Emergence of mcr-3 carrying Escherichia coli in Diseased Pigs in South Korea

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Abstract: We examined the prevalence and molecular characteristics of mcr-3 carrying colistin-resistant Escherichia coli among cattle, pig, and chicken isolates in South Korea. Among a total of 185 colistin-resistant E. coli isolates determined in this study (47 from cattle, 90 from pigs, and 48 from chicken), PCR amplification detected mcr-3 genes in 17 isolates predominantly from diseased pigs. The mcr-3 genes were characterized as mcr-3.1 in 15 isolates and mcr-3.5 in 2 isolates. The mcr-3 gene was transferred to the E. coli J53 recipient strain from more than 50% of the mcr-3-carrying isolates. The mcr-3.1 and mcr-3.5 genes were identified predominantly in IncHI2 and IncP plasmids, respectively. Multi-locus sequence typing analysis revealed eight previously reported sequence types (ST), including ST1, ST10, and ST42. We identified isolates with similar pulsed-field gel electrophoresis patterns from diseased pigs in three farms. Besides, the isolates carried various virulence factors and demonstrated resistance to multiple antimicrobials, including β-lactams and quinolones. Further, the mcr-3.5 encodes three amino acid substitutions compared with mcr-3.1. To the best of our knowledge, this is the first report of pathogenic E. coli carrying mcr-3.5 in South Korea, which implies that mcr-3 variants may have already been widely spread in the pig industry.

Keywords: colistin; Escherichia coli; mcr-3 gene; plasmid; resistance

1. Introduction

Colistin is considered one of the last-resort antimicrobial agents against multi-drug resistant Gram-negative bacterial infections. The emergence of mcr-harboring colistin-resistant Escherichia coli presented a serious public health risk. Among the ten mcr genes identified so far i.e., mcr-1 up to mcr-10, the mcr-3 genes have been distributed worldwide [1]. Recent studies in South Korea (Korea), identified mcr-3-carrying E. coli isolates from food-producing animals [2,3]. However, both studies investigated limited numbers of isolates from specific provinces. Besides, although the mcr-3 gene is subjected to constant evolution due to the impacts of unknown selective pressure in the environment, animals, and humans [4], no attempt has been made so far to determine the mcr-3 variants in bacteria isolated in Korea. Consequently, we undertook this study to provide new knowledge on the prevalence and molecular characteristics of mcr-3 variants in E. coli isolated from food-producing animals throughout Korea between 2005 and 2018.
2. Materials and Methods

2.1. Identification of Colistin-Resistant E. coli

*E. coli* isolates were recovered from healthy and diseased animals (i.e., cattle, chicken, and pigs), and their carcasses during a nationwide surveillance study on antimicrobial susceptibility conducted between 2005 and 2018. The minimum inhibitory concentration (MIC) of colistin was determined by the broth microdilution method [5] in KRNV5F Sensititre Panel following the manufacturer’s instruction (Trek Diagnostic Systems, Waltham, MA, USA). The MIC values were interpreted according to the EUCAST breakpoint (>2 µg/mL). PCR amplification was performed to investigate the mcr-3 gene carriage of isolates exhibiting colistin resistance using primer pairs and PCR conditions described previously [6].

2.2. Conjugation Assay

Conjugation was performed using a filter mating method with azide-resistant *E. coli* J53 as the recipient strain [7]. The transconjugants were confirmed by PCR detection of the mcr-3 genes and were investigated for their MICs as described above.

2.3. Molecular Characterization of mcr-3 carrying E. coli

A PCR-based replicon typing kit (Diatheva, Fano, Italy) and a multiplex PCR assay [8] were used to identify the plasmid replicon types and virulence factor genes, respectively. Pulsed-field gel electrophoresis of mcr-3 positive isolates was conducted using genomic DNA prepared in agarose blocks, digested with XbaI enzyme (TaKaRa, Shiga, Japan), as described previously [9]. The banding profiles were analyzed using Bionumerics software and the genetic relatedness of the isolates was calculated using the unweighted pair-group method. Besides, molecular typing of mcr-3 carrying isolates was carried out according to the protocols specified at the *E. coli* multilocus sequence typing website [10].

2.4. Whole-Genome Sequencing

Whole-genome sequencing was conducted to investigate the immediate genetic environment and amino acid sequences of mcr-3 genes (PacBio RSII platform, Pacific Biosciences, Menlo Park, CA, USA). Complete sequences of the chromosomes and plasmids of strain V01-E02-025, V01-E02-51, and V01-R02-053 have been deposited into GenBank under the accession no. (CP049943, CP049944), (CP049299, CP049300), and (CP049086, CP049087), respectively.

