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# β-lactamase characterization of baseline Gram-negative pathogens from a Phase 3 trial of ceftazidime-avibactam (CAZ-AVI) for the treatment of nosocomial pneumonia

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## Amended Abstract

**Background:** The efficacy and safety of ceftazidime-avibactam was assessed for treating adult patients with hospital-acquired pneumonia (HAP), including ventilator-associated pneumonia (VAP), in a randomised, multi-centre, double-blind, double-dummy, parallel-group Phase 3 trial (REPROVE; NCT01808092). Here, the β-lactamase content of select Gram-negative isolates recovered from patients enrolled in this Phase 3 trial was characterized.

**Methods:** Of the total 817 randomised patients, 209 patients from 16 countries had baseline isolates that met the MIC screening criteria for β-lactamase content determination. Selected isolates were screened for extended-spectrum β-lactamase (ESBL), oxacillinase, class C β-lactamase (plasmid AmpC; pAmpC), and carbapenemase genes. Isolates underwent microarray-based assay complemented by PCR/sequencing. Relative transcription levels of chromosomal AmpC (cAmpC) were assessed by RT-PCR, and *Pseudomonas aeruginosa* isolates underwent OprD protein and efflux-pump gene expression analysis. Isolates from China were only evaluated by *in silico* DNA sequence analysis.

**Results:** A total of 235 Gram-negative isolates recovered from the randomised patient populations met the screening criteria. This total included 26 patients with 2 baseline isolates each that met the screening criteria. *Enterobacteriaceae* (9 species) comprised 69.8% of the isolates (mostly *Klebsiella pneumoniae* [36.2%], *Escherichia coli* [11.1%], and *Enterobacter cloacae* [10.2%]) followed by *Pseudomonas* spp. (30.2%). A total of 47.6% (78/164) of *Enterobacteriaceae* isolates harboured at least 2 β-lactamase-encoding genes. Most *Enterobacteriaceae* (63.4%; 104/164) carried $bla_{CTX-M}$ alone or in combination with other ESBL/pAmpC/carbapenemases and/or narrow-spectrum enzymes. The CTX-M-encoding genes were predominantly from group 1 (69.2%; 72/104) or group 9 (29.8%; 31/104). Carbapenemase-encoding genes (4 $bla_{KPC-2}$, 2 $bla_{NDM-1}$, 2 $bla_{NDM-5}$, 2$bla_{IMP-4}$, and 1 $bla_{OXA-48}$) were noted in 6.7% (11/164) of *Enterobacteriaceae*, and these isolates were from China (8), India (2), and Romania (1). *Enterobacter* spp. often overexpressed cAmpC (43.8%; 14/32). *P. aeruginosa* (outside China) resulted in 32.4% (11/34) showing decreased expression of OprD and 11.8% (4/34) showing overexpression of cAmpC in the absence of any other examined resistance mechanism; however, 38.2% (13/34) exhibited OprD loss or cAmpC overexpression in combination with various other resistance mechanisms such as efflux over-expression or presence of other β-lactamase genes, including $bla_{VIM-1}$ (1 isolate from Ukraine). One isolate from Russia had $bla_{VIM-2}$, and 1 isolate from the Czech Republic had $bla_{PSE}$. Resistance mechanisms were not detected in 4 *Pseudomonas* spp. isolates. Only 16.2% (6/37) of the *Pseudomonas* spp. isolates from China had β-lactamase genes ($bla_{CTX-M-3}$ [1], $bla_{PSE}$ [1], $bla_{IMP-25}$ [2], and $bla_{PER-1}$ plus $bla_{OXA-246}$ [2]). The remaining *P. aeruginosa* isolates (83.8%; 31/37) presumably had intrinsic resistance mechanisms that were not analysed further.

**Conclusions:** CTX-M, mainly group 1 enzymes, prevailed among HAP/VAP *Enterobacteriaceae* isolates that met the screening criteria. Carbapenemase-producing *Enterobacteriaceae* were concentrated in the Asia-Pacific region. *Pseudomonas* spp. organisms rarely carried β-lactamase-encoding genes investigated in this study.

