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Carriage and within-host diversity of mcr-1.1-harboring Escherichia coli from pregnant mothers: inter- and intra-mother transmission dynamics of mcr-1.1

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Abstract

Exchange of antimicrobial resistance genes via mobile genetic elements occur in the gut which can be transferred from mother to neonate during birth. This study is the first to analyze transmissible colistin resistance gene, \textit{mcr}, in pregnant mothers and neonates. Samples were collected from pregnant mothers (rectal) and septicaemic neonates (rectal & blood) and analyzed for presence of \textit{mcr}, its transmissibility, genome diversity, and exchange of \textit{mcr} between isolates within an individual and across different individuals (not necessarily mother-baby pairs). \textit{mcr-1.1} was detected in rectal samples of pregnant mothers (n=10, 0.9%), but not in neonates. All \textit{mcr}-positive mothers gave birth to healthy neonates from whom rectal specimen were not collected. Hence, transmission of \textit{mcr} between these mother-neonate pairs could not be studied. \textit{mcr-1.1} was noted only in \textit{Escherichia coli} (phylogroup A & B1), and carried few resistance and virulence genes. Isolates belonged to diverse sequence types (n=11) with two novel STs (ST12452, ST12455). \textit{mcr-1.1} was borne on conjugative IncHI2 bracketed between IS\textit{Apl}1 on Tn6630, and the plasmids exhibited similarities in sequences across the study isolates. Phylogenetic comparison showed that study isolates were related to \textit{mcr}-positive isolates of animal origin from Southeast Asian countries. Spread of \textit{mcr-1.1} within this study occurred either via similar \textit{mcr}-positive clones or similar \textit{mcr}-bearing plasmids in mothers. Though this study could not build evidence for mother-baby transmission, but presence of such genes in the maternal specimen may enhance the chances of transmission to neonates.
Keywords: Colistin-resistant *Escherichia coli*, pregnant mother and neonatal gut carriage, *mcr-1.1*-bearing IncHI2, transmission dynamics of *mcr-1.1*, Illumina & MinION nanopore sequencing.

**Introduction:**

The human intestine accommodates a complex dynamic microbial community including antimicrobial-resistant Gram-negative bacteria harboring resistance genes such as *bla*\(_{TEM}\), *bla*\(_{CTX-M}\), *qnrS*, etc. in both healthy and sick populations [1,2]. The intestine allows for the inter- and intra-species exchange of antimicrobial resistance genes (ARGs) primarily via different mobile genetic elements (MGEs) [3]. MGEs (plasmids, transposons, etc.) aids exchange of genes. Exchange of genes in the gut can occur due to antibiotic exposure or even without its use [3,4]. However, the use of antibiotics exerts selective pressure that facilitates overgrowth of resistant bacteria.

Early in life, the newborn acquires microbes from the mother and the immediate environment [3,5,6]. Antibiotics consumption during pregnancy increases the risk of selection of antibiotic-resistant bacteria in the maternal microbiota which in turn may be passed on to the newborn during birth [2]. The acquisition of resistance genes from the mother or the hospital environment can have serious consequences for the newborn particularly those who are preterm or low birth weight [7]. Translocation of the bacteria, which includes those that are resistant, from the neonatal gut via the myentric lymph node to the bloodstream can lead to sepsis [8].

Members of the Enterobacterales family such as *Escherichia coli*, commensals of the human gut, can serve as a common reservoir of different ARGs [3,9]. They have been found to exchange antibiotic-resistant plasmids and virulence genes among themselves [3].
comparison to the Bacteroides or Firmicutes, *E. coli* are fewer in gut but have the ability to acquire and exchange genes with other commensal and transient bacteria [10]. Several resistance genes (*bla*NDM, *bla*CTX-M, *bla*CMY, *qnr*S, etc) conferring resistance to different groups of antibiotics have been noted in *E. coli*. [3,11].

