harbored multiple antibiotic-resistant genes. In addition, molecular characterization revealed that 78.3% of all the XDR isolates carried wzy-K2 serotype gene. Notably, 4 (80%) hypervirulent and 14 (77.8%) classical isolates were found in this study, which contained wzy-K2 gene. Conclusion: The study highlighted the emergence of five XDR hypervirulent isolates, the occurrence of hypervirulent isolates is particularly worrisome due to the confluence of both hypervirulence and XDR.

Keywords: Resistant Hypervirulent; Al-Najaf province; Extensively Drug

1. Introduction

Klebsiella pneumoniae mainly has two path types that pose a danger to our health: classical (cKp) and hypervirulent (hvKp). Hypervirulent *K. pneumoniae* is linked to high morbidity and mortality because it may cause serious, community-acquired illnesses in young, healthy people. These infections include liver lesions, pneumonitis, and meningitis. The hyper-mucoviscous phenotype of these strains is evident using the string test because of their enhanced iron acquisition and capsular substance production in comparison to standard *K. pneumonia* (1). Most hv*K. pneumoniae* isolates are mainly found in Asia (1) as well as the continents of America, Africa, Australia, and Europe (2). The hypervirulent strains characterized by hypermucoviscosity colonies appear when the growth of the bacteria on blood agar. This phenotype defined as a "string test" was done as an official lab test to recognize hypervirulent from classical *K. pneumoniae* where hypervirulent isolates yield a positive result for the test (3). Hypervirulent *K. pneumoniae* demonstrates heightened virulence characteristics such as increased synthesis of capsule polysaccharide, resistance to macrophage action, and distant metastases (4). The capsule is an important virulence component that makes hypervirulent *K. pneumoniae* resistant to complements and capable of forming biofilms, which imparts non-sensitive to antibacterial drugs (5). Infections caused by hypervirulent *K. pneumoniae* have increased dramatically in recent years, and studies have shown that classical *K. pneumoniae* strains can easily acquire the hypervirulence phenotype by obtaining the virulence plasmid by conjugation. This results in hyper-mucoviscosity and the production of capsule polysaccharide (6,7).

The capsular serotypes K1 and K2 make up the vast bulk of these hypervirulent *K. pneumoniae*. Their ability to efficiently acquire iron and boost capsule synthesis, both of which provide the typical hyper-mucoviscous phenotype, seems to be the root of their pathogenicity. PCRs using particular primers for the identification of the genes wzx-K1, wzy-K2,

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and magA were carried out to identify the capsular serotypes K1 and K2 (8).

Generally, most antimicrobials are effective against hypervirulent *K. pneumoniae* isolates; nevertheless, these strains have an innate resistance to ampicillin. However, along with the worldwide dissemination of mobile genetic elements conferring antimicrobial resistance, MDR and XDR hypervirulent *K. pneumoniae* isolates are progressively being reported, most often in countries with an epidemic dispersion of hypervirulent *K. pneumoniae* (7,9). With a national occurrence of hypervirulent *K. pneumoniae*, one study in Najaf has revealed the emergence of hypervirulent *K. pneumoniae* isolates that have acquired extensive antibiotic resistance, including resistant to carbapenem (10).

2. Materials and methods

Sources of K. pneumoniae Isolates:

This a cross-sectional analysis was performed at the Private Laboratories and Public Health Laboratory in Najaf province during six months from September to February 2022. The study population consisted of 121 *K. pneumoniae* clinical isolates isolated from patients clinically suspected by the physician to have infections, and the samples immediately transported to the microbiology laboratory, all *K. pneumoniae* isolates collected in this study had been confirmed and identified depending on Morphological characteristics, Microscopically examination (gram-stain), and Biochemical tests according to the standard method described by MacFaddin 's instructions(11). *K. pneumoniae* isolates were also identified by the VITEK-2 compact system.