## Introduction

- A Phase 3, randomised, multi-centre, double-blind, double-dummy, parallel-group, comparative trial to determine the efficacy, safety, and tolerability of ceftazidime-avibactam administered intravenously as a 2-hour infusion (2 g-0.5 g, every 8 hours) compared to meropenem administered intravenously as a 30-minute infusion (1 g every 8 hours) was conducted (REPROVE; NCT01808092)
- The study presented here reports the characterisation of β-lactamase content among baseline *Enterobacteriaceae* and *Pseudomonas aeruginosa* isolates that met the predefined MIC criteria for extended-spectrum β-lactamase (ESBL) and/or carbapenemase production. The transcriptional levels of intrinsic AmpC and efflux pumps were also measured in selected isolates

## Materials and Methods

### *Patients and clinical isolates*

- In total, 817 patients were randomized into the Phase 3 study
- Randomised patients were from 23 countries, and 209 patients from 16 countries had baseline isolates that met the MIC screening criteria for β-lactamase content determination and were included in this study (Table 1)

### *Antimicrobial susceptibility testing and MIC screening criteria*

- Clinical isolate susceptibility testing was centrally performed using the broth microdilution method and following the Clinical Laboratory Standard Institute (CLSI) guidelines[1, 2]
- Ceftazidime was tested in combination with avibactam at a fixed concentration of 4 mg/L
- Ceftazidime-avibactam (≤8 mg/L for susceptible and ≥16 mg/L for resistant) and comparator breakpoints published by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were applied for all *Enterobacteriaceae* and *P. aeruginosa*[3, 4]
- *Enterobacteriaceae* displaying ceftriaxone and/or ceftazidime MIC results of ≥2 mg/L and *P. aeruginosa* with ceftazidime MIC results of ≥16 mg/L were selected for further characterisation of narrow and ESBL genes and for *ampC* expression[5]
- *Enterobacteriaceae* isolates exhibiting imipenem/meropenem MIC results ≥2 mg/L were tested for the presence of carbapenemase-encoding genes. *P. aeruginosa* isolates exhibiting imipenem/meropenem MIC results ≥8 mg/L were tested for the presence of carbapenemase-encoding genes, transcription levels of the efflux pump genes, and OprD analysis[5]

### *Screening of β-lactamase and AmpC expression*

- Isolates that met the MIC screening criteria were subjected to a microarray-based assay Check-MDR CT101 kit according to the manufacturer's instructions (Check-Points, Wageningen, Netherlands), which is capable of detecting CTX-M groups 1, 2, 8+25 and 9, TEM, SHV, ACC, ACT/MIR, CMY, DHA, FOX, KPC, and NDM encoding genes[5]
- Supplemental multiplex PCR assays were used to detect additional ESBL- ($bla_{GES}$, $bla_{VEB}$, $bla_{PER}$), and oxacillinase enzymes ($bla_{OXA-2}$-, $bla_{OXA-10}$-, and $bla_{OXA-13}$-groups, $bla_{OXA-18}$, and $bla_{OXA-45}$), and carbapenemase-encoding genes ($bla_{IMP}$, $bla_{VIM}$, $blav_{NDM-1}$, $bla_{OXA-48}$, $bla_{GES}$, $bla_{NMC-A}$, $bla_{SME}$, $bla_{IMI}$)[5]
- All amplicons generated were sequenced on both strands (Sanger method); nucleotide and amino acid sequences were analysed using the Lasergene software package (DNASTAR, Madison, WI)
- The transcription levels of the chromosomal *ampC* and efflux pumps (MexAB, MexCD, MexEF, and MexXY) genes were determined using quantitative real-time PCR assays (qRT-PCR)
- During the quantification process for the target mRNA gene, a normalized expression analysis method was applied and relative comparison to susceptible control strains was completed
- A given strain was considered to overexpress *ampC* or efflux pump genes when at least a 5-fold greater difference of transcripts was detected as compared with a species-specific wild-type reference control strain
- Isolates from China were only evaluated by *in silico* whole genome DNA sequence analysis