In 2015, plasmid-mediated colistin resistance gene, *mcr-1*, emerged in China and *E. coli* served as the reservoir of this gene [12,13]. Other bacteria such as *Salmonella sp.*, *Shigella sp.*, *Klebsiella sp.*, *Enterobacter cloacae*, etc. were also reported to harbor *mcr* genes [14]. *mcr-1* has been found in multiple plasmid types, *viz*. IncI2, IncHI2, IncX4, IncP, and IncF, signifying efficient spread of this gene among various organisms and countries [15]. Apart from livestock, poultry and aquaculture, occurrence of *mcr* genes has been detected in humans [14] both in isolates causing infection and in colonizers, with a recent report of *mcr-1* gene in gut of healthy human [15,16]. The presence of transmissible *mcr* gene in the healthy human gut, particularly in pregnant mother might predispose neonates to bacteria harboring the gene [3]. This would result in emergence of resistance in neonates even in the absence of colistin exposure, ultimately leading to treatment failure.

Reports of *mcr* and their transmission dynamics in human gut is limited, more so in pregnant mothers and neonatal population. With increased use of colistin as a last line resort and incidences of sepsis due to resistant bacteria, an understanding of colistin-resistant carriage and transmission in the gut is essential. Herein, we studied i) carriage of *mcr* in the gut of mothers and sick newborns (not necessarily mother-baby pairs), and among neonatal blood isolates, ii) inter- and intra-patient transmission dynamics of *mcr* and comparison of *mcr*-bearing organisms with susceptible isolates from the same sample, and iii) phylogenetic relationships among the study isolates and with similar isolates across different Southeast Asian countries.

**Materials and methods-**
Study design, collection of rectal swabs and blood cultures at clinical site:

In this study, samples collected during a multi-centric study named ‘Burden of Antibiotic Resistance in Neonates from Developing Societies (BARNARDS)’ [2,17] has been used. BARNARDS study involved pregnant mothers and sick newborns who were admitted in IPGMER and SSKM Hospital, Kolkata. Rectal samples from all pregnant mothers but only sick neonates (no healthy neonates) were collected, so the mother-baby pairs were only present for sick neonates. Herein, we focused in analyzing carriage of the transmissible colistin resistance gene, \textit{mcr}, and its transmission dynamics within the samples collected during the BARNARDS study.

During the study period (July to November, 2017), women in labor or immediately postpartum, were recruited and rectal swabs were collected following consent. Neonates (inborn and outborn) when suspected with sepsis were enrolled, rectal swabs were collected and cultured isolates from blood were sent to ICMR-NICED. As a part of the BARNARDS study, socio-demographic information of the mothers and neonates were collected [17]. A schematic representation of the study design has been depicted in Figure 1.

Processing of rectal swabs and blood cultures, and detection of transmissible colistin resistance genes, \textit{mcr}:

Processing of rectal swabs and blood culture have been described in Figure 1 and in supplementary methods. Briefly, rectal swabs were plated on vancomycin (10 mg/L) (MP Biomedicals, California, USA) supplemented chrome agar (CA) (BD BBL, MD, USA) and incubated (37°C, 18-24 hours). Cultures from primary inoculum of each plates and cultured isolates from neonatal blood were screened for \textit{mcr} genes by polymerase chain reaction (PCR) as described previously [18]. Sample positive for \textit{mcr} genes were further enriched in
Enterobacteriaceae Enrichment (EE) Mossel broth (37°C, 18-24 hours) (BD BBL) and plated on to CA supplemented with/without colistin sulphate (2 mg/L) (MP Biomedicals).

Colonies with different colors were picked from plates with/without colistin, and again screened for \textit{mcr} genes. Amplified PCR products were Sanger sequenced and stocked for further analysis [12]. Any \textit{mcr}-negative colony with similar color as the \textit{mcr}-positive ones were collected from CA plates (without colistin) to check clonality with \textit{mcr}-positive colonies.

**Identification and susceptibility of \textit{mcr}-positive/\textit{mcr}-negative isolates:**

Identification and susceptibility testing of all \textit{mcr}-positive/\textit{mcr}-negative isolates were done by Vitek2 compact system. (BioMérieux, Marcy l'Etoile, France). For minimum inhibitory concentration (MIC) of colistin, broth microdilution (BMD) was carried out using colistin sulphate, following CLSI guidelines. Results were interpreted according to CLSI (2020) [19].