Antimicrobial Sensitivity Testing

Antimicrobial sensitivity testing of K. pneumoniae isolates was performed by using disk diffusion methods (Kirby-Baure method), The resistance profile to 25 antibiotics disks including: piperacillin(100µg), ticarcillin(75µg), piperacillin-tazobactam(10µg), ticarcillin-clavulanic acid(75µg), cefoxitin(30µg) ceftazidime(30µg), cefotaxime(30µg), ceftriaxone(30µg), aztreonam(30µg), cefepime(30µg), meropenem(10µg), imipenem(10µg), gentamicin(10µg), amikacin(30µg), tobramycin(10µg), netilmicin (30µg), ciprofloxacin(5µg) , levofloxacin(30µg), oflaxacin(5µg), lomefloxacin(10µg), moxofloxacin(5µg), chloramphenicol(30µg),trimethoprim(5µg), Sulfonamides (300µg), colistin(25µg). All susceptibility results were interpreted according to the standard values performed by CLSI 2021(12). Following antibiotic susceptibility testing, the multiple antimicrobial resistance profiles of tested K. pneumoniae isolates were determined based on the standardized international terminology proposed by CDC center and the European Centre for Disease Prevention and Control (ECDC) definitions (13) and according to the CLSI (2022) recommendations using the Kirby-Bauer method. They were categorized into groups according to their classification as MDR, XDR or PDR. MAR index is calculated following the prescription of Krumperman 1983(14).

Hypermucoviscosity Testing (HMV)

After deteaction of XDR *K. pneumoniae* hypermucoviscosity testing (HMV) was performed to identify the hypervirulent (hypermucoviscous) isolates By inoculating bacteria on MCA and incubating at 37 °C for 24 hours, we were able to identify mucoid isolates of *K. pneumoniae*. After incubation the colony touched by a loop and lifted vertically away from surface of MCA, colonies formed a string ≥ 5 mm in length show HMV isolates (11).

Molecular detection of Resistance genes:

DNA was extracted from the isolates by using the protocol kit of the manufacturing company (Favorgen,Taiwan), hvKp were screening for detection the presence main ESBL, AmpC, carbapenemases, AMEs, 16S-RMTase, PMQR, capsular serotypes, and class I integrons genes by PCR, the primers sequences were mentioned in appendix I Table (1). Amplicons were separated by agarose gel electrophoresis in 1.5 % (w/v) agarose gel, stained with ethidium bromide. The positive results were detection when the DNA band base pairs of sample equal to the target product size. PCR conditions had performed in T3000 thermocycler (Biometra).

3. Results

The results indicated that out of all the 121 *K. pneumoniae* clinical isolates following antimicrobial susceptibility tested, present study classified 23 (19%) isolates as XDR based upon disk diffusion methods (Kirby-Baure method) and VITEK-2-system test (base on MICs).A retrospective investigation was conducted on all 23 XDR *K. pneumoniae* to identify the hypervirulent (hypermucoviscous) isolates. In order to see if isolate showed a hypermucoviscous phenotype, the string test was performed in this study. String test to detect hypervirulent isolates of *K. pneumoniae* is shown in Figure (1).

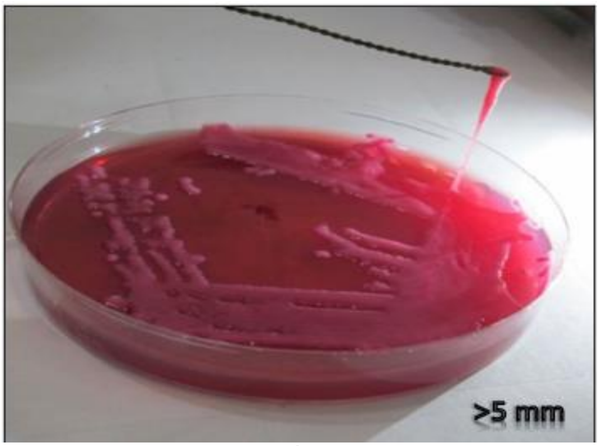

Figure (1): Culture of XDR hypervirulent K. pneumoniae isolate KP6 recovered from urinary tract infection of outpatient on MacConkey agar illustrating a positive string test (formation of viscous strings of > 5mm in length when colony was touched with loop).