**Table 1.** Baselines isolates meeting MIC screening criteria

| Species/Region | Number of isolates |
|---|---|
| *Citrobacter freundii* | 1 |
| *Citrobacter koseri* | 1 |
| *Enterobacter aerogenes* | 8 |
| *Enterobacter cloacae* | 24 |
| *Escherichia coli* | 26 |
| *Klebsiella pneumoniae* | 85 |
| *Morganella morganii* | 1 |
| *Proteus mirabilis* | 8 |
| *Serratia marcescens* | 10 |
| *Pseudomonas aeruginosa* | 71 |
| **Total** | 235 |
| Region/Country of origin | |
| Latin America | |
| Brazil | 12 |
| Mexico | 2 |
| Europe | |
| Bulgaria | 1 |
| Czech Republic | 56 |
| France | 6 |
| Hungary | 3 |
| Poland | 2 |
| Romania | 2 |
| Russia | 9 |
| Ukraine | 14 |
| Asia-Pacific | |
| China | 97 |
| India | 6 |
| Japan | 10 |
| Philippines | 7 |
| Korea | 5 |
| Vietnam | 3 |
| **Total** | 235 |

## Results

- Among the 235 Gram-negative total isolates recovered from randomised patients that met the screening criteria, 26 patients each had 2 baseline isolates of different species
- *Enterobacteriaceae* (9 species) comprised 69.8% of the isolates (mostly *Klebsiella pneumoniae* [36.2%], *Escherichia coli* [11.1%], and *Enterobacter cloacae* [10.2%]), followed by *P. aeruginosa* (30.2%) (Table 1)
- Ceftazidime-avibactam and meropenem inhibited growth of 95.1% (156/164) and 92.7% (152/164) of *Enterobacteriaceae*, respectively, at their susceptibility breakpoints (Table 2)
- Ceftazidime tested alone was not active (14.6% susceptible) against this collection of *Enterobacteriaceae*
- A total of 47.6% (78/164) of *Enterobacteriaceae* isolates harboured at least 2 β-lactamase-encoding genes
- Most *Enterobacteriaceae* (63.4%; 104/164) carried $bla_{CTX-M}$ alone or in combination with other ESBL/pAmpC/carbapenemases and/or narrow-spectrum enzymes
- The CTX-M-encoding genes were predominantly from group 1 (CTX-M-1/3/15/32-like: 69.2%; 72/104) or group 9 (CTX-M-9-like: 29.8%; 31/104) (Table 3)
- *Enterobacter* spp. often overexpressed cAmpC (43.8%; 14/32)
- Carbapenemase-encoding genes (4 $bla_{KPC-2}$, 2 $bla_{NDM-1}$, 2 $bla_{NDM-5}$, 2 $bla_{IMP-4}$, and 1 $bla_{OXA-48}$) were noted in 6.7% (11/164) of *Enterobacteriaceae*, and these isolates were from China (8), India (2), and Romania (1) (Table 3)
- A total of 32.4% (11/34) and 11.8% (4/34) of *P. aeruginosa.* (outside China) showed decreased expression of OprD or overexpression of cAmpC, respectively, in the absence of any other resistance mechanism (Table 3)

**Table 3.** Summary of β-lactamase enzymes detected among baseline *Enterobacteriaceae* pathogens recovered from patients in the nosocomial pneumonia Phase 3 trials for ceftazidime-avibactam