**Molecular typing of \textit{mcr}-positive/\textit{mcr}-negative isolates:**

More than one colony of same color with \textit{mcr} gene was stocked from each sample and were subjected to repetitive extragenic palindromic elements-PCR (rep-PCR) [20]. Additionally, all \textit{mcr}-negative isolates (of same color as the \textit{mcr}-positive ones) were subjected to rep-PCR to check for similar clones. Clonality among \textit{mcr}-positive isolates were determined by pulsed-field gel electrophoresis (PFGE) in a CHEF-DRIII apparatus (Bio-Rad Laboratories, Hercules, and CA) using XbaI macro digestion and visually interpreted as per Tenovar criteria [21]. With FP Quest software v4.5 (Biorad laboratories Inc, Hercules, California, USA), a dendrogram was prepared using Dice coefficient and UPGMA (unweighted pair group method using arithmetic averages). Tolerance and optimization was set at 1.5% and isolates with ≥90% similarity were considered identical. The phylogenetic classification of the isolates was performed \textit{via} phylogroup multiplex PCR as described previously [22].
Transmissibility of plasmid-mediated *mcr*:

Transfer of *mcr* was carried out in *Az* E. coli J53 (recipient) by solid-mating conjugation method. Transconjugants (TCs) were selected on Luria Bertani (LB) (BD BBL) agar plates supplemented with sodium azide (100 mg/L) (Sigma-Aldrich, St Louis, MO, USA) and colistin (2 mg/L). TCs retrieved were subjected to confirmation of *mcr* and other resistance genes by PCR, followed by assessment of colistin MIC by BMD.

Following this, plasmid typing was performed using PCR-based replicon typing (PBRT) [23] in both wild-type (WT) and TCs.

Whole genome sequencing (WGS) of *mcr*-positive/ *mcr*-negative isolates:

Genomic DNA from *mcr*-positive/ *mcr*-negative isolates was processed for paired-end WGS on an Illumina MiSeq. Selected isolates (based on gDNA quantity) were subject to additional long-read sequencing on a MinION (Oxford Nanopore Technology, UK) as described previously [24]. Further analysis of resistance, virulence, plasmid types and sequence types was carried out by different online pipelines (Supplementary methods).

Comparison of *mcr* bearing plasmids within the study isolates-

Complete plasmid sequences of *mcr*-positive isolates were extracted using Bandage (v0.8.1), annotated using Prokka (v1.14.5) and mobile element finder (MGE) (v1.0.2). Sequences were aligned using Geneious (https://www.geneious.com/) and EasyFig (v2.2.5). Bacterial plasmid database (PLSDB) [25] was used to search for similar plasmid sequences and the plasmid with the greatest similarity (as determined by the match with the highest % nucleotide identity match to the *mcr* plasmid sequence from this study) was used as a reference sequence for sequence alignment analysis using GSAlign (v1.0.22).
Comparative phylogenetic analysis of mcr-positive/mcr-negative isolates-

A core genome alignment and maximum likelihood phylogenetic tree of isolates was performed using Roary (v3.12.0) and IQ-TREE (v2.0) with the GTR substitution model and 1,000 bootstrap replicates. Phylogenetic trees were mid-rooted and annotated using iTOL(v5.7). Snippy (v4.6.0) was used to assess the number of single nucleotide polymorphisms (SNPs) between isolates of the same ST using an available hybrid genome as the local reference genome with --mincov 10 applied and trimmed paired end fastq used as input (--R1 --R2).

Phylogenomic comparison of study isolates with mcr-1 from Southeast Asia-

A literature search was conducted during July-August, 2021, WGS data of mcr-bearing E. coli from Southeast Asia was retrieved and fastq were assembled into genomes as described [24]. All genomes were screened for the presence of mcr-1. ClermonTyping was used to determine in silico phylogroups [26,27]. Prokka (v1.14.5) was used to annotate the assemblies and Panaroo (v1.2.8; --clean-mode [moderate], --core parameters applied) was used to create the core genome alignment. IQ-TREE (v2.0) was used to generate the maximum likelihood phylogenetic tree with parameters as described previously [24].