3. Results and discussion

A total of 14,631 *E. coli* isolates were obtained from healthy and diseased animals (i.e., cattle, chicken, and pigs), and their carcasses during 2005–2018 (Table 1). The overall prevalence of colistin-resistant *E. coli* was less than 5%. In our previous study [11], colistin resistance was identified only in 1.3% of isolates recovered from food-producing animals between 2005 and 2015. This study demonstrated that the colistin resistance rate was maintained below 2% for three consecutive years after 2015. Indeed, the prevalence of colistin-resistant isolates in this study was consistent with previous reports in Poland [12] but lower than other reports from Japan (48%) [13], China (42%) [14], and Cambodia (20%) [1].

Among a total of 185 colistin-resistant *E. coli* isolates determined in this study (47 from cattle, 90 from pigs, and 48 from chicken), PCR amplification detected mcr-3 genes in 17 isolates: 2 from healthy pigs, 1 from a pig carcass, and 14 from diseased pigs (Table 1). mcr-1 was the major colistin resistance determinant in *E. Couple* isolated from livestock, especially chickens in Korea since 2013 [11]. However, this study exhibited the emergence of mcr-3 in livestock, especially in pigs since 2011. Notably, the majority of the mcr-3 carrying isolates from diseased pigs were found between 2014 and 2018, highlighting a recent rise in prevalence compared to previous years. Recent studies reported a 38% [1] and 13% [15] prevalence of mcr-3 among colistin-resistant pig isolates in Cambodia and Brazil,
respectively. In addition, Fukuda et al. [13] identified the \textit{mcr}-3 gene in 8% of \textit{E. coli} isolated from diseased pigs in Japan. \textit{mcr}-3-carrying plasmids can stably persist by lowering fitness cost [16], suggesting careful monitoring of the \textit{mcr}-3 gene in Korean livestock.
Table 1. Prevalence of mcr-3 in colistin-resistant *Escherichia coli* obtained from food-producing animals and animal carcasses from Korea.

| Year (No. of Isolates) | Prevalence (%) of mcr-3 Gene (no. of mcr-3 Positive Isolates/no. of Colistin-Resistant Isolates) | Cattle (n = 47) | Pigs (n = 90) | Chicken (n = 48) | Total (n = 185) |
|------------------------|-----------------------------------------------------------------------------------------------|----------------|--------------|-----------------|----------------|
|                        |                                                                                             | Healthy        | Diseased     | Healthy         | Diseased       | Healthy         | Diseased     | Healthy         | Diseased       | Healthy         | Diseased       |                  |
| 2005 (n = 693)          |                                                                                             | 0 (0/1)        | 0 (0/0)      | 0 (0/0)        | 0 (0/1)        | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/1)        |
| 2006 (n = 744)          |                                                                                             | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        |
| 2007 (n = 744)          |                                                                                             | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        |
| 2008 (n = 559)          |                                                                                             | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        |
| 2009 (n = 641)          |                                                                                             | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        |
| 2010 (n = 1101)         |                                                                                             | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        |
| 2011 (n = 1276)         |                                                                                             | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        |
| 2012 (n = 1242)         |                                                                                             | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        |
| 2013 (n = 1078)         |                                                                                             | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        |
| 2014 (n = 1329)         |                                                                                             | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        |
| 2015 (n = 1169)         |                                                                                             | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        |
| 2016 (n = 1794)         |                                                                                             | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        |
| 2017 (n = 1218)         |                                                                                             | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        |
| 2018 (n = 1043)         |                                                                                             | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)      | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        | 0 (0/0)        |
| Total                  |                                                                                             | 0 (0/43)       | 0 (0/2)      | 0 (0/2)        | 0 (0/47)       | 3.9 (2/51)     | 12.5 (1/8)   | 45.2 (14/31)   | 18.9 (17/90)   | 0 (0/22)       | 0 (0/12)       | 0 (0/14)       | 0 (0/48)       | 9.2 (17/185)   |
The mcr-3 genes were characterized as mcr-3.1 in 15 isolates and mcr-3.5 in 2 isolates (Table 1). Both of the mcr-3.5 isolates were identified in 2018. To the best of our knowledge, this is the first report of mcr-3.5-carrying E. coli in Korea, while these genes have been identified in E. coli from pigs and other sources in Europe and other Asian countries [17–19]. The mcr-3 gene was transferred to E. coli J53 recipient strain from 53% of the mcr-3-carrying isolates as indicated by filter mating assay (Table 2), which is lower than Belayneh et al. [2]. Agreeing with Zurfluh et al. [19], all mcr-3-carrying isolates were multi-drug resistant (MDR). Notably, the two mcr-3.5-carrying isolates from diseased pigs were resistant to ceftriaxone. In addition, MDR in five mcr-3-carrying isolates was transferred to a recipient E. coli. Although we did not investigate other antimicrobial resistance genes, the coexistence of multiple resistant genes in the same or different plasmids could confer resistance to a broad range of antimicrobials [20].

PCR analysis presented five replicon types (IncP1, HI2, II-α, IncM, and IncN). The mcr-3.1 gene was identified predominantly in the IncHI2 plasmid, which is associated with the spread of MDR, including β-lactams and quinolones [14,21]. Agreeing with Li et al. [16], the mcr-3.5 genes belonged to IncP plasmid. The less frequent plasmid replicon types in our study, such as IncI1-α, IncM, IncN, and IncP, were reported to co-harbor genes resistant to aminoglycosides, β-lactams, quinolones, and tetracyclines in Enterobacteriaceae [17,21,22].

Multi-locus sequence typing analysis revealed eight previously reported ST types: four ST1s, four ST10s, two ST42s, two ST641s, and each of ST29, ST101, ST3523, and ST4532 (Table 2). E. coli ST1, ST42, and ST641 isolates from diseased pigs of three farms showed similar patterns in the pulse-field gel electrophoresis results (Figure S1). Besides, ST1 and ST10 isolates carrying mcr-3.1 gene were identified from farms located in different provinces. ST1, ST10, ST101, and ST641 E. coli isolates have already been identified in food-producing animals in several countries [11,12,15], suggesting its widespread distribution. Thus, a combination of clonal expansion and dissemination of plasmids carrying mcr-3 variants contributed to the rise in the prevalence of mcr-3 carrying E. coli.

We identified a total of 13 different virulence factor genes, with up to 5 of those in a single isolate (Table 2). The predominant virulence factors include the fimbrial adhesins (F18), the heat-labile (LT) or heat-stable (Stb) enterotoxins, and virulence factors involved in diffuse adherence of E. coli (AIDA). Mcr-3 positive strains isolated from diseased pigs were associated with enterotoxigenic E. coli (47.1%) and Shiga toxin-producing E. coli (23.5%), both expressing fimbrial adhesion (F18). These toxins and fimbrial genes are associated with porcine diarrhea and edematous disease [23,24].

Whole-genome sequencing demonstrated that plasmids pK18EC051 (GenBank accession no. CP049300, 270.3 kb) and pK15EC053 (CP049087, 96.2 kb) shared a similar mcr-3.1-carrying region with plasmid pZR10 from pigs in China, but a gene encoding for 5-nitroimidazole based antimicrobials (nimC) was excluded from downstream of mcr-3.1 gene in pK15EC053 (Figure 1). In contrast, only diacylglycerol kinase (dgkA) and transposase encoding genes were identified in the immediate downstream and upstream of the mcr-3.5 gene, respectively, in pK18EC025 (CP049944, 60.2kb). The mcr-3.5 variant in pK18EC025 differed from the mcr-3.1 gene variant found in this study (pK18EC051 and pK15EC053) as well as the original mcr-3 variant from China (pWJ1) by three amino acid substitutions (M23V, A457E, and T488I). Although the MIC of colistin was not altered by these substitutions, Yang et al. [16] demonstrated that mcr-3.5 has higher fitness than mcr-3.1.

In conclusion, the proportion of mcr-3-carrying isolates is increasing in diseased pigs, presumably due to the horizontal and clonal dissemination. Therefore, active surveillance of mcr-carrying isolates is vital for preventing the spread of colistin resistance. In addition, a guideline that ensures prudent use of antimicrobials in pigs is urgently needed.
Table 2. Characteristic of the mcr-3 positive *Escherichia coli* from healthy and diseased pigs, and pig carcasses in Korea.

| Isolates   | Source | Farm ID | Year | Province | MIC of Colistin (µg/mL) | MCR-3 Variant Type | Resistance Pattern | Transfer-Ability | Replicon Type of Transconjugant Plasmid | Multilocus Sequence Type | PULSOTYPE | Virulence Factors |
|------------|--------|---------|------|----------|------------------------|-------------------|-------------------|-----------------|------------------------------------------|------------------------|-----------|------------------|
| V08-R02-015 | diseased | GB-1    | 2011 | Gyeongbuk | 16                     | mcr-3.1            | AMP CHL CIP GEN NAL STR FIS TET SXT | /               |                                         | 3523                   | A         | F18/LT/STb/EAST  |
| V04-A02-010 | healthy  | CN-1    | 2012 | Chungnam  | 8                      | mcr-3.1            | AMP CHL GEN NAL STR FIS TET SXT | +               | HI2                                      | 4532                   | B         |                  |
| V05-S02-016 | carcass  | CN-2    | 2013 | Chungnam  | 8                      | mcr-3.1            | AMP CHL GEN NAL STR FIS TET SXT | /               |                                         | 101                    | C         | EAST             |
| 14D084     | diseased | GB-2    | 2014 | Gyeongbuk | 16                     | mcr-3.1            | AMP GEN NAL FIS TET SXT | +               | HI2, I1-α                                | 1                      | D         | F18/Stx2e/AIDA  |
| 14D084-2   | diseased | GB-2    | 2014 | Gyeongbuk | 16                     | mcr-3.1            | AMP CHL GEN NAL STR FIS TET SXT | /               |                                         | 1                      | D         | F18/Stx2e/AIDA  |
| 14D085     | diseased | GB-3    | 2014 | Gyeongbuk | 16                     | mcr-3.1            | AMP CHL GEN NAL STR FIS TET SXT | /               |                                         | 1                      | D         | F18/Stx2e/AIDA  |
| V01-R02-019 | diseased | CB-4    | 2015 | Chungbuk  | 16                     | mcr-3.1            | AMP CHL GEN NAL STR FIS TET SXT | +               | HI2                                      | ND e                    | E         |                  |
| V01-R02-020 | diseased | CB-4    | 2015 | Chungbuk  | 8                      | mcr-3.1            | AMP CHL GEN NAL STR FIS TET SXT | /               |                                         | 10                    | - d       |                  |
| V01-R02-053 | diseased | GG-1    | 2015 | Gyeonggi   | 16                     | mcr-3.1            | AMP CHL GEN NAL STR FIS TET SXT | +               | M                                        | 1                      | D-1       | F18/Stx2e/AIDA  |
| V01-A02-017 | healthy  | GN-3    | 2018 | Gyeongnam | 16                     | mcr-3.1            | AMP CHL GEN NAL STR FIS TET SXT | /               |                                         | 10                    | L         | LT/STb/EAST      |
| V01-E02-088 | diseased | GN-4    | 2018 | Gyeongnam | >16                    | mcr-3.1            | AMP CHL GEN NAL STR FIS TET SXT | +               | HI2, I1-α, N                             | 29                    | M         | eae/paa          |
| V01-E02-090 | diseased | GN-4    | 2018 | Gyeongnam | 16                     | mcr-3.1            | AMP CHL GEN NAL STR FIS TET SXT | +               | P1, I1-α                                 | 42                    | N         | F18/LT/STb/EAST  |
| V01-E02-025 | diseased  | GB-4  | 2018 Gyeongbuk | 8  | mcr-3.5 | AMP CHL XNL STR FIS TET SXT AMC AMP FOX CHL CIP GEN NAL STR FIS TET SXT | + P1 | 42 | N | F18/LT/STb/EAST |
|-------------|-----------|-------|----------------|----|---------|---------------------------------------------------------------|-----|----|---|----------------|
| V01-E02-049 | diseased  | GN-5  | 2018 Gyeongnam | >16 | mcr-3.1 | /                                                             |     | 10 | L | F18/LT/STb/EAST/AIDA |
| V01-E02-050 | diseased  | GN-5  | 2018 Gyeongnam | 8  | mcr-3.1 | STR FIS TET SXT AMP CHL GEN SXT STR FIS TET SXT AMP CHL GEN | + HI2 | 641 | O | STb/EAST/AIDA |
| V01-E02-051 | diseased  | GN-5  | 2018 Gyeongnam | 8  | mcr-3.1 | STR FIS TET SXT AMP CHL GEN SXT STR FIS TET SXT AMP CHL GEN | + HI2 | 641 | O | STb/EAST/AIDA |

a AMC, amoxicillin/clavulanic acid; AMP, ampicillin; FOX, cefoxitin; XNL, ceftiofur; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; FIS, sulfisoxazole; TET, tetracycline; SXT, trimethoprim/sulfamethoxazole. b The underlined resistance markers were transferred to the recipient *E. coli* J53 strain by conjugation. c Not determined. d *XbaI* macrorestriction analysis yielded no DNA banding patterns in V01-R02-020 *E. coli* strain due to constant autodigestion of the genomic DNA during agarose plug preparation, and thus, a cluster formed by this strain is excluded.
Supplementary Materials: The following are available online at www.mdpi.com/2076-2607/8/10/1538/s1. Figure S1: XbaI-digested pulsed-field gel electrophoresis patterns of mcr-3 carrying E. coli strains isolated from healthy pigs, pig carcasses, and diseased pigs in Korea. XbaI macrorestriction analysis yielded no DNA banding patterns in V01-R02-020 E. coli strain due to constant autodigestion of the genomic DNA during agarose plug preparation, and thus, a cluster formed by this strain is excluded. (ND, not determined).

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