| Pathogen (No; % of all Enterobacteriaceae) | Results[a] | No. of isolates | Pathogen (No; % of all Enterobacteriaceae) | Results[a] | No. of isolates |
|---|---|---|---|---|---|
| *K. pneumoniae* (85; 51.8) | CTX-M-14 | 2 | *Enterobacter* spp. (32; 19.5) | cAmpC | 12 |
| | CTX-M-14, DHA-1, SHV-27 | 1 | | cAmpC, OXA-1 | 1 |
| | CTX-M-14, SHV-12, OXA-1 | 1 | | CTX-M-3 | 3 |
| | CTX-M-15 | 6 | | CTX-M-14 | 1 |
| | CTX-M-15, OXA-1 | 25 | | CTX-M-15, OXA-1, cAmpC | 1 |
| | CTX-M-15, OXA-1, SHV-107 | 4 | | CTX-M-15, OXA-1 | 4 |
| | CTX-M-15, OXA-1, SHV-12 | 1 | | IMP-4, DHA-1, SHV-12 | 1 |
| | CTX-M-15, OXA-1, SHV-38 | 1 | | OXA-1 | 1 |
| | CTX-M-15, SHV-187, OXA-1 | 1 | | SHV-12 | 1 |
| | CTX-M-15, SHV-31, OXA-1 | 1 | | Unknown | 7 |
| | CTX-M-27, DHA-1, SHV-31 | 1 | *E. coli* (26; 15.9) | CMY-42 | 1 |
| | CTX-M-3 | 3 | | CMY-42, DHA-1 | 1 |
| | CTX-M-3, OXA-1 | 2 | | CTX-M-3, SHV-2 | 1 |
| | CTX-M-3, SHV-28 | 2 | | CTX-M-14 | 2 |
| | CTX-M-55/57 | 1 | | CTX-M-15, DHA-1 | 1 |
| | CTX-M-65 | 1 | | CTX-M-15, OXA-1 | 9 |
| | CTX-M-9 | 2 | | CTX-M-24 | 1 |
| | CTX-M-9, SHV-5 | 1 | | CTX-M-27 | 6 |
| | DHA-1, OXA-1 | 3 | | CTX-M-55/57 | 1 |
| | DHA-1, OXA-1, SHV-33 | 4 | | CTX-M-65 | 1 |
| | DHA-1, SHV-12 | 1 | | CTX-M-104 | 1 |
| | IMP-4 | 1 | | NDM-5, CMY-42 | 1 |
| | KPC-2 | 1 | *P. mirabilis* (8; 4.9) | CMY-2 | 2 |
| | KPC-2, CTX-M-15, SHV-28, OXA-1 | 1 | | CTX-M-14 | 1 |
| | KPC-2, CTX-M-65 | 1 | | CTX-M-14, CMY-2 | 1 |
| | KPC-2, SHV-12 | 1 | | CTX-M-14, DHA-1, SHV-12 | 1 |
| | NDM-1, CTX-M-15 | 1 | | CTX-M-44, OXA-2 | 1 |
| | NDM-1, CTX-M-3, SHV-12 | 1 | | TEM-2 | 1 |
| | NDM-5, SHV-108 | 1 | | Unknown | 1 |
| | OXA-1 | 1 | *Morganella* spp. (1; 0.6) | CTX-M-14 | 1 |
| | OXA-48, CTX-M-15 | 1 | *Citrobacter* spp. (2; 1.2) | CTX-M-1 | 1 |
| | LAP-2 | 1 | | cAmpC | 1 |
| | SHV-12 | 3 | *S. marcescens* (10; 6.1) | cAmpC | 1 |
| | SHV-26 | 1 | | CTX-M-14 | 4 |
| | SHV-5 | 1 | | CTX-M-14, CMY-2 | 1 |
| | SHV-71 | 1 | | PSE-4 | 1 |
| | Unknown | 4 | | Unknown | 3 |

[a] Known non-ESBL enzymes are not reported here; cAmpC, over-expression of chromosomal AmpC; Unknown, screened β-lactamases were not detected (isolates from China were only subjected to *in silico* DNA sequence analysis i.e. transcription levels of *ampC*, efflux pump genes, and OprD analysis were not performed)

- A total of 38.2% (13/34) exhibited OprD loss and cAmpC overexpression or OprD loss or cAmpC overexpression in combination with various other resistance mechanisms (Table 3)
- Five IMP- and 2 VIM-producing *P. aeruginosa* were detected (Table 4)
- These isolates were recovered from Ukraine ($bla_{VIM-1}$ and $bla_{IMP-1}$), China ($bla_{IMP-25}$), Vietnam ($bla_{IMP-26}$), Mexico ($bla_{IMP-56}$) and Russia ($bla_{VIM-2}$)
- Other ESBL-encoding genes detected in *P. aeruginosa* were $bla_{CTX-M-3}$ (1), $bla_{PSE}$ (2), and $bla_{PER-1}$ plus $bla_{OXA-246}$ (2) (Table 4)
- Only 16.2% (6/37) of the *P. aeruginosa* isolates from China had acquired β-lactamase genes (Table 4), the remaining isolates (83.8%; 31/37) from China presumably had intrinsic resistance mechanisms that were not analysed further