Results-

Identification of bacterial isolates with mcr:

mcr-1 was found in rectal sample of ten pregnant mothers (n=10/1169), indicating a low prevalence (0.9%). These 10 mothers had healthy babies and hence the paired neonatal sample was not collected. None of the other sick neonates from whom rectal samples were collected harbored mcr-1. Nucleotide sequencing of mcr-1 revealed it to be mcr-1.1 and was found only
in *Escherichia coli*. *E. coli* lacking *mcr*-1.1 from those samples that harbored *mcr*-positive isolates were also analyzed. Fourteen *mcr*-1.1 *E. coli* and twenty-five *mcr*-negative *E. coli* were isolated from 10 mothers (Table 1). Few mothers had more than one isolates carrying *mcr*-1.1. Other colonies found in the sample were *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, but none carried *mcr*-1.

**Socio-demographic details of mothers possessing *mcr*-1.1-positive *E. coli***:
Mothers who harbored *mcr*-1.1 gave birth to term, singleton, healthy babies, with no birth complications. Since, the babies were healthy, rectal swabs were not collected from these neonates and hence, not assessed for the presence of *mcr*-1.1. No mother received antibiotic treatment, nor visited traditional, or private healthcare or hospitalized or travelled outside the local province prior to enrolment. Most mothers (n=7) were from urban areas while three mothers were from rural areas.

**Antibiotic susceptibility of *E. coli* isolates collected from *mcr*-1.1-positive maternal sample**: 
Both *mcr*-positive and *mcr*-negative isolates were susceptible to most of the antibiotics tested but were resistant to ampicillin and trimethoprim-sulfomethoxazole (Table 1). Few were additionally resistant to cefuroxime, ceftriaxone, cefoperazone/sulbactam, cefepime, nalidixic acid, and ciprofloxacin. However, *mcr*-positive isolates were resistant to colistin (MIC: 8-16 mg/L), while *mcr*-negative isolates were in susceptible range (0.015-2 mg/L) (Table 1).

**Molecular typing of *E. coli* from *mcr*-1.1-positive maternal sample**: 
Eleven distinct *mcr*-positive isolates and 25 distinct *mcr*-negative isolates as per rep-PCR, were proceeded with WGS.
PFGE revealed that \textit{mcr}-positive isolates were clonally distinct (Figure 1) with few indistinguishable isolates such as IN-MR674R1, IN-MR680R1 & IN-MR683R1; and IN-MR750R1 and IN-MR750R4 (Figure 2).

\textit{mcr}-positive isolates belonged to diverse STs (11 STs among 14 isolates), of which ST12452 and ST12455 were novel (Table 1). \textit{mcr}-negative isolates were also diverse (22 STs), with three novel STs (ST12453, ST12454, and ST12457) (Table 1). STs such as ST181, ST394 and ST2705 were common in both types of \textit{E. coli}.

\textbf{Comparison of resistance, virulence, plasmid profiles and phylogroups among both types of \textit{E. coli} based on WGS:}

\textit{mcr}-positive isolates harbored different \(\beta\)-lactamase resistance genes; fluoroquinolone-resistant genes, and various aminoglycoside-, sulphonamide-, macrolide-, chloramphenicol-, tetracycline- and trimethoprim-resistant genes (Table 1). Most of the \textit{mcr}-negative isolates (n=17, 68\%) did not bear \(\beta\)-lactamase genes but had a multidrug resistance gene, \textit{mdf(A)}. Few had \(\beta\)-lactamase genes (\textit{bla}_{TEM-1}, \textit{bla}_{CTX-M-15}) (n=8), along with fluoroquinolone-resistant genes (Table 1). None of the study isolates harbored carbapenemases.

Carriage of virulence genes within \textit{mcr}-positive and \textit{mcr}-negative isolates was nearly same (Table 1). \textit{gad} (glutamate decarboxylase) was prevalent among both group of isolates, followed by \textit{terC} (tellurite resistance gene). Presence of different serum and complement resistance genes were noted in many \textit{mcr}-positive and few \textit{mcr}-negative isolates (Table 1). However, IN-MR569R1 and few \textit{mcr}-negative didn’t show presence of any virulence genes at all. Most of the study isolates belonged to phylogroup A (\textit{mcr}-positive=8 and \textit{mcr}-negative=11) and B1 (\textit{mcr}-positive=4 and \textit{mcr}-negative=10) (Table 1).

