β-Lactam Resistance in Upper Respiratory Tract Pathogens Isolated from a Tertiary Hospital in Malaysia

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Abstract: The rise of antimicrobial resistance (AMR) among clinically important bacteria, including respiratory pathogens, is a growing concern for public health worldwide. Common causative bacteria for upper respiratory tract infections (URTIs) include Streptococcus pneumoniae and Haemophilus influenzae, and sometimes Staphylococcus aureus. We assessed the β-lactam resistant trends and mechanisms of 150 URTI strains isolated in a tertiary care hospital in Kuala Lumpur Malaysia. High rates of non-susceptibility to penicillin G (38%), amoxicillin-clavulanate (48%), imipenem (60%), and meropenem (56%) were observed in S. pneumoniae. Frequent mutations at STMK and SRNVP motifs in PBP1a (41%), SSNT motif in PBP2b (32%), and STMK and LKSG motifs in PBP2x (41%) were observed in S. pneumoniae. H. influenzae remained highly susceptible to most β-lactams, except for ampicillin. Approximately half of the ampicillin non-susceptible H. influenzae harboured PB P3 mutations (56%) and only blatEM was detected in the ampicillin-resistant strains (47%). Methicillin-susceptible S. aureus (MSSA) strains were mostly resistant to penicillin G (92%), with at least two-fold higher median minimum inhibitory concentrations (MIC) for all penicillin antibiotics (except ticarcillin) compared to S. pneumoniae. H. influenzae and S. pneumoniae. Almost all URTI strains (88–100%) were susceptible to cefcapene and floxoxef. Overall, β-lactam antibiotics except penicillins remained largely effective against URTI pathogens in this region.

Keywords: antimicrobial resistance; β-lactamase; Haemophilus influenzae; penicillin resistance; penicillin-binding proteins; Staphylococcus aureus; Streptococcus pneumoniae

1. Introduction

Respiratory tract infections (RTIs) are generally classified into either upper or lower respiratory tract infections [1]. Based on national surveillance conducted by the Ministry of Health (MOH) Malaysia, RTIs accounted for approximately 68% of the patients admitted to government hospitals due to diseases of the respiratory system [2]. Among the RTIs, pneumonia is of particularly high risk to susceptible hosts in healthcare settings [3] and is the world’s leading infectious cause of death for infants under five, accounting for 16% of child deaths [4]. In Malaysia, pneumonia is one of the most common nosocomial infections, second only to clinical sepsis cases [5]. Common bacterial pathogens causing pneumonia are Streptococcus pneumoniae, Haemophilus influenzae, and sometimes Staphylococcus aureus.
4. Non-viral pneumonia is and has been the main cause of death (99%) among patients’ death due to RTIs in Malaysian hospitals [6].

Antibiotic treatment is often contraindicated for healthy individuals with uncomplicated acute RTIs. However, in RTIs caused by bacteria, amoxicillin or penicillin antibiotics are often the drugs of choice, followed by macrolides or fluoroquinolones in cases of penicillin allergy [4,7,8]. In Malaysia, higher rates of antibiotic prescription for upper respiratory tract infections (URTIs) were observed in private clinics (46.7%) compared to public clinics (27.8%) [9]. Most of the antibiotics prescribed were penicillin and macrolides, with broad-spectrum antibiotics being the preferred option [9]. The higher-than-usual antibiotic prescription rates for URTIs, which are mainly caused by viruses, may increase the risk of bacterial pathogens developing antimicrobial resistance (AMR) [9]. The first-line drugs that are commonly used for the treatment of acute RTIs mainly comprise β-lactam antibiotics [4,7,8]. However, β-lactam resistance among the clinically important bacteria, including those that cause RTIs, has been on a global rise [10]. Therefore, continuous efforts to monitor the AMR trends and to explore new antibacterial agents are essential to reduce the impact of AMR on public health [11]. This study aimed to investigate the β-lactam resistance trends and mechanisms among S. pneumoniae, H. influenzae, and S. aureus strains isolated from URTI patients.

2. Results

The patient’s demographic data and S. pneumoniae serotypes are summarized in Table 1. Table 2 summarizes the minimum inhibitory concentration (MIC) data for all 150 bacterial strains.

Table 1. Summary of demographic data, source of specimen and serotype of the URTI strains (n = 150).

|                      | S. pneumoniae (n) (%) | H. influenzae (n) (%) | MSSA (n) (%) |
|----------------------|----------------------|----------------------|-------------|
| **Gender**           |                      |                      |             |
| Female               | 27 (54)              | 21 (42)              | 18 (36)     |
| Male                 | 23 (46)              | 29 (58)              | 32 (64)     |
| **Age Range**        |                      |                      |             |
| <1 year              | 7 (14)               | 5 (10)               | 12 (24)     |
| 1–17                 | 43 (86)              | 45 (90)              | 10 (20)     |
| 18–59                | 0 (0)                | 0 (0)                | 18 (36)     |
| ≥60                  | 0 (0)                | 0 (0)                | 10 (20)     |
| **Source of Specimen** |                    |                      |             |
| Bronchoalveolar lavage | 2 (4)               | 0 (0)                | 0 (0)       |
| Nasopharyngeal swab  | 46 (92)              | 50 (100)             | 50 (100)    |
| Sputum               | 2 (4)                | 0 (0)                | 0 (0)       |
| **Serotype**         |                      |                      |             |
| 3                    | 1 (2)                | -                    | -           |
| 34                   | 1 (2)                | -                    | -           |
| 11A/D/F              | 3 (6)                | -                    | -           |
| 15A/F                | 1 (2)                | -                    | -           |
| 19A                  | 2 (4)                | -                    | -           |
| 19F                  | 11 (22)              | -                    | -           |
| 23A                  | 3 (6)                | -                    | -           |
| 23F                  | 2 (4)                | -                    | -           |
| 6A/6B                | 11 (22)              | -                    | -           |
| 6C                   | 3 (6)                | -                    | -           |
| Non-typeable         | 12 (24)              | -                    | -           |

1 Polymerase chain reaction (PCR) serotyping was only performed on S. pneumoniae strains (n = 50).
Table 2. Summary of MIC data for *S. pneumoniae* (*n* = 50), *H. influenzae* (*n* = 50), and methicillin-susceptible *S. aureus* (MSSA) (*n* = 50).