Based on the results of string test, the hypermucoviscous phenotype was detected in 5 (21.7%) isolates (KP6, KP7, KP10, KP14, and KP18) and identified as hypervirulent XDR *K. pneumoniae*, while the remaining 18 isolates (78.3%) were string test negative and designated as classical XDR *K. pneumoniae* (Figure 2). The clinical and microbiological characteristics of the hypervirulent *K. pneumoniae* isolates are shown in Table (2).

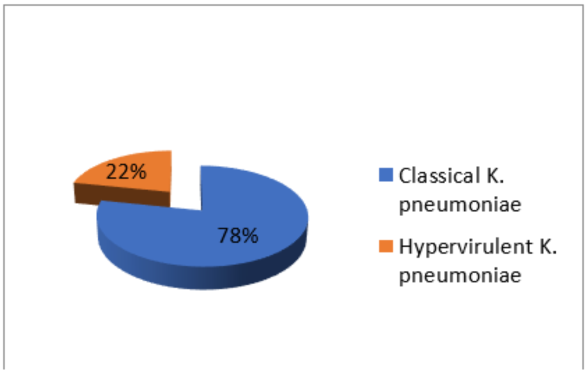

Figure (2): Percentage of hypervirulent XDR K. pneumoniae clinical isolates

Among the five hypervirulent *K. pneumoniae* isolates, three were resistant to imipenem and meropenem, indicating that these isolates were carbapenem resistant- hypervirulent *K. pneumoniae*, in addition one isolate were intermediate resistant to imipenem and susceptible to meropenem and one isolate exhibited susceptibility to both antibiotics. Fortunately, all the hypervirulent isolates in this study were sensitive to colistin except Kp14 isolate. Present data support the concept that antibiotic resistance is high among hypervirulent isolates, evaluation of MAR index of the isolates showed MAR value ranging from 0.64 to 0.92.

Diverse resistance determinants were detected among four hypervirulent XDR *K. pneumoniae* isolates. The distribution of resistance genes are listed in Table (2). Overall, these isolates had a high prevalence rate of carbapenemase-related resistance genes; specifically the carbapenemase gene bla_{NDM} was present in three isolates, KP6, KP7, and KP10. The PCR analyses demonstrated that four isolates were rich in ESBL genes. The $bla_{\mathrm{CTX\text{-}M}}$, bla_{SHV}, and bla_{TEM} genes were identified in KP6 isolate. The bla_{SHV} gene was found to coexist with bla_{OXA} and bla_{TEM} in KP7 isolate. In addition, KP10 isolate co-carried $bla_{\mathrm{CTX\text{-}M}}$, bla_{SHV}, bla_{TEM} and bla_{OXA} and KP18 isolate co-carried bla_{SHV} and bla_{OXA}. For amplification of AmpC gene encoding bla_{CMY} β-lactamase, two isolates (KP6 and KP10) gave amplification product with this gene. Furthermore, three isolates (KP6, KP7, KP10) possessed two AME genes namely *aph(3')-I* and *aac(6')-Ib* and one isolate (KP18) had one AME gene, *aph(3')-I*. Of the PMQR genes, *qnr* genes were present in three isolates. The *qnrB* gene was detected in two isolates (KP6 and KP10) and *qnrA* was detected in one isolate (KP7). Interestingly, all antibiotic resistant gene tested was not observed in the hypervirulent XDR *K. pneumoniae* isolate KP14.