Both \textit{mcr}-positive and \textit{mcr}-negative isolates possessed different plasmid replicons such as \textit{IncFIA}, \textit{IncFIB}, \textit{IncFII}, \textit{IncX1}, \textit{IncX2}, \textit{IncX4}, p0111, \textit{IncN}, \textit{IncY}, \textit{IncHI1A}, \textit{IncHI1B}, \textit{IncI}-
I (alpha), Col, and Col440I (Table 1). However, mcr-positive isolates additionally harbored IncHI2, IncHI2A and IncX4.

Transmissibility and characterization of mcr-1.1 plasmid:
Conjugal transfer of mcr-1.1 was successful with co-transfer of blaTEM, and qnrS in various combinations among transconjugants (TCs). Colistin MIC of TCs and wild-type isolates were found to be similar (Table 1).

mcr-1.1 was carried on IncHI2 plasmid (~216kb-240kb), except for one (IN-MR569R1) which harbored mcr-1.1 in IncX4 (~33kb) (Table 1). Plasmid types found in TCs as evaluated by PBRT corroborated with WGS data. All IncHI2 plasmids belonged to pST4 (Table 1). Out of 14 mcr-positive isolates, 13 complete plasmids including the IncX4 plasmid were assembled from long read sequencing. Two groups of IncHI2 plasmids were noted, ~216 kb (plasmid 1, P1) found in multiple isolates collected from three samples and the other ~240kb (plasmid 2, P2) found in six different samples (Table 1 & Figure 3.a & 3.b). All P1s (n=7) when aligned against pCFSAN061771 (Egypt, accession: CP042898.1), were found to be identical to each other (Figure 3.a) with an average nucleotide identity (ANI) of 93.07% to pCFSAN061771 (455 single nucleotide variants, SNVs; 8 insertions and 5 deletions). P1 from IN-MR750R1 had an ANI >99.88% to pCFSAN061771 and was slightly larger (~217kb) (Figure 3.a), showing some variations. Similarly, all P2s (n=6) when aligned against a plasmid of an E. coli from Saudi Arabia (NZ_CP022735.1) exhibited >99.9% ANI. P2s from different mothers were within 50 pairwise SNVs (Figure 3.b).

mcr-1.1 in P1 and P2 was bracketed between ISAplI upstream and downstream within a composite transposon, Tn6330, with variations in orientation and truncation of ISAplI in few isolates (Figure 4). Genetic environment of mcr-1.1 of study isolates when compared with global
mcr-1, revealed a variation in the arrangement of the genes i.e., ISApl1-mcr-1.1-ISApl1-pap2 instead of ISApl1-mcr-1.1-pap2-ISApl1 (Figure 4).

mcr-1.1 when present in IncX4 plasmid (~33kb) did not have ISApl1 or other IS element in the vicinity, rather IS26 was noted in the same plasmid (Figure 4). IncX4 plasmid when aligned with other previously described plasmid sequences, produced multiple identical hits on PLSDB with mcr-1 isolates from China, Laos, and Vietnam.

Transmission of mcr-1.1 within gut microbiome:

Within the study population, intra- and inter-gut transmission of mcr-1.1 was studied. We compared isolates in terms of their typing patterns (PFGE), STs, SNPs, and mcr-1.1-bearing plasmids in individual or different maternal samples. We analyzed transmission of mcr-1.1 from two aspects: i) clonal spread, where mcr-positive isolates of same STs were isolated from different mothers, and ii) plasmid-mediated spread, where mcr-carrying similar plasmids belonging to different STs were isolated from individual or different mothers (Figure 5). When assessing the clonal spread, mcr-negative isolates belonging to similar STs of mcr-positive isolates were also compared.

Isolates IN-MR674R1, IN-MR680R1, IN-MR683R1 collected from three mothers admitted within a time frame of 0-1 days (Figure 5) belonged to ST2705, phylogroup A, and shared >90% similarity (PFGE) (Table 1 & Figure 2). These isolates clustered together in core genome phylogenetic tree and IN-MR674R1, IN-MR680R1 were 22 SNPs distant from each other (Figure 6). These results indicated a possible inter-mother transfer of clonal isolates with mcr-1.1.

To understand the transmission of mcr-1.1 through plasmids, we compared mcr-1.1-bearing IncHI2 plasmids in individual/ different mothers. Sequences of mcr-1.1-bearing IncHI2 plasmid within five mothers (IN-MR361, IN-MR362, IN-MR725, IN-MR727 & IN-MR750) showed significant similarities. IN-MR361 (ST181) and IN-MR362 (ST12452)
despite belonging to different STs, carried a similar mcr-1.1 plasmid (240kb) with identical genetic environment (Table 1 & Figure 3.b, 4, 5). Likewise, mcr-1.1-bearing IncHI2 plasmid of E. coli (ST1286) isolated from IN-MR727 also showed similarity with plasmids of E. coli from IN-MR725 & IN-MR750 (Table 1, Figure 3.a, 4, 5). These mothers were distantly related to each other as per core genome SNP phylogeny (Figure 6) but harbored a similar plasmid (Figure 5). Both horizontal transmission of mcr-1 through other E. coli bacteria among different mothers or independent acquisition of similar plasmids at different points of time beyond the hospital environment are possible. However, this cannot be definitively assessed by the findings of this study. In some mothers more than one diverse E. coli isolate belonging to different STs were found- IN-MR725 (ST101, ST2178, ST9421) & IN-MR750 (ST394, ST515), exhibiting similar mcr-1.1-bearing IncHI2 plasmids (~216kb) with same genetic environment (Table 1, Figure 3a, 4, 5). This indicates a possible transmission of plasmid within an individual host (intra-mother).

Three other independent acquisitions of mcr-1.1-bearing plasmid/ bacteria were noted, one with mcr-1.1 in IncX4 plasmid, and the other two with mcr-1.1 in IncHI2 having a genetic environment distinct from others that are discussed above (Figure 5).

Few mcr-negative isolates identical to mcr-positive isolates in terms of STs and core genome were also isolated but they lack IncHI2 and IncX4 (Table 1, Figure 5 & Figure 6).

**Phylogenomic comparison of E. coli mcr-1 carriage in Southeast Asia:**

Core genome phylogeny was built involving genomic data from Southeast Asian mcr-1-positive E. coli (n=106) collected between 2011-2019. This included isolates from twenty studies along with twelve mcr-positive isolates of this study (Figure 7). The isolates belonged to diverse sources including blowflies (n=20), chicken (meat and stool, n=30), dairy cattle farm, (n=1), dogs (n=1), environment (n=1), human carriage (n=29), human clinical samples (n=12), migratory birds (n=1), pigs (n=9) and sheep (n=2) (Figure 7). Isolates were distributed in over
70 STs, with ST10, ST48, ST156, ST410 and ST648 being the most frequent. *mcr*-positive isolates of this study were found dispersed throughout the phylogeny indicating a wide diversity of *mcr-1* *E. coli* isolates across the species.

Southeast Asian *mcr-1* collection harbored *blaCTX-M* (n=40), *blaNDM* variant (n=20), *blaOXA-48* (n=1), *tet (X4)* (n=3), *mcr-3* (n=3) in addition to the *mcr-1* gene. Whilst none of the study isolates within this study contained carbapenemase or *tet(X4)*, although few possessed other ARGs. Though different plasmid replicons were seen in the analyzed genomes, *mcr-1* was principally present in IncHI2 or IncX4. Isolates possessed either IncHI2 or IncX4 but never both. Some isolates from Vietnam did not show presence of any plasmid, indicating occurrence of chromosomal *mcr-1* gene.

Analyzed isolates belonged to phylogroup A (n=57, 54%) followed by phylogroup B1 (n=30, 28%), F (n=7), C (n=6), D (n=5), and one case of B2. Likewise, study isolates also belonged to phylogroup A and B1.

Study strains showed closeness with strains from various countries of Southeast Asia viz. Bangladesh, China, Thailand, Singapore, Vietnam, and Laos (Figure 7). Most of the study isolates showed resemblances with *E. coli* isolated from chicken (n=5) or human carriage (n=4) and few from pig and blow flies. This shows that *mcr*-positive isolates circulate among different origins (mainly food animals), emphasizing its presence within the food chain.

**Discussion**

The passage of antibiotic-resistant organisms from mother to the neonate is of concern. The pristine and immature gut of the neonate, particularly for those who are premature and low-birth weight, can allow the translocation of resistant organisms to the bloodstream leading to sepsis [28]. *mcr-1.1* was isolated from maternal rectal samples only, with very low prevalence (0.9%). No *mcr* was noted in gut samples or in cultured blood from neonates suspected with
sepsis. Mothers with *mcr-1.1* had healthy babies and since samples were not collected from healthy neonates, the presence of *mcr* in the healthy neonatal gut could not be assessed. Hence, possibility of mother-to-baby transmission of *mcr*-harboring *E. coli* could not be studied.

Carriage of *mcr* in this study is much lower than that reported from China (2.08%-6.2%), Switzerland and France, but higher than Netherlands (0.35%) [29]. Colistin resistance is low in India and a study on neonatal blood isolates over a period of 12-years from the same unit also exhibited low prevalence of colistin resistance (2.8%) with no *mcr* genes [30]. Such low resistance may be attributed to the limited use of colistin in clinical settings. Absence of selection pressure (colistin) probably restricted spread of *mcr-1* in clinical settings as studies from India documented fewer reports of *mcr-1* from human clinical isolates compared to food items such as meat, poultry and environmental isolates [31,32]. From 2019, restrictions in colistin usage in India within animal industries, have attributed to low carriage of *mcr-1* [32].

In India, *mcr-1* was reported first in *Klebsiella pneumoniae* [33] and later in *E. coli*, *Aeromonas* sp., *A. baumannii*, etc. [34-36]. Presence of different bacteria such as *K. pneumoniae*, *P. aeruginosa*, *A. baumannii* along with *E. coli* was found in the maternal samples. However, *mcr-1.1* was found only in *E. coli*, implicating its restricted spread.

In this study, *mcr*-positive isolates belonged to phylogroup A and B1, were highly susceptible and carried few virulence genes. *mcr-1 E. coli* from South Asian countries, also exhibited prevalence of similar phylogroups. Though primarily restricted to the gut, A and B1 isolates do cause sepsis in debilitated or immunocompromised patients by translocating from gut to blood through the immature/compromised gut barrier [37]. A and B1 *E. coli* being commensals, possess virulence genes necessary for colonization [27], and harbor different ARGs [38] which is also noted among the global *mcr-1* isolates, but study isolates exhibited low carriage of ARGs.
*mcr*-positive isolates were diverse and belonged to various STs as also observed in previous studies [39,40]. Occurrence of *mcr-1.1* among novel STs as found in this study, exhibited the emergence of new colistin-resistant clones. This highlighted the fact that spread of *mcr-1.1* is not through any particular clone or lineage. Southeast Asian *mcr-1* isolates were also diverse irrespective of source of origin. Study isolates exhibited resemblances with isolates from Southeast Asian countries (Bangladesh, Vietnam, Singapore, Laos, etc.).

*mcr-1.1* was detected in IncHI2 and IncX4 replications, though several other replications were present in the *mcr*-positive study isolates. Plasmids of this study showed similarities with plasmids from Egypt and Saudi Arabia (IncHI2), and China, Laos and Vietnam (IncX4), highlighting proficiency of these plasmids for the spread of *mcr* gene. Analysis of Southeast Asian *mcr-1* isolates also exhibited prevalence of these two plasmids harboring *mcr-1* gene. IncI2, IncHI2, IncP, IncX4 are the predominant carriers of *mcr-1* worldwide [27,39,40]. The study isolates shared similar plasmid backbone of replicon type IncHI2. Some differences were noted due to the inversion/deletion of certain sequences. Association of IncHI2-pST4 with *mcr-1.1* in the study isolates, corroborated with others [41,42], implying IncHI2-pST4 to be a dominant plasmid lineage contributing to the horizontal transfer of *mcr-1.1*.

IncHI2 and IncX4 plasmids were conjugative and associated with various MGEs such as IS*ApII*, Tn6330 (IncHI2) and IS26 (IncX4). IS26 being a hotspot for plasmid fusion has intensified the spread of *mcr-1*-harboring IncX4 plasmids in absence of IS*ApII* or any transposon [43]. IS*ApII*-mcr-1.1-pap2-IS*ApII* is the known genetic background of the gene reported so far [44], but a different genetic environment: IS*ApII*-mcr-1.1-IS*ApII*-pap2 has been detected within the study isolates which has not been reported previously.

Clonal spread of *mcr-1.1*E. coli (ST2705) was observed in three mothers who were admitted in hospital during the same time highlighting the possibility of an inter-mother transmission of *mcr-1.1*-harboring *E. coli*. Further similar *mcr-1.1* plasmids were isolated from
five maternal samples in diverse *E. coli*. Each isolate belonged to different STs, but their plasmid sequences revealed significant similarities. Prevalence of different clones with identical plasmids in several mothers indicate that the spread of *mcr-1.1* is plasmid-mediated. However, this study could not definitively underline the fact whether presence of similar plasmid within different mothers was due to horizontal transmission through bacteria in the hospital environment or independent acquisition of similar plasmids from other sources beyond the hospital environment. In contrast, the transmission of the plasmid in the gut is more evident in cases where similar *mcr-1.1* plasmid has been isolated from different STs in individual mother. Other studies documented spread of *mcr-1* via plasmids occurring between diarrhoeal patients and from animals to human [39,40,45]. We hypothesize that when more than one *mcr-* positive *E. coli* of distinct STs with similar plasmids were isolated from an individual mother (intra-mother transmission), transfer of *mcr-1.1* via plasmids was more probable than an independent acquisition of two separate distinct *mcr-* possessing isolates.

Presence of *mcr-1.1* in healthy pregnant mothers with no exposure to antibiotics, might be due to the presence and persistence of *mcr-1* gene in the food chain. In a recent study, *mcr-1* has been found to enhance commensal lifestyle of *E. coli*, which led to maintenance of *mcr-1*-positive *E. coli* within the gut even in the absence of antibiotic pressure [46]. Similarities of *mcr-1*-harboring maternal study isolates with isolates from chicken (food animal) indicated presence and circulation of *mcr*-positive isolates within the food chain.

In conclusion, studies highlighting prevalence of *mcr-1.1* in pregnant mothers are rare with no studies explaining within-host diversity of *mcr-1.1*-harbouring *E. coli*. Presence of *mcr*-positive isolates with highly similar plasmids in the gut of healthy mothers (individual mother or among different mothers) indicated the involvement of plasmids. The only limitation of this study lies in the fact that transmission of *mcr-1.1* from mothers to their respective neonate could not be studied as their babies were healthy and hence not included in the study.
Presence of mcr-1.1 in susceptible *E. coli* of healthy individuals is worrisome since they remain undetected and may serve as a focal point for spread of colistin resistance in community and in newborns from their colonized mothers.

**Ethics approval and consent to participate**-

The study protocol was approved by the Institutional Ethics Committee of the ICMR-National Institute of Cholera and Enteric Diseases (A-1/2016-IEC.). Patient consent was taken prior to enrolment to the study. Patient information was anonymized and de-identified prior to analysis.

**Acknowledgements**-

We thank the team at the Bill & Melinda Gates Foundation. We acknowledge the staff of the Department of Gynaecology & obstetrics, and Neonatology, who cared for the mothers and neonates. We also extend our thanks to all medical technicians, multi-tasking staffs, data entry operator and nurses for their assistance throughout the BARNARDS study. We thank the team of curators for the databases hosted on PubMLST (https://pubmlst.org/databases/) for curating the *Escherichia coli* data and making it publicly available.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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**Data availability statement**

Whole genomes sequences of isolates from this study have been submitted to NCBI database under BioProject number: PRJNA808864.
References:


Figure 1: Schematic flow diagram of the study plan, summarizing sampling/laboratory/sequencing workflows.

Figure 2: Molecular typing and