**Table 4.** Summary of β-lactamase enzymes and resistance mechanisms detected among baseline *P. aeruginosa* recovered from patients enrolled in the nosocomial pneumonia Phase 3 trials for ceftazidime-avibactam

| Pathogen (no; % of all isolates) | Results[a] | No. of isolates |
|---|---|---|
| *P. aeruginosa* (71; 30.2) | cAmpC | 4 |
| | cAmpC, MexCD, OXA-2 | 1 |
| | cAmpC, MexXY | 2 |
| | cAmpC, PSE-1 | 1 |
| | cAmpC, OprD loss | 3 |
| | cAmpC, OprD loss, MexXY | 1 |
| | CTX-M-3 | 1 |
| | IMP-1, OprD loss | 1 |
| | IMP-25 | 1 |
| | IMP-25, OXA-1 | 1 |
| | IMP-26, OprD loss | 1 |
| | IMP-56, cAmpC | 1 |
| | OprD loss | 11 |
| | OprD loss, MexAB, MexXY | 1 |
| | PER-1, OXA-246 | 2 |
| | PSE-1 | 2 |
| | VIM-1, cAmpC | 1 |
| | VIM-2 | 1 |
| | Unknown | 35 |

[a] Known non-ESBL enzymes are not depicted here; cAmpC or Mex indicates over-expression of the corresponding gene(s) was detected; Unknown, screened β-lactamases were not detected (isolates from China were only subjected to *in silico* DNA sequence analysis i.e. transcription levels of *ampC*, efflux pump genes, and OprD analysis were not performed)

## Conclusions

- Ceftazidime-avibactam was highly potent, and 95.1% of all *Enterobacteriaceae* isolates that met the MIC screening criteria were susceptible to ceftazidime-avibactam based on current interpretive criteria
- CTX-M, mainly group 1 enzymes, prevailed among HAP/VAP *Enterobacteriaceae* isolates that met the MIC screening criteria
- Carbapenemase-producing *Enterobacteriaceae* were concentrated in the Asia-Western Pacific region
- *P. aeruginosa* organisms rarely carried acquired β-lactamase-encoding genes investigated in this study and resistance phenotypes are presumably conferred by permeability issues (efflux pumps and/or porins)

**Table 2.** MIC results for ceftazidime, ceftazidime-avibactam, and meropenem obtained against ex-China pathogens responsible for nosocomial pneumonia in Phase 3 trials for ceftazidime-avibactam that met the MIC screening criteria for molecular characterization