Table (2): Characteristics of the hypervirulent XDR *K. pneumoniae* isolates (n= 5)

Characteristic	Isolate code No.				
	KP6	KP7	KP10	KP14	KP18
Sample source	PHL	PLs	PLs	PLs	PLs
Clinical sample	Urine	Urine	Burn	Ear	Burn
Hospitalization	Outpatient	Outpatient	Inpatient	Inpatient	Inpatient
Patient gender	Male	Female	Male	Male	Female
Patient age	61	19	26	31	35
Resistance pattern	PLR, TI, TPZ, TIM, FOX, CAZ, CTX, CRO, FEP, ATM, IMP, MEM, AK, CN, TOB, CIP, LEV, LOM, OFL, MFX, TMP, STX, C	PLR, TI, TIM, FOX, CAZ, CTX, CRO, ATM, CN, TOB, NET, CIP, LEV, TMP, STX, C	PLR, TI, TPZ, TIM, FOX, CAZ, CTX, CRO, FEP, ATM, IMP, MEM, TOB, NET, CIP, LEV, LOM, OFL, MFX,C	PLR, TI, TIM, FOX, CAZ, CTX, CRO, ATM, CN, TOB, CIP, LEV, LOM, OFL, MFX, TMP, STX, C	PLR, TI, TPZ, TIM, FOX, CAZ, CTX, CRO, FEP, ATM, IMP, MEM, AK, CN, TOB, CIP, LEV, LOM, OFL, MFX, TMP, STX, C
MAR index	0.8	0.64	0.76	0.72	0.92
Resistance gene profile	bla_{NDM}, bla_{KPC}, $bla_{\mathrm{CTX\text{-}M}}$, bla_{SHV}, bla_{TEM}, bla_{CMY}, *aph(3')-I*, *aac(6')-Ib*, *ant(3'')-I*, *qnrB*,	bla_{NDM}, $bla_{\mathrm{CTX\text{-}M}}$, bla_{SHV}, bla_{OXA}, bla_{TEM}, *aph(3')-I*, *aac(6')-Ib*, *qnrA*,	bla_{NDM}, bla_{KPC}, $bla_{\mathrm{CTX\text{-}M}}$, bla_{SHV}, bla_{OXA}, bla_{TEM}, bla_{CMY}, *ant(3'')-I*, *aac(6')-Ib*, *qnrB*,	No gene	bla_{SHV}, bla_{OXA}, *aph(3')-I*,

PHL: Public Health Laboratory, PL: Private Laboratories, CTX: Cefotaxime, CAZ: Ceftazidime, CRO: Ceftriaxone, FEP: Cefepime, FOX: Cefoxitin, IPM: Imipenem, MEM: Meropenem, OFX: Ofloxacin, LVE: Levofloxacin, LOM: Lomefloxacin, MFX: Moxofloxacin AK: Amikacin, TOB: Tobramycin, CN: Gentamicin, NET: Netilmicin STX: Sulfamethoxazole, TMP: Trimethoprim, C: Chloramphenicol, CLT: colistin , CIP: Ciprofloxacin, ATM: Aztreonam, TZP: Piperacillin/ Tazobactam, TIM: Ticarcillin/Clavulanic Acid, TI: Ticarcillin, PLR: Piperacillin

Capsular Genotypes of XDR K. pneumoniae:

To determine the capsular genotypes of the 23 XDR *K. pneumoniae*, present study performed *cps* genotyping by using the multiplex PCR assays of K-serotype-specific alleles at the *wzy* loci, including serotypes K1 (*magA*),) and K2. The distribution of capsular types among all XDR *K. pneumoniae* isolates are shown in Table (2). Capsular serotyping results of the XDR isolates showed that 18 (78.3%) isolates were typeable serotypes and 5 (71.7%) were untypeable serotypes. All the 18 typeable serotypes isolates tested were positive for the *wzy*-K2 gene, while all the XDR isolates were negative for the *wzy*-K1 gene.

Notably, 4 (80%) of the five hypervirulent *K. pneumoniae* isolates were found in this work, which contained *wzy*-K2 gene. Furthermore, *wzy*-K2 gene was also positively associated with classical *K. pneumoniae*, 14 (77.8%) out of the 18 isolates that belonged to capsular serotype K2 were classical isolates.

Table (3): The distribution of capsular serotype genes (**wzy**-K1 and **wzy**-K2) among XDR *K. pneumoniae* isolates (n=23)

Serotype gene	No.(%) of hvKp isolates(n=5)	Isolate code No.	No. (%) of cKp isolates (n=18)	Isolate code No.
wzy-K2 gene	4 (80)	KP6, KP10, KP14, KP18	14 (77.8)	KP1, KP2, KP3, KP4, KP5, KP8, KP9, KP11, KP12, KP13, KP15, KP21, KP22, KP23
No gene	1 (20)	KP7	4 (22.2)	KP16, KP17, KP19 ,KP20
Total	5(100)	_	18 (100)	_

hvKp: Hypervirulent K. pneumonia , cKp: Classical K. pneumoniae. Note, wzy-K1 was not found in any isolate.

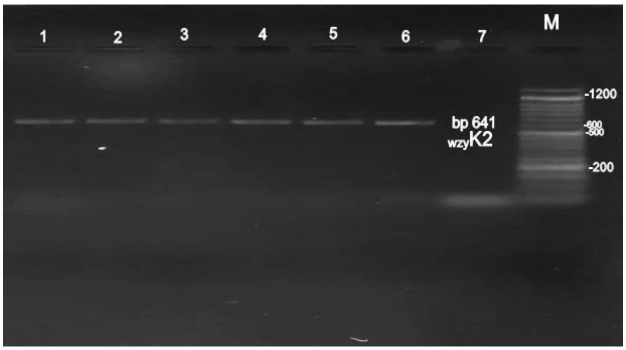

Figure (3): Ethidium bromide-stained agarose gel of multiplex PCR amplified products from extracted DNA of K. pneumoniae isolates and amplified with two genes primers. The electrophoresis was performed at 80 volt for 1.5 hr. Lane (M), DNA molecular size marker (1500 bp ladder), Lanes (K1, 2,3, 4, 5, and 6) show positive results with wzy-K2(641bp), and the wzy-K1(1283bp) gene was not found in any isolate.

4. Discussion

Occurrence of XDR Hypervirulent K. pneumoniae Isolates

Present investigation guessed that hypervirulent *K. pneumoniae* isolates are currently present in Najaf. To test this guess, present study piloted a retrospective investigation of 23 XDR *K. pneumoniae* isolates recovered from Najaf. According to previous designation criteria for hypervirulent *K. pneumoniae*, 5 (21.7%) of XDR *K. pneumoniae* isolates were identified as string test positive in present study (Table 2). The proportion was higher than that reported in previous study conducted in Najaf, with a prevalence of 18.2% (10). This percentage is lower than that reported in several countries, however in study conducted in Russia reported that 53.6% of *K. pneumoniae* isolates were hypervirulent (15). In China, 46.6% of *K. pneumoniae* isolates collected from mechanically ventilated patients with hypervirulence-positive (16). A study in Egypt showed that 32.1% of *K. pneumoniae* isolates collected from various patients had the hypervirulence phenotype (17). Lower rates were found in India, 13.9% ; Iran, 15.1%; Canada 8.2%; China, 6.6% ; USA, 6.35%; and Spain, 2.8% (18, 19, 20, 21, 22, 23). Moreover, another study conducted in Iran reported that 35.7% of *K. pneumoniae* isolates were hypermucoviscous (24).

XDR-hypervirulent *K. pneumoniae* isolates are clinically important since they can causes untreatable life-threatening infections and they should be given priority in clinical settings (25). Several studies reported that XDR-hypervirulent *K. pneumoniae* isolates caused fatal infections in clinical settings (25, 26). The five isolates were obtained from clinical samples as follows: 2 from urine, 2 from burn exudate and 1 from ear. Three of the five isolates were hospital acquired, two of which were associated with burn infection and one was related with ear infection, while the remaining two were community acquired and were associated with UTI. Contrarily, hypervirulent *K. pneumoniae* strains tend to be picked up in the community, while their ability to infect healthy people may aid in their spread inside healthcare facilities. As a result, hypervirulent *K. pneumoniae* infections are becoming more common and related with rising mortality (27). In study conducted by Al-Hasnawi 2020 (10) in Najaf, 25% of carbapenem resistant-hypervirulent *K. pneumoniae* isolates were obtained from burn infections. In Egypt, 30% of carbapenem resistant hypervirulent *K. pneumoniae* isolates were obtained from UTIs (17).

Classical *K. pneumoniae* isolates have typically exhibit resistance to the currently available antibiotics. Fortunately,except for ampicillin, hypervirulent *K. pneumoniae* isolates are sensitive to the vast majority of antimicrobials used in medicine (25, 28). Contrary to the previous view that the prevalence of antibiotic resistant hypervirulent *K. pneumoniae* is low, the results of present study indicated that no difference in the occurrence of antibiotic resistance between hypervirulent and classical *K. pneumoniae* isolates. Furthermore, antibiotics susceptibility data found that the emergence of XDR-hypervirulent *K. pneumoniae* isolates in Najaf (Table2). Emerging reports of multidrug-resistant and extensively drug-resistant hypervirulent *K. pneumoniae* isolates provide cause for concern (24,29). The combination of hypervirulence in *K. pneumoniae* and antibiotic resistance phenotypes (including MDR, XDR, and carbapenem resistance) pose a serious danger to public health (30,31). However, along with the worldwide dissemination of mobile genetic elements such as transposons or plasmids that carry various antibiotic resistance genes, MDR and XDR hypervirulent *K. pneumoniae* isolates are increasingly being detected in several countries and begun to emerge in the past few years [32,33,34]. A report in China showed that 17% of hypervirulent *K. pneumoniae* isolates exhibited resistance to all the antibiotics tested, except amikacin and carbapenems [35]. Present data emphasize that XDR hypervirulent *K. pneumoniae* is present in Najaf, the study hypothesize that such isolates may be have outbreak potential.

Capsular Serotypes of XDR Hypervirulent K. pneumoniae

The presence of polysaccharide capsule is one of the most important virulence factors associated with *K. pneumoniae* that protects isolates from lethal serum factors and phagocytosis. Among the 79 known capsular serotypes of *K. pneumoniae*, K1 and K2 are the most frequently observed types associated with the main virulent isolates [21,36]. In this study, all the 23 XDR *K. pneumoniae* were tested by PCR for the detection of *wzy*-K1 and *wzy*-K2 serotype genes. Only 78.3% of isolates tested positive for the *wzy*-K2 gene, while the *wzy*-K1 gene was not detected. However, 72.7% of the studied isolates were not related to any serotype tested (Table 3). This is the first report, to our knowledge, of K2-capsular-type XDR *K. pneumoniae* isolates in Najaf.

Despite the strongly association of the K1 and K2 serotypes with hypervirulent *K. pneumoniae*, these serotypes have been found in many of classical *K. pneumoniae* isolates as well. At the same time, some hypervirulent *K. pneumoniae* isolates have a non-K1 or -K2 serotype [19,37]. In this study, the frequency of K2 among hypervirulent isolates was 80%.

This result much higher than previous studies by Russo *et al.* [3] which found that 20% of hypervirulent *K. pneumoniae* isolates were carried K2 gene in China, and Liu *et al.*, [26] in Iran which found that 7 (24.1%) of hypervirulent *K. pneumoniae* isolates showed the K2 serotype. Previous studies, from China, reported the occurrence of K2 serotypes in 68.75% and 42.9% of hypervirulent *K. pneumoniae* clinical isolates related with several types of invasive infections [38,39]. Variable rates of the K2 serotype were seen in several of the hypervirulent *K. pneumoniae* isolates reported in Europe and the United States [40,41].

In addition, K2 capsular serotype has also been found in classical *K. pneumoniae*, the study found that 77.8% of isolates carried *wzy*-K2 gene. However, present study documented that K2 capsular serotype is common in hypervirulent and classical *K. pneumoniae* isolates tested; in addition 20% of isolates have a non-K2 serotype. These results indicate that K2 and K1 may be not enough for detect hypervirulent *K. pneumoniae* isolates and need other genes to determine these isolates.

5. Conclusion

The study highlighted the emergence of five XDR hypervirulent isolates, the occurrence of hypervirulent isolates is particularly worrisome due to the confluence of both hypervirulence and XDR.

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