| Antimicrobial Agent                      | MIC Range          | MIC<sub>50</sub> | MIC<sub>90</sub> | MIC Range          | MIC<sub>50</sub> | MIC<sub>90</sub> | MIC Range          | MIC<sub>50</sub> | MIC<sub>90</sub> | MIC Range          | MIC<sub>50</sub> | MIC<sub>90</sub> |
|------------------------------------------|--------------------|------------------|------------------|--------------------|------------------|------------------|--------------------|------------------|------------------|--------------------|------------------|------------------|
| Ampicillin                               | ≤0.125–128         | 16               | 64               | 0.5–>256           | 4                | >256             | ≤0.125–128         | 32               | 64               |
| Penicillin G                             | ≤0.03–8            | 2                | 4                | 0.25–>64           | 1                | >64              | ≤0.03–>64          | 32               | >64              |
| Piperacillin                              | ≤0.03–16           | 2                | 8                | ≤0.03–>64          | ≤0.03            | >64              | 0.5–>128           | 64               | >128             |
| Ticarcillin                              | 1–>64              | ≥64              | >64              | 0.25–>64           | 2                | >64              | 2–32               | 16               | 32               |
| Aminocillin-clavulanate (2:1)            | <0.03/0.015–32/16  | 2/1              | 8/4              | 0.125/0.06–64/32   | 2/1              | 32/16           | ≤0.125/0.06–8/4   | 2/1              | 4/2              |
| Cefotaxime-clavulanate<sup>2</sup>       | ≤0.03–1            | 0.06             | 0.5              | ≤0.03–0.5          | ≤0.03            | 0.25             | 0.25–2             | 1                | 2                |
| Ceftazidime-clavulanate<sup>2</sup>      | ≤0.03–32           | 0.125            | 2                | ≤0.03–4           | 0.25             | 2                | 16–256             | 64               | 128              |
| Piperacillin-tazobactam<sup>3</sup>      | ≤0.03–2            | 0.5              | 1                | ≤0.03–1           | ≤0.03            | 1                | 0.5–64             | 16               | 64               |
| Ticarcillin-clavulanate<sup>4</sup>      | 1–>64              | ≥64              | >64              | 0.125–16          | 1                | 2                | 0.5–16             | 8                | 8                |
| Cefmetazole                              | 0.125–64           | 4                | 32               | 1–8               | 8                | 8                | 0.5–2              | 1                | 2                |
| Cefoxitin                                | 32–>64             | >64              | >64              | 1–>64             | 4                | >64              | 2–4               | 4                | 4                |
| Cefcapene                                | ≤0.03–8            | 0.5              | 2                | ≤0.03–1           | ≤0.03            | 0.125            | 0.125–1           | 0.5              | 1                |
| Cefoperazone                             | ≤0.03–32           | 4                | 32               | ≤0.03–16          | 0.125            | 8                | 0.25–8            | 4                | 8                |
| Ceftazidime                              | ≤0.03–2            | 0.125            | 2                | ≤0.03–4           | <0.03            | 0.25             | 0.25–2            | 1                | 2                |
| Ceftazidime                              | ≤0.03–64           | 2                | 16               | 0.06–64           | 1                | 8                | 32–256            | 128              | 256              |
| Ceftriaxone                              | ≤0.03–4            | 0.5              | 2                | ≤0.03–0.5         | ≤0.03            | 0.25             | 2–4               | 2                | 4                |
| Cefepime                                 | ≤0.03–2            | 0.25             | 1                | 0.06–2            | 0.25             | 0.5              | 0.5–4             | 2                | 2                |
| Imipenem                                 | 0.06–32            | 0.5              | 16               | 0.5–32            | 2                | 4                | <0.03             | ≤0.03            | ≤0.03            |
| Meropenem                                | ≤0.03–2            | 0.5              | 1                | ≤0.03–0.5         | 0.125            | 0.5              | ≤0.03–0.06         | 0.06             | 0.06             |
| Flomoxef                                 | 0.125–16           | 0.5              | 16               | 0.5–4             | 1                | 4                | 0.06–0.5          | 0.25             | 0.25             |
| Oxacillin<sup>5</sup>                    | -                  | -                | -                | -                 | -                | -                | <0.125–0.5         | 0.25             | 0.5              |

<sup>1</sup> All MIC values are indicated in µg/mL; <sup>2</sup> Fixed concentration of clavulanate (4 µg/mL); <sup>3</sup> Fixed concentration of tazobactam (4 µg/mL); <sup>4</sup> Fixed concentration of clavulanate (2 µg/mL); <sup>5</sup> Broth microdilution assay was only performed for MSSA strain.
2.1. *Streptococcus Pneumoniae*

*S. pneumoniae* showed the highest rates of resistance to imipenem (44%) and meropenem (38%) (Figure 1). Among the two major *S. pneumoniae* serotypes observed in this study, serotype 19F showed higher rates of non-susceptibility to penicillin (55%), imipenem (91%), and meropenem (91%) compared to serotype 6A/6B (36%, 55%, 45%, respectively). The potential non-susceptibility to ampicillin was predicted in 74% of the *S. pneumoniae* strains (penicillin MIC > 0.06 μg/mL) [8]. Simultaneous resistance to two-or-more β-lactam antibiotics was observed in 34% of the strains (Supplementary Table S1).

High-level resistance (MIC$_{50}$ ≥ 64 μg/mL) to ticarcillin, ticarcillin-clavulanate, and cefoxitin was observed among the *S. pneumoniae* strains (Table 2). Penicillin antibiotics showed relatively higher MIC$_{50}$ values (2–64 μg/mL), compared to other groups of β-lactam. Generally, β-lactam combination agents remained highly active against *S. pneumoniae* (MIC$_{50}$ ≤ 0.5 μg/mL), except amoxicillin-clavulanate and ticarcillin-clavulanate. Similarly, most third and fourth-generation cephalosporins (cefcapene, cefotaxime, ceftriaxone, and cefepime) and the oxacephem antibiotic flomoxef recorded low MIC$_{50}$ values (≤0.5 μg/mL). Cefotaxime-clavulanate showed highest activity against the *S. pneumoniae* (MIC$_{50}$ = 0.5 μg/mL).

Thirty-seven ampicillin non-susceptible *S. pneumoniae* strains were examined for amino acid substitutions in three conserved motifs of the *pbp* genes (Table 3). Seven strains failed to produce amplicons for all three *pbp* genes, probably due to primer-binding site alteration. The KTG motif in the penicillin-binding protein PBP1a and SVVK in PBP2b remained highly conserved. All but five mutation profiles (M01, M07, M09, M11, and M14) conferred reduced susceptibility to penicillin G (MIC ≥ 4 μg/mL). M12 and M13...
profiles were penicillin-resistant *S. pneumoniae* (PRSP). Mutations beyond the conserved motifs were identified in PBPla (block substitution of Thr-Ser-Gln-Phe \(T^{574}SQF\) by Asn-Thr-Gly-Tyr \(N^{574}TGY\); \(n = 16\)) and PBPlb (Ser-411-Pro, Asn-421-Tyr, Thr-425-Lys, and Gln-426-Ala/Leu; \(n = 11\)).

### 2.2. *Haemophilus Influenzae*

The majority of the *H. influenzae* strains were resistant to ampicillin (72%) and non-susceptible to ceftazidime (20%), amoxicillin-clavulanate (18%), cefotaxime (6%), and imipenem (6%). All strains remained susceptible to piperacillin-tazobactam, ceftriaxone, cefepime, and meropenem. Simultaneous resistance/non-susceptibility to two to three \(\beta\)-lactam antibiotics was observed in approximately one-third of the strains \((n = 16)\) (Supplementary Table S2).

High-level resistance was not common among the *H. influenzae* strains (Table 2). The MIC\(_{50}\) values of amoxicillin-clavulanate, piperacillin-tazobactam, ceftriaxone, cefepime, imipenem, and meropenem were within the susceptible range (Clinical and Laboratory Standards Institute, CLSI interpretive criteria). Cefmetazole (MIC\(_{50}\) = 8 \(\mu g/mL\)), ampicillin (MIC\(_{50}\) = 4 \(\mu g/mL\)), and cefoxitin (MIC\(_{50}\) = 4 \(\mu g/mL\)) were relatively less active against the *H. influenzae* strains. The majority of the \(\beta\)-lactam combination agents, third and fourth generation cephalosporins, piperacillin, and meropenem remained highly active against *H. influenzae* (MIC\(_{50}\) ≤ 0.25 \(\mu g/mL\)). *H. influenzae* were mostly inhibited by low concentrations of cefotaxime-clavulanate, cefcapene, ceftriaxone, cefepime, and meropenem (MIC\(_{90}\) ≤ 0.5 \(\mu g/mL\)). Contrarily, high concentrations of penicillin were required to inhibit the growth of most of the *H. influenzae* strains (MIC\(_{90}\) > 64 \(\mu g/mL\)).

Thirty-nine ampicillin non-susceptible *H. influenzae* strains were examined for \(\beta\)-lactamase (bla) genes and \textit{ftsI} mutations (Table 4). The \textit{bla}_{TEM} gene was detected in 44\% \((n = 17)\) of the strains, while \textit{bla}_{ROB} was totally absent. The \textit{bla}_{TEM}-positive strains were further classified as \(\beta\)-lactamase positive ampicillin-resistant (BLPAR; harbours wild type \textit{ftsI} gene) \((n = 7)\) or \(\beta\)-lactamase positive amoxicillin-clavulanate resistant (BLPACR; harbours \textit{ftsI} gene mutation) \((n = 10)\). Approximately one-third (31\%) of the ampicillin-resistant strains lacked \(\beta\)-lactamase but harboured a mutated \textit{ftsI} gene (\(\beta\)-lactamase negative ampicillin-resistant, BLNAR). The amino acid substitutions in the \textit{ftsI} gene were generally more diverse in BLNAR strains than in BLPACR strains.

### 2.3. Methicillin-Susceptible *Staphylococcus aureus*

Susceptibility to oxacillin (MIC ≤ 2 \(\mu g/mL\)) and cefoxitin (MIC ≤ 4 \(\mu g/mL\)) confirmed the methicillin-susceptible phenotype of the *S. aureus* strains (Table 2). The majority of the methicillin-susceptible *S. aureus* (MSSA) strains showed a penicillin-resistant phenotype (92\%). Higher MIC values of penicillinase-labile agents such as ampicillin, piperacillin, and ticarcillin (16 ≤ MIC\(_{50}\) ≤ 64 \(\mu g/mL\)) were observed among penicillin-resistant MSSA. High MIC values were recorded for ceftazidime (MIC\(_{50}\) = 128 \(\mu g/mL\)) and ceftazidime-clavulanate (MIC\(_{50}\) = 64 \(\mu g/mL\)). Carbapenems (MIC\(_{50}\) ≤ 0.06 \(\mu g/mL\)) generally had lower MIC\(_{50}\) values than \(\beta\)-lactam combination agents (1 ≤ MIC\(_{50}\) ≤ 64 \(\mu g/mL\)) and cephems (1 ≤ MIC\(_{50}\) ≤ 128 \(\mu g/mL\)), except cefcapene. Most MSSA strains remained highly susceptible to carbapenems, cefcapene and flomoxef (MIC\(_{90}\) ≤ 1 \(\mu g/mL\)).
Table 3. Mutations identified in the penicillin-binding proteins (PBPs) in *S. pneumoniae*.

| Mutation Profile | No. of Strains | Ampicillin MIC Range (µg/mL) | Penicillin G MIC Range (µg/mL) | Penicillin-Binding Protein (PBP) motifs<sup>2,3</sup> | PBP1a | PBP2b | PBP2x |
|------------------|----------------|-----------------------------|-------------------------------|-----------------------------------------------|-------|-------|-------|
|                  |                |                             |                               | STMK (370–373) | SRNVP (428–432) | KTG (557–559) | SVVK (385–388) | SSNT (442–445) | KTGTA (614–618) | STMK (337–340) | HSSN (395–397) | LKSG (546–549) |
| M01              | 1              | 4                           | 1                             | —                | —                | —                | —                | —                | —                | —                | L—                | V—                |
| M02              | 3              | 16–64                       | 4                             | -S—              | ——T              | —                | —                | —                | —                | —                | -A—               | —                |
| M03              | 3              | <0.125–64                   | 1–4                           | -S—              | ——T              | —                | —                | —                | —                | —                | -A—               | —                |
| M04              | 1              | 16                          | 4                             | —                | —                | —                | —                | —                | —                | —                | —                | —                |
| M05              | 3              | 16–32                       | 4                             | -A—              | ——T              | —                | —                | —                | —                | —                | —                | —                |
| M06              | 3              | 4–32                        | 2–4                           | -S—              | ——T              | —                | —                | —                | —                | —                | -A—               | —                |
| M07              | 1              | 2                           | 0.25                          | —                | —                | —                | —                | —                | —                | —                | —                | —                |
| M08              | 2              | 4–16                        | 0.25–4                        | —                | —                | —                | —                | —                | —                | —                | -A—               | —                |
| M09              | 2              | 2–32                        | 0.5–2                         | —                | —                | —                | —                | —                | —                | —                | —                | -A—               | —                |
| M10              | 1              | 16                          | 4                             | -A—              | ——T              | —                | —                | —                | —                | —                | —                | —                |
| M11              | 1              | 16                          | 2                             | -A—              | ——T              | —                | —                | —                | —                | —                | -A—               | —                |
| M12              | 1              | 64                          | 8                             | -S—              | ——T              | —                | —                | —                | —                | —                | -A—               | —                |
| M13              | 1              | 128                         | 8                             | —                | —                | —                | —                | —                | —                | —                | -A—               | —                |
| M14              | 1              | 32                          | 2                             | —                | —                | —                | —                | —                | —                | —                | -A—               | —                |
| $               | 6              | <0.125–16                   | 0.125–4                       | —                | —                | —                | —                | —                | —                | —                | —                | —                |

<sup>1</sup> Arbitrarily designated mutation profiles; $: Absence of amino acid substitution at all sites.  
<sup>2</sup> The wild-type gene sequences from *S. pneumoniae* R6 (NCBI GenBank accession number: NC_003098) were used as the reference for mutation analysis.  
<sup>3</sup> A: Alanine; F: Phenylalanine; G: Glycine; H: Histidine; K: Lysine; L: Leucine; M: Methionine; N: Asparagine; P: Proline; R: Arginine; S: Serine; T: Threonine; V: Valine.
Table 4. Summary of ftsI mutation sites and classification of ampicillin-resistant H. influenzae.

| Amino Acid Substitution Sites 1,2 | Group 3 | Ampicillin MIC Range (µg/mL) | No. (%) of Strains |
|-----------------------------------|---------|-----------------------------|--------------------|
| **BLNAR**                         |         |                             |                    |
| Asp-350-Asn; Met-377-Ile; Gly-490-Glu; Ala-502-Val; Asn-526-Lys | Ilb     | 8                           | 1 (9)              |
| Asp-350-Asn; Gly-490-Glu; Ala-502-Val; Asn-526-Lys | Ilb     | 2–4                         | 5 (46)             |
| Asp-350-Asn; Gly-490-Glu; Ala-502-Val; Asn-526-Lys | Ilb     | 4                           | 2 (18)             |
| **BLPAR**                         |         |                             |                    |
| Asp-350-Asn; Gly-490-Glu; Asp-350-Asn; Met-377-Ile; Ala-502-Val; Asn-526-Lys | Ila     | 128                         | 1 (9)              |
| Ile-449-Val; Asn-526-Lys; Ala-530-Ser | IId     | 4–>256                      | 3 (27)             |
| Asp-350-Asn                         | M       | >256                        | 6 (55)             |

1 BLNAR: β-lactamase negative ampicillin-resistant strain; BLPARC: β-lactamase positive amoxicillin-clavulanate resistant; Ala: Alanine; Asp: Aspartic acid; Asn: Asparagine; Glu: Glutamic acid; Gly: Glycine; Ile: Isoleucine; Lys: Lysine; Met: Methionine; Ser: Serine; Val: Valine; M: Miscellaneous group. 2 The wild-type sequence of ftsI gene from H. influenzae Rd KW20 (L42023) was used as the reference for mutation analysis. 3 Classification of strains based on key mutation sites [12–14].

2.4. In Vitro Efficiency of Flomoxef and Cefcapene

The susceptibility of H. influenzae to penicillin G was negatively correlated with both flomoxef and cefcapene, while that of amoxicillin-clavulanate was only negatively correlated with cefcapene (Figure 2). In MSSA, susceptibility to penicillin G was positively correlated with flomoxef (Figure 3a) but negatively correlated with cefcapene (Figure 3b). Similarly, a positive relationship was only observed between piperacillin-tazobactam and flomoxef in MSSA (Figure 3). For S. pneumoniae strains, none of the β-lactam antibiotics selected for comparison showed an obvious correlation with both flomoxef and cefcapene (data not shown). An overall comparison including all URTI strains revealed negative correlations between penicillin G and piperacillin-tazobactam with flomoxef, but the opposite with cefcapene (Figure 4).
Figure 2. Scatter plots comparing the in vitro activity of amoxicillin-clavulanate, penicillin G, flomoxef and cefcapene against *H. influenzae*. (a) compares the Log_{10} MIC values of amoxicillin-clavulanate (AMC) and penicillin G (PCG) against that of flomoxef, while (b) compares the Log_{10} MIC values of AMC and PCG against that of cefcapene. The dotted lines indicate the linear correlation between the comparators.

Figure 3. Scatter plots comparing the in vitro activity of penicillin G, piperacillin-tazobactam, flomoxef and cefcapene against MSSA. (a) compares the Log_{10} MIC values of penicillin G (PCG) and piperacillin-tazobactam (TZP) against flomoxef, while (b) compares the Log_{10} MIC values of PCG and TZP against cefcapene. The dotted lines indicate the linear correlation between the comparators.
Figure 4. Scatter plots comparing minimum inhibitory concentration (MIC) values of selected β-lactam antibiotics for 150 bacterial strains. (a) compares the MIC values of amoxicillin-clavulanate (AMC), penicillin G (PCG) and piperacillin-tazobactam (TZP) against that of flomoxef, while (b) compares the MIC values of AMC, PCG and TZP against that of cefcapene. The dotted lines indicate the linear correlation between the comparators.

A cross-species comparison between flomoxef and cefcapene showed that both antimicrobial agents inhibited the growth of more than half of the organisms at very low concentrations (Table 5). Flomoxef was most active against MSSA (MIC ≤ 1 µg/mL) while cefcapene was most active against H. influenzae (MIC ≤ 1 µg/mL). By using referral MIC breakpoints, all H. influenzae and MSSA strains were predicted as susceptible to both flomoxef and cefcapene. Most of the S. pneumoniae strains showed susceptible phenotypes for cefcapene (98%) and flomoxef (88%).

Table 5. Comparison of flomoxef and cefcapene minimum inhibitory concentration values among different bacterial species.

| Antimicrobial Agent | MIC Range (µg/mL) | Susceptibility Phenotype | S. pneumoniae (n) (%) | H. influenzae (n) (%) | MSSA (n) (%) |
|---------------------|--------------------|--------------------------|-----------------------|----------------------|-------------|
|                     | ≤ 1                | Susceptible              | 29 (58)               | 25 (50)              | 50 (100)    |
|                     | 2–8                | Susceptible              | 15 (30)               | 25 (50)              | 0 (0)       |
|                     | ≥ 16               | Resistant                | 6 (12)                | 0 (0)                | 0 (0)       |
| Flomoxef 1          |                    |                          |                       |                      |             |
|                     | ≤ 1                | Susceptible              | 40 (80)               | 50 (100)             | 50 (100)    |
|                     | 2                  | Intermediate             | 9 (18)                | 0 (0)                | 0 (0)       |
|                     | ≥ 4                | Resistant                | 1 (2)                 | 0 (0)                | 0 (0)       |
| Cefcapene 2         |                    |                          |                       |                      |             |
| 1 The MIC breakpoints for moxalactam (CLSI) were used as the reference for interpretive criteria of flomoxef.  
2 The MIC breakpoints for ceftriaxone (CLSI) were used as the reference for interpretive criteria of cefcapene.

3. Discussion

A total of 150 bacterial strains comprised of three common URTI organisms were examined. All H. influenzae and S. pneumoniae were isolated from paediatric patients, while MSSA was isolated from patients with a wider age range. This sampling outcome was not unexpected as both H. influenzae and S. pneumoniae are the two most common causative agents for RTIs in children. In Malaysia, the Hib conjugate vaccine for H. influenzae has been included in the National Immunization Programme. Pneumococcal vaccine was not listed among the mandatory vaccines in our country. Despite the implementation of
these vaccination programmes, the rates of *S. pneumoniae* and *H. influenzae* remain high among children and cause more severe infections. Due to the high prevalence and reduced susceptibility of *S. pneumoniae* and *H. influenzae* strains isolated from children worldwide, therefore we focused on the AMR trends and mechanisms of these two organisms in paediatric patients for this study [15–17]. Contrarily, paediatric RTIs caused by MSSA are less frequent. From the year 2012 to 2015, a total of 7434 *S. aureus* infections had been reported in UMMC and 76.6% of the cases were caused by MSSA, among which only 22.2% involved paediatric patients (unpublished data). This record shows that although MSSA infections occurred more frequently than its methicillin-resistant counterpart, it is somehow less studied. Therefore, there was limited AMR data for MSSA in this region.

The relatively high rates of non-susceptibility to penicillin, amoxicillin-clavulanate and carbapenems in *S. pneumoniae* are a cause of public health concern among children. Previous studies conducted in the Southeast Asian region have documented relatively high rates of penicillin non-susceptibility (>50%) among paediatric populations [18–20]. Our findings indicated that *S. pneumoniae* serotype 19F was commonly associated with non-susceptibility to penicillin and carbapenem resistance, in agreement with the previous notion that this serotype often shows greater AMR tendency [18,19,21].

We observed the most frequent mutations at Thr-371-Ala/Ser (STMK motif) and Pro-432-Thr (adjacent to SRN motif) in PBP1a, corresponding to the common mutation sites in penicillin non-susceptible *S. pneumoniae* [22,23]. The block mutation identified at amino acid positions 574–577 in PBP1a has been previously associated with intermediate- to high-level penicillin resistance [24,25]. The most frequent mutation in PBP2b occurred at Thr-445-Ala (adjacent to the SSN motif). All strains with Thr-445-Ala substitution showed penicillin MIC ≥ 0.25 µg/mL, consistent with a recent work reporting this mutation to reduce PBP binding affinity by 60% and resulting in raised penicillin MIC (>0.25 µg/mL) [26]. Additional mutations beyond the active sites of PBP2b observed in this study had been reported to decrease β-lactams reactivity and is commonly present in strains with penicillin MIC > 0.5 µg/mL [27]. The most common mutations in PBP2x (Thr-338-Ala in STMK motif and Leu-546-Val adjacent to KSG motif) were identified in *S. pneumoniae* strains exhibiting raised penicillin MICs (2–8 µg/mL), consistent with previous reports associating these mutations with higher-level β-lactam resistance [28–30]. Similar to other non-susceptible *S. pneumoniae* reported globally, His-395-Leu adjacent to the SSN motif in PBP2x was not associated with Thr-338-Ala (in STMK motif) and conferred a lower penicillin MIC (1 µg/mL) [28]. The only strain harbouring Met-339-Phe alongside Thr-338-Ala in the STMK motif of PBP2x did not show high penicillin and cefotaxime MIC values (2 and 0.5 µg/mL, respectively), although mutation at this site was associated with drastically decreased acylation efficiency of β-lactams and results in higher penicillin and cefotaxime MICs (≥4 and ≥2 µg/mL, respectively) [30–32]. The combined effect of the mutations at all three PBPs has resulted in the raised penicillin MICs (≥0.25 µg/mL) observed in this study, thus accounting for the high MIC50/90 values of ticarcillin, ticarcillin-clavulanate and cefoxitin which are known to be less active against penicillin-resistant *S. pneumoniae* [33,34].

*H. influenzae* remained highly susceptible to most of the β-lactam antibiotics tested, except ampicillin and cefamycin. The high rate of ampicillin resistance among the URTI strains observed in this study could be the combined effect of β-lactamase production and penicillin-binding protein 3 (PBP3) alteration. The high prevalence of BLNAR and BLPACR in this study indicates that PBP3 alteration resembles the main resistance mechanism against ampicillin in local *H. influenzae* strains. This is noteworthy since β-lactamase production has been the main ampicillin resistance mechanism for *H. influenzae* globally except Japan [35]. The majority of the PBP3-altered strains in our study were classified as subgroups II, with mutations that conferred only low-level β-lactam resistance (low-rPBP3) [13,36]. This observation concurred with the previous notion that low-rPBP3 predominates in *H. influenzae* isolated in most parts of the world except Japan and South Korea [37,38]. The high prevalence of PBP3 alteration among our ampicillin-resistant *H. influenzae* strains could have explained the reduced susceptibility to cefamycin since
PBP3 mutations are also known to confer non-susceptibility to second and third-generation cephalosporins [38].

The high rate of penicillin resistance among the MSSA strains in this study reflects the common AMR trends of *S. aureus* in Malaysia. Indeed, the national surveillance data recorded a consistently high rate of penicillin resistance (≥80%) among the clinical *S. aureus* isolated yearly [39]. This is not surprising given the high prevalence of the *bla*Z gene in local *S. aureus* populations, including those associated with nasal carriage [40]. The higher MIC values for penicillinase-labile penicillin (ampicillin, piperacillin, and ticarcillin) among the MSSA strains are common in penicillin-resistant but oxacillin-susceptible *S. aureus* [41]. Ceftazidime (with or without clavulanate) showed high MIC values, which is a predictable observation since ceftazidime is known to be less active against *S. aureus* [42]. The higher activity of penicillinase-stable agents, including the β-lactam combination agents (except ceftazidime-clavulanate and piperacillin-tazobactam), cephems (except ceftazidime), and carbapenems are expected in MSSA [43]. In contrast with *mecA*-mediated resistance, *bla*Z-mediated penicillin resistance in *S. aureus* rarely produces an effect on other β-lactam antibiotics even at high inoculum size [44–46].

Flomoxef and cefcapene, currently not available in Malaysia, showed well in vitro activity against the URTI pathogens examined in this study. Both antibiotics were highly effective against *H. influenzae* that were non-susceptible to penicillin and amoxicillin-clavulanate. Furthermore, cefcapene showed greater activity against penicillin-resistant MSSA, while flomoxef was highly effective against URTI strains with reduced susceptibility to penicillin and piperacillin-tazobactam. Both cefcapene (third-generation cephalosporin) and flomoxef (previously a fourth-generation cephalosporin but currently grouped in oxacephem (cephalosporin group IIIC)) exhibit broad-spectrum activity against Gram-positive and Gram-negative bacteria [47]. However, third-generation cephalosporins are more active against Gram-negative bacteria especially Enterobacteriaceae, Neisseria spp. and *H. influenzae* [48]. Indeed, cefcapene showed lower MIC<sub>50/90</sub> values in *H. influenzae* compared to *S. pneumoniae* and MSSA in this study. Although fourth-generation cephalosporins are similar to the third generation, members of this group possess zwitterionic compounds that penetrate through the outer membrane of Gram-negative bacteria more rapidly [49]. Furthermore, fourth-generation cephalosporins show a higher affinity to PBPs compared to β-lactamase in both Gram-positive and Gram-negative organisms [50]. Our results showed that the PBP alterations that conferred penicillin resistance might not be effective against flomoxef, hence the negative correlation between the susceptibility of these two agents among the URTI organisms.

In short, both cefcapene and flomoxef were highly active against bacterial strains that were increasingly resistant to β-lactams commonly used to treat URTIs and pneumonia. Based on the Malaysian National Antimicrobial Guideline 2019, penicillin is recommended for the treatment of URTIs, while amoxicillin-clavulanate is recommended for the treatment of both upper and lower RTIs [51]. However, our study shows increased non-susceptibility to penicillin and amoxicillin-clavulanate among the common URTI pathogens. The MIC<sub>50/90</sub> values of flomoxef reported in this study were comparable with, sometimes slightly lower than, similar studies conducted in China and Japan, indicating its higher activity against local URTI strains [52–54]. Similar to findings reported in Korea, cefcapene showed lower MIC<sub>50/90</sub> values compared to other cephalosporins [55]. Both cefcapene and flomoxef have been clinically proven to be effective and well-tolerated in patients with RTIs [56–59].

Although our study was a single-site study that involved only small subsets of strains, the data provided herein may improve current knowledge on the extent of non-susceptibility to β-lactams and the associated resistance mechanisms in local URTI pathogens. Nonetheless, multiple centres and larger sample sizes should be included in future studies to provide a more comprehensive understanding of the β-lactam resistance trends and mechanisms among the important URTI pathogens in this region. Another limitation of the current study was that only selected genes encoding for penicillin-binding proteins
(pbp and ftsI) and β-lactamases (blaTEM and blaROB) were investigated. Although these genes represent the most common β-lactam resistance mechanisms in S. pneumoniae and H. influenzae, other molecular mechanisms could have been accounted for the raised MIC values observed in the strains that lacked these genes (or mutations).

4. Materials and Methods

4.1. Bacterial Strains

A total of 150 non-duplicated bacterial strains comprised of S. pneumoniae (n = 50), H. influenzae (n = 50), and methicillin-susceptible S. aureus (MSSA) (n = 50) were revived from the bacterial stock cultures collection in the diagnostic laboratory of University Malaya Medical Centre (UMMC). All strains were isolated from respiratory tract specimens of patients admitted to the UMMC with URTI from 2013 to 2015. Both H. influenzae and S. pneumoniae were collected from only paediatric patients (aged 0–17) while S. aureus was collected from patients of all age groups. All bacterial strains were isolated from bronchoalveolar lavage, nasopharyngeal swab and sputum specimens of the patients. Bacterial strains isolated from other sites were excluded from the study. The isolation and initial identification of the bacterial strains were part of the routine microbiological examination procedures in the hospital’s diagnostic laboratory. The identity of the bacterial strains was further confirmed using polymerase chain reaction (PCR) protocols adapted from published studies [43,60,61]. S. pneumoniae strains were further subjected to PCR serotyping using previously described protocols [62].

4.2. Minimum Inhibitory Concentrations and Comparison of In Vitro Activity

The MICs of twenty β-lactam antibiotics were determined using the broth microdilution method based on CLSI guidelines [41]. The antimicrobial agents examined in this study include penicillins (ampicillin, penicillin G, piperacillin, and ticarcillin), β-lactam combination agents (amoxicillin-clavulanate, cefotaxime-clavulanate, ceftazidime-clavulanate, piperacillin-tazobactam, and ticarcillin-clavulanate), cephems (cefmetazole, cefoxitin, cefcapene, cefoperazone, cefotaxime, ceftazidime, ceftriaxone, cefepime, and flomoxef) and carbapenems (imipenem and meropenem). The methicillin susceptibility of the S. aureus was further confirmed via determining the MIC of oxacillin in addition to the β-lactams aforementioned. The MIC values of the antimicrobial agents were interpreted according to the available breakpoints in CLSI guidelines [41]. The median MIC value for each antimicrobial agent is represented as MIC$_{50}$, and MIC$_{90}$ indicates a concentration that effectively inhibited 90% of the tested strains. Scatter plots were generated using log$_{10}$ MIC values to compare the activity of flomoxef and cefcapene against the common β-lactam antibiotics recommended for the treatment of RTIs based on the Malaysian National Antibiotics Guideline [51].

4.3. Molecular Detection of Penicillin Resistance-Conferring Genes

S. pneumoniae and H. influenzae strains with non-susceptible phenotypes for ampicillin were examined for potential amino acid substitution in the genes encoding for penicillin-binding proteins (pbp1a, pbp2b, and pbp2x in S. pneumoniae and ftsI, also known as pbp3, in H. influenzae). The primer sequences and PCR conditions for the amplification of pbp and ftsI genes were adapted from published studies [12,22,63]. The PCR products were then purified using MEGAquick-spin™ Plus Total Fragment DNA Purification Kit (iNtRON Biotechnology, South Korea), and sent for sequencing by a commercial sequencing service provider (First BASE Laboratories, Malaysia). The DNA sequences obtained were then analysed using MEGA-X software [64].

The presence of β-lactamase genes blaTEM (526 bp) and blaROB (692 bp) in the H. influenzae strains were investigated using PCR primers and reaction-mix content adapted from a published study [65].
5. Conclusions

In conclusion, URTI-associated *S. pneumoniae*, *H. influenzae* and MSSA remained largely susceptible to most of the β-lactams but showed increased non-susceptibility to penicillin antibiotics. High-level ampicillin resistance in *H. influenzae* was mainly mediated by the *bla*<sub>TEM</sub> gene. Multiple-sites mutation in the PBPs was responsible for the penicillin non-susceptible phenotypes of *S. pneumoniae* and *H. influenzae*. Cefcapene and flomoxef showed comparable in vitro activity with cephalosporins and carbapenems, thus could be considered as alternative options for the empirical treatment of URTIs.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pathogens10121602/s1, Table S1: Antimicrobial resistance (AMR) profiles of *S. pneumoniae* strains (*n* = 50); Table S2: Antimicrobial resistance (AMR) profiles of *H. influenzae* strains (*n* = 50).

Author Contributions: Conceptualization, C.S.J.T., L.H.T., K.C.L. and S.A.; formal analysis, S.T.N., A.N.M., C.W.C. and L.C.C.; funding acquisition, S.A., L.H.T. and K.C.L.; investigation, S.T.N., A.N.M. and K.A.J.; methodology, C.S.J.T. and S.A.; supervision, C.S.J.T. and S.A.; visualization, S.T.N. and A.N.M.; writing—original draft, S.T.N. and A.N.M.; writing—review and editing, C.S.J.T., S.A., C.W.C., L.C.C., K.A.J., K.C.L. and L.H.T. All authors have read and agreed to the published version of the manuscript.

Funding: This work was partially supported by the Universiti Malaya Research Fund (RU005-2020) and Shionogi Singapore Pte. Ltd.

Institutional Review Board Statement: This study was approved by the Medical Ethics Committee of University Malaya Medical Centre (UMMC) on 6 June 2014 (MEC-ID: 1073.21) and had conformed to the principles embodied in the Declaration of Helsinki.

Informed Consent Statement: This study did not involve human subjects. Only secondary data were collected hence patient’s consent was not required.

Data Availability Statement: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Acknowledgments: This is a collaborative study between the Universiti Malaya (UM) and Shionogi Singapore Pte. Ltd. We thank both UM and Shionogi for providing the materials and facilities for this study.

Conflicts of Interest: The authors declare that they have no conflict of interest.

References

1. Centre for Clinical Practice at NICE (UK). Respiratory Tract Infections—Antibiotic Prescribing: Prescribing of Antibiotics for Self-Limiting Respiratory Tract Infections in Adults and Children in Primary Care; National Institute for Health and Clinical Excellence: London, UK, 2008.
2. Ministry of Health Malaysia. Ministry of Health Hospitals Admissions Data for Diseases of the Respiratory System. 2018. Available online: http://www.datagov.my/data/en_US/dataset/kemasukan-ke-hospital-kkm-bagi-diseases-of-the-respiratory-system-malaysia/resource/0c6d18ca-5a0e-4a3c-9050-5de4d20c5afa (accessed on 24 January 2021).
3. World Health Organization. Prevention of Hospital-Acquired Infections: A Practical Guide, 2nd ed.; Ducel, G., Fabry, J., Nicolle, L., Eds.; World Health Organization: Geneva, Switzerland, 2002.
4. World Health Organization. Pneumonia Fact Sheets: World Health Organization. 2016. Available online: http://www.who.int/en/news-room/fact-sheets/detail/pneumonia (accessed on 10 February 2021).
5. Hughes, A.J.; Ariffin, N.; Huat, T.L.; Abdul Molok, H.; Hashim, S.; Sarijo, J.; Abd Latif, N.H.; Abu Hanifah, Y.; Kamarulzaman, A. Prevalence of nosocomial infection and antibiotic use at a university medical center in Malaysia. Infect. Control Hosp. Epidemiol 2005, 26, 100–104. [CrossRef]
6. Ministry of Health Malaysia. Ministry of Health Hospitals Mortality Data for Diseases of the Respiratory System. 2018. Available online: http://www.datagov.my/data/en_US/dataset/kematian-di-hospital-kkm-bagi-diseases-of-the-respiratory-system-malaysia/resource/3d0f4e99-5e75-4d5c-b54c-e8803420d69# (accessed on 24 January 2021).
7. Kronman, M.P.; Zhou, C.; Mangione-Smith, R. Bacterial prevalence and antimicrobial prescribing trends for acute respiratory tract infections. Pediatrics 2014, 134, e956. [CrossRef]
8. Centers for Disease Control and Prevention, CDC. Appropriate Antibiotic Use: Community Atlanta, Georgia, United States of America Centers for Disease Control and Prevention. 2015. Available online: http://www.cdc.gov/antibiotic-use/community/for-hcp/index.html (accessed on 7 February 2021).
9. Teng, C.L.; Tong, S.F.; Khoo, E.M.; Lee, V.; Zailinawati, A.H.; Mimi, O.; Chen, W.S.; Nordin, S. Antibiotics for URTI and UTI—Prescribing in Malaysian primary care settings. *Aust. Fam. Physician* 2011, 40, 325–329.

10. Huttner, A.; Harbarth, S.; Carlet, J.; Cosgrove, S.; Goossens, H.; Holmes, A.; Jarlier, V.; Voss, A.; Pittet, D. Antimicrobial resistance: A global view from the 2013 World Healthcare-Associated Infections Forum. *Antimicrob. Infect. Control* 2013, 2, 31. [CrossRef]

11. Ruiz, J. Antimicrobial resistance, from bench-to-publicside. *Microbes Infect. Chemother.* 2021, 1, e1182. [CrossRef]

12. Dabernet, H.; Delmas, C.; Seguy, M.; Pelissier, R.; Faucon, G.; Bennamani, S.; Pasquier, C. Diversity of beta-lactam resistance-concurring amino acid substitutions in penicillin-binding protein 3 of *Haemophilus influenzae*. *Antimicrob. Agents Chemother.* 2002, 46, 2208–2218. [CrossRef]

13. García-Cobos, S.; Campos, J.; Lázaro, E.; Román, F.; Cercenedo, E.; García-Rey, C.; Pérez-Vázquez, M.; Oteo, J.; de Abajo, F. Ampicillin-resistant non-beta-lactamase-producing *Haemophilus influenzae* in Spain: Recent emergence of clonal isolates with increased resistance to cefotaxime and ceftriaxone. *Antimicrob. Agents Chemother.* 2007, 51, 2564–2573. [CrossRef]

14. Ubukata, K.; Shibasaki, Y.; Yamamoto, K.; Chiba, N.; Hasegawa, K.; Takeuchi, Y.; Sunakawa, K.; Inoue, M.; Konno, M. Association of amino acid substitutions in penicillin-binding protein 3 with beta-lactam resistance in beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. *Antimicrob. Agents Chemother.* 2001, 45, 1693–1699. [CrossRef]

15. Vasoo, S.; Singh, K.; Hsu, L.Y.; Chiew, Y.F.; Chow, C.; Lin, R.T.; Tambyah, P.A. Increasing antibiotic resistance in *Streptococcus pneumoniae* colonizing children attending day-care centres in Singapore. *Respirology* 2011, 16, 1241–1248. [CrossRef]

16. Ito, M.; Hotomi, M.; Maruyama, Y.; Hatano, M.; Sugimoto, H.; Yoshizaki, T.; Yamanaka, N. Clonal spread of beta-lactamase-producing amoxicillin-clavulanate-resistant (BLPACR) strains of non-typeable *Haemophilus influenzae* among young children attending a day care in Japan. *Int. J. Pediatr. Otorhinolaryngol.* 2010, 74, 901–906. [CrossRef]

17. Phongsamart, W.; Srifeungfung, S.; Dejsirilert, S.; Chatsuwan, T.; Nunthapisud, P.; Treerauthaweeraphong, V.; Rungnobhakhun, P.; Chokephaibulkit, K. Serotype distribution and antimicrobial resistance of *Streptococcus pneumoniae* isolates in Malaysia. *PLoS ONE* 2011, 6, e19547. [CrossRef]

18. Le, C.-F.; Palanisamy, N.K.; Mohd Yusof, M.Y.; Sekaran, S.D. Capsular Serotype and Antibiotic Resistance of *Streptococcus pneumoniae* Isolates in Malaysia. *Antimicrob. Agents Chemother.* 2000, 44, 2193–2196. [CrossRef]

19. Vasoo, S.; Singh, K.; Hsu, L.Y.; Chiew, Y.F.; Chow, C.; Lin, R.T.; Tambyah, P.A. Increasing antibiotic resistance in *Streptococcus pneumoniae* colonizing children attending day-care centres in Singapore. *Respirology* 2011, 16, 1241–1248. [CrossRef]

20. Ito, M.; Hotomi, M.; Maruyama, Y.; Hatano, M.; Sugimoto, H.; Yoshizaki, T.; Yamanaka, N. Clonal spread of beta-lactamase-producing amoxicillin-clavulanate-resistant (BLPACR) strains of non-typeable *Haemophilus influenzae* among young children attending a day care in Japan. *Int. J. Pediatr. Otorhinolaryngol.* 2010, 74, 901–906. [CrossRef]

21. Soh, S.W.; Poh, C.L.; Lin, R.V. Serotype distribution and antimicrobial resistance of *Streptococcus pneumoniae* isolates from pediatric patients in Singapore. *Antimicrob. Agents Chemother.* 2000, 44, 2193–2196. [CrossRef]

22. Goh, S.L.; Kee, B.P.; Abdul Jabar, K.; Chua, K.H.; Nathan, A.M.; Bruyne, J.; Ngoi, S.T.; Teh, C.S.J. Molecular detection and genotypic characterisation of *Streptococcus pneumoniae* isolates from children in Malaysia. *Pathog. Glob. Health* 2020, 114, 46–54. [CrossRef]

23. Diawara, I.; Nayme, K.; Katfy, K.; Barguigna, A.; Kettani-Halabi, M.; Belabbes, H.; Timinouni, M.; Zerouali, K.; Elmdaghri, N. Analysis of amino acid motif of penicillin-binding proteins 1a, 2b, and 2x in invasive *Streptococcus pneumoniae* nonsusceptible to penicillin isolated from pediatric patients in Casablanca, Morocco. *BMC Res. Notes* 2018, 11, 632. [CrossRef]

24. Chewapreecha, C.; Marttinen, P.; Croucher, N.J.; Salter, S.J.; Harris, S.R.; Mather, A.E.; Hanage, W.P.; Nosten, F.H.; Turner, C.; et al. Comprehensive Identification of Single Nucleotide Polymorphisms Associated with Beta-lactam Resistance in Clinical *Streptococcus pneumoniae* Nonsusceptible to Penicillin Isolated from Children in Malaysia. *Microb. Drug Resist.* 2021, 45, 311–319. [CrossRef]

25. Job, V.; Carapito, R.; Vernet, T.; Dessen, A.; Zapun, A. Common alterations in PBP1a from resistant *Streptococcus pneumoniae* decrease its reactivity toward beta-lactams: Structural insights. *J. Biol. Chem.* 2008, 283, 4886–4894. [CrossRef]

26. Varghese, R.; Neeravi, A.; Subramanian, N.; Baskar, P.; Anandhan, K.; Veeraraghavan, B. Analysis of Amino Acid Sequences of Penicillin-Binding Proteins 1a, 2b, and 2x in Invasive *Streptococcus pneumoniae* Nonsusceptible to Penicillin Isolated from Children in India. *Microb. Drug Resist.* 2021, 10, e1004547. [CrossRef]

27. Calvez, P.; Breukink, E.; Roper, D.I.; Dib, M.; Contreras-Martel, C.; Zapun, A. Substitutions in PBP2b from β-Lactam-resistant *Streptococcus pneumoniae* Have Different Effects on Enzymatic Activity and Drug Reactivity. *J. Biol. Chem.* 2017, 292, 2854–2865. [CrossRef] [PubMed]

28. Granger, D.; Boily-Larouche, G.; Turgeon, P.; Weiss, K.; Roger, M. Genetic analysis of pbp2x in *clinical Streptococcus pneumoniae* isolates in Quebec, Canada. *J. Antimicrob. Chemother.* 2005, 55, 832–839. [CrossRef]

29. Asahi, Y.; Takeuchi, Y.; Ubukata, K. Diversity of substitutions within or adjacent to conserved amino acid motifs of penicillin-binding protein 2X in cephalosporin-resistant *Streptococcus pneumoniae* isolates. *Antimicrob. Agents Chemother.* 1999, 43, 1252–1255. [CrossRef]

30. Nagai, K.; Davies, T.A.; Jacobs, M.R.; Appelbaum, P.C. Effects of amino acid alterations in penicillin-binding proteins (PBPs) 1a, 2b, and 2x on PBP affinities of penicillin, ampicillin, amoxicillin, cefotinone, cefuroxime, cefprozil, and cefaclor in 18 clinical isolates of penicillin-susceptible, -intermediate, and -resistant pneumococci. *Antimicrob. Agents Chemother.* 2002, 46, 1273–1280. [CrossRef]
31. Chesnél, L.; Pernot, L.; Le Claire, D.; Champelovier, D.; Croizé, J.; Dideberg, O.; Vernet, T.; Zapun, A. The structural modifications induced by the M339F substitution in PBP2x from Streptococcus pneumoniae further decreases the susceptibility to beta-lactams of resistant strains. *J. Biol. Chem.* 2003, 278, 44448–44456. [CrossRef]

32. Sanbongi, Y.; Ida, T.; Ishikawa, M.; Osaki, Y.; Kataoka, H.; Suzuki, T.; Kondo, K.; Ohnawa, F.; Yonezawa, M. Complete sequences of six penicillin-binding protein genes from 40 Streptococcus pneumoniae clinical isolates collected in Japan. *Antimicrob. Agents Chemother.* 2004, 48, 2244–2250. [CrossRef]

33. Doi, Y.; Chambers, H.F. Penicillins and beta-lactamase inhibitors. In *Mandell, Douglas, Bennett’s Principles and Practice of Infectious Diseases*, 8th ed.; Bennett, J.E., Dolin, R., Blaser, M.J., Eds.; W.B. Saunders: Philadelphia, PA, USA, 2015, pp. 263–277.

34. Strachan, S.A.; Friedland, I.R. Therapy for penicillin-resistant Streptococcus pneumoniae. *J. Med. Microbiol.* 1995, 43, 237–238. [CrossRef]

35. Jacobs, M.R. Worldwide trends in antimicrobial resistance among common respiratory tract pathogens in children. *Pediatr. Infect. Dis. J.* 2003, 22, S109–S119. [CrossRef]

36. Yokota, S.-i.; Ohkoshi, Y.; Sato, K.; Fuji, N. High prevalence of beta-lactam-resistant *Haemophilus influenzae* type b isolates derived from respiratory tract specimens in Japanese children. *Int. J. Infect. Dis.* 2009, 13, 584–588. [CrossRef]

37. Ubukata, K.; Chiba, N.; Morozumi, M.; Iwata, S.; Sunakawa, K. Longitudinal surveillance of *Haemophilus influenzae* isolates from pediatric patients with meningitis throughout Japan, 2000–2011. *J. Infect. Chemother.* 2013, 19, 34–41. [CrossRef]

38. Skaare, D.; Allum, A.G.; Anthonisen, I.L.; Jenkins, A.; Lia, A.; Strand, L.; Tveten, Y.; Kristiansen, B.E. Mutant ftsl genes in the emergence of penicillin-binding protein-mediated beta-lactam resistance in *Haemophilus influenzae* in Norway. *Clin. Microbiol. Infect.* 2010, 16, 1117–1124. [CrossRef]

39. Institute of Medical Research. National Surveillance of Antimicrobial Resistance, Malaysia. 2019. Available online: https://www.imr.gov.my/MyOHAR/index.php/site/archive_rpt (accessed on 10 February 2021).

40. Zarizal, S.; Yeo, C.C.; Faizal, G.M.; Chew, C.H.; Zakaria, Z.A.; Jamil Al-Obaidi, M.M.; Syafinaz Amin, N.; Mohd Nasir, M.D. Ceftepime, cefpirome, and cefaclidine binding affinities for penicillin-binding proteins in *Pseudomonas aeruginosa* and *Escherichia coli* K-12. *Antimicrob. Agents Chemother.* 2018, 62, e00768-17. [CrossRef]

41. CLSI. Performance standards for antimicrobial susceptibility testing: Twenty-fifth informational supplement. In *CLSI Document M100-S25*; CLSI: Wayne, PA, USA, 2015.

42. Richards, D.M.; Brogden, R.N. Ceftazidime. *Drugs* 1985, 29, 105–161. [CrossRef]

43. Brakstad, O.G.; Aasbakk, K.; Maeland, J.A. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the nuc gene. *J. Clin. Microbiol.* 1992, 30, 1654–1660. [CrossRef]

44. Song, K.H.; Jung, S.I.; Lee, S.; Park, S.; Kim, E.S.; Park, K.H.; Park, W.B.; Cheo, P.G.; Kim, Y.K.; Kwak, Y.G.; et al. Inoculum effect of high concentrations of methicillin-susceptible *Staphylococcus aureus* on the efficacy of cefazolin and other beta-lactams. *J. Infect. Chemother.* 2018, 24, 212–215. [CrossRef]

45. Saeki, M.; Shinagawa, M.; Yakuwa, Y.; Nirasawa, S.; Sato, Y.; Yanagihara, N.; Takahashi, S. Inoculum effect of high concentrations of methicillin-resistant *Staphylococcus aureus* induced by the M339F substitution in PBP2x from *Escherichia coli*. *J. Med. Microbiol.* 2015, 64, 44448–44456. [CrossRef]

46. Brys, R.; Asztalos, J.; Chantot, J.-F. Parenteral cephalosporin classification. *Expert Opin. Investig. Drugs* 1994, 3, 145–171. [CrossRef]

47. Arumugham, V.B.; Gujarathi, R.; Cascella, M. Third Generation Cephalosporins. In *StatPearls; StatPearls Publishing LLC.*: Treasure Island, FL, USA, 2021.

48. Hancock, R.E.; Bellido, F. Antibacterial in vitro activity of fourth generation cephalosporins. *J. Chemother.* 1996, 8 (Suppl. S2), 31–36.

49. Pucci, M.J.; Boice-Sowek, J.; Kessler, R.E.; Dougherty, T.J. Comparison of ceftepime, cefpirome, and cefaclidine binding affinities for penicillin-binding proteins in *Escherichia coli* K-12 and *Pseudomonas aeruginosa* SC8329. *Antimicrob. Agents Chemother.* 1991, 35, 2312–2317. [CrossRef]

50. Ministry of Health Malaysia. National Antimicrobial Guideline 2019. In *Programme PS.*; Malaysia Pharmaceutical Services Programme: Selangor, Malaysia, 2019.

51. Cui, L.; Li, Y.; Lv, Y.; Xue, F.; Liu, J. Antimicrobial resistance surveillance of flomoxef in China. *J. Infect. Chemother.* 2015, 21, 402–404. [CrossRef]

52. Yang, Q.; Zhang, H.; Cheng, J.; Xu, Z.; Hou, X.; Xu, Y. Flomoxef showed excellent in vitro activity against clinically important gram-positive and gram-negative pathogens causing community- and hospital-associated infections. *Diagn. Microbiol. Infect. Dis.* 2015, 81, 269–274. [CrossRef]

53. Takakura, M.; Fukuda, Y.; Nomura, N.; Mitsuayama, J.; Yamaoka, K.; Asano, Y.; Sawamura, H.; Katsuragawa, K.; Hashido, H.; Matsukawa, Y.; et al. Antibacterial susceptibility surveillance of *Haemophilus influenzae* isolated from pediatric patients in Gifu and Aichi prefectures (2009–2010). *Jpn. J. Antibiots.* 2012, 65, 305–321. [PubMed]

54. Choo, E.J.; Kwak, Y.G.; Lee, M.S.; Jeong, J.Y.; Choi, S.H.; Kim, N.J.; Kim, Y.S.; Woo, J.H.; Ryu, J. In vitro Antimicrobial Activity of Ceftaroline against Clinical Isolates. *Infect. Chemother.* 2005, 37, 133–137.
56. Kanegae, H.; Yamada, H.; Yamaguchi, T.; Kuroki, S.; Katoh, O. Clinical studies on flomoxef in respiratory tract infections. Jpn. J. Antibiot. 1987, 40, 1803–1808. [PubMed]

57. Sato, H.; Narita, A.; Nakazawa, S.; Suzuki, H.; Mastumoto, K.; Nakanishi, Y.; Nakazawa, S.; Niino, K.; Nakada, Y. The study of flomoxef in the pediatric field. Jpn. J. Antibiot. 1987, 40, 1349–1363. [PubMed]

58. Saito, A.; Hiraga, Y.; Watanabe, A.; Saito, A.; Shimada, K.; Kobayashi, H.; Odagiri, S.; Miki, F.; Soejima, R.; Oizumi, K.; et al. Comparative clinical study of cefcapene pivoxil and cefteram pivoxil in chronic respiratory tract infections by a double-blind method. J. Int. Med. Res. 2004, 32, 590–607. [CrossRef]

59. Wang, H.L.; Huo, L.; Wang, Z.S.; Zhao, J.Y.; Xue, X.; Ge, G.Z.; Ren, J.L.; Xia, T.; Han, X.W.; Yue, H.M.; et al. Multicenter, double-blind, randomized controlled clinical trial of cefcapene pivoxil hydrochloride tablets in the treatment of acute bacterial infections. Chin. J. Clin. Pharmacol. 2012, 1, R978.11.

60. Enright, M.C.; Spratt, B.G. A multilocus sequence typing scheme for Streptococcus pneumoniae: Identification of clones associated with serious invasive disease. Microbiology 1998, 144 (Pt 11), 3049–3060. [CrossRef]

61. Torigoe, H.; Seki, M.; Yamashita, Y.; Sugaya, A.; Maeno, M. Detection of Haemophilus influenzae by loop-mediated isothermal amplification (LAMP) of the outer membrane protein P6 gene. Jpn. J. Infect. Dis. 2007, 60, 55–58. [PubMed]

62. Dias, C.A.; Teixeira, L.M.; Carvalho, M.D.G.; Beall, B. Sequential multiplex PCR for determining capsular serotypes of pneumococci recovered from Brazilian children. J. Med. Microbiol. 2007, 56, 1185–1188. [CrossRef] [PubMed]

63. Zhou, X.; Liu, J.; Zhang, Z.; Liu, Y.; Wang, Y.; Liu, Y. Molecular characteristics of penicillin-binding protein 2b, 2x and 1a sequences in Streptococcus pneumoniae isolates causing invasive diseases among children in Northeast China. Eur. J. Clin. Microbiol. Infect. Dis. 2016, 35, 633–645. [CrossRef] [PubMed]

64. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol. Biol. Evol. 2018, 35, 1547–1549. [CrossRef] [PubMed]

65. Tenover, F.C.; Huang, M.B.; Rasheed, J.K.; Persing, D.H. Development of PCR assays to detect ampicillin resistance genes in cerebrospinal fluid samples containing Haemophilus influenzae. J. Clin. Microbiol. 1994, 32, 2729–2737. [CrossRef] [PubMed]