| | | Number of isolate at each MIC (mg/L) and cumulative % | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Organism** | **Agent[a]** | **≤0.06** | **0.12** | **0.25** | **0.5** | **1** | **2** | **4** | **8** | **16** | **32** | **64** | **128** | **256** | **>256** |
| *Enterobacteriaceae* (164)[b] | CAZ | 2 (1.2) | 3 (3.0) | 8 (7.9) | 4 (10.4) | 7 (14.6) | 10 (20.7) | 14 (29.3) | 16 (39.0) | 9 (44.5) | 19 (56.1) | 24 (70.7) | 48 (100.0)[d] | | |
| | CAZ-AVI | 18 (11.0) | 43 (37.2) | 37 (59.8) | 34 (80.5) | 12 (87.8) | 7 (92.1) | 4 (94.5) | 1 (95.1) | 1 (95.7) | 1 (96.3) | 0 (96.3) | 0 (96.3) | 0 (96.3) | 6 (100.0)[e] |
| | MER | 128 (78.0) | 16 (87.8) | 5 (90.9) | 2 (92.1) | 1 (92.7) | 0 (92.7) | 1 (93.3) | 1 (93.9) | 10 (100.0)[d] | | | | | |
| *E. coli* (26) | CAZ | | | | 1 (3.8) | 1 (7.7) | 5 (26.9) | 1 (30.8) | 5 (50.0) | 2 (57.7) | 2 (65.4) | 5 (84.6) | 4 (100.0)[d] | | |
| | CAZ-AVI | 5 (19.2) | 11 (61.5) | 4 (76.9) | 3 (88.5) | 0 (88.5) | 1 (92.3) | 0 (92.3) | 0 (92.3) | 0 (92.3) | 1 (96.2) | 0 (96.2) | 0 (96.2) | 0 (96.2) | 1 (100.0) |
| | MER | 24 (92.3) | 1 (96.2) | 0 (96.2) | 0 (96.2) | 0 (96.2) | 0 (96.2) | 0 (96.2) | 0 (96.2) | 1 (100.0)[d] | | | | | |
| *Enterobacter spp.* (32) | CAZ | | | 2 (6.3) | 0 (6.3) | 1 (9.4) | 1 (12.5) | 3 (21.9) | 4 (34.4) | 1 (37.5) | 4 (50.0) | 6 (68.8) | 10 (100.0)[d] | | |
| | CAZ-AVI | 1 (3.1) | 4 (15.6) | 13 (56.3) | 10 (87.5) | 3 (96.9) | 0 (96.9) | 0 (96.9) | 0 (96.9) | 0 (96.9) | 0 (96.9) | 0 (96.9) | 0 (96.9) | 0 (96.9) | 1 (100.0) |
| | MER | 28 (87.5) | 2 (93.8) | 0 (93.8) | 1 (96.9) | 0 (96.9) | 0 (96.9) | 0 (96.9) | 0 (96.9) | 1 (100.0)[d] | | | | | |
| *K. pneumoniae* (85) | CAZ | | 2 (2.4) | 3 (5.9) | 2 (8.2) | 1 (9.4) | 1 (10.6) | 6 (17.6) | 6 (24.7) | 6 (31.8) | 13 (47.1) | 13 (62.4) | 32 (100.0)[d] | | |
| | CAZ-AVI | 7 (8.2) | 22 (34.1) | 16 (52.9) | 17 (72.9) | 9 (83.5) | 6 (90.6) | 3 (94.1) | 1 (95.3) | 0 (95.3) | 0 (95.3) | 0 (95.3) | 0 (95.3) | 0 (95.3) | 4 (100.0) |
| | MER | 66 (77.6) | 6 (84.7) | 2 (87.1) | 1 (88.2) | 1 (89.4) | 0 (89.4) | 1 (90.6) | 1 (91.8) | 7 (100.0)[d] | | | | | |
| Other (21)[c] | CAZ | 2 (9.5) | 1 (14.3) | 3 (28.6) | 1 (33.3) | 4 (52.4) | 3 (66.7) | 4 (85.7) | 1 (90.5) | 0 (90.5) | 0 (90.5) | 0 (90.5) | 2 (100.0)[d] | | |
| | CAZ-AVI | 5 (23.8) | 6 (52.4) | 4 (71.4) | 4 (90.5) | 0 (90.5) | 0 (90.5) | 1 (95.2) | 0 (95.2) | 1 (100.0) | | | | | |
| | MER | 10 (47.6) | 7 (81.0) | 3 (95.2) | 0 (95.2) | 0 (95.2) | 0 (95.2) | 0 (95.2) | 0 (95.2) | 1 (100.0)[d] | | | | | |
| *P. aeruginosa* (71) | CAZ | | | | | 3 (4.2) | 10 (18.3) | 6 (26.8) | 10 (40.8) | 6 (49.3) | 10 (63.4) | 12 (80.3) | 14 (100.0) | | |
| | CAZ-AVI | | | | 1 (1.4) | 5 (8.5) | 18 (33.8) | 17 (57.7) | 14 (77.5) | 3 (81.7) | 5 (88.7) | 2 (91.5) | 0 (91.5) | 2 (94.4) | 4 (100.0) |
| | MER | 1 (1.4) | 3 (5.6) | 6 (14.1) | 4 (19.7) | 4 (25.4) | 6 (33.8) | 16 (56.3) | 5 (63.4) | 26 (100.0)[d] | | | | | |

[a] CAZ, ceftazidime; CAZ-AVI, ceftazidime-avibactam; MER, meropenem; [b] Includes 26 *E. coli*, 32 *Enterobacter* spp., 85 *K. pneumoniae*, 8 *P. mirabilis*, 2 *Citrobacter* spp., 10 *S. marcescens*, and 1 *M. morganii*; [c] Includes 8 *P. mirabilis*, 2 *Citrobacter* spp., 10 *S. marcescens*, and 1 *M. morganii*; [d] Represents MIC values of >64 mg/L for CAZ or >8 mg/L for MER; [e] Two NDM (NDM-1 and NDM-2) isolates from India and 2 IMP-4 and 2 NDM (NDM-1 and NDM-5) isolates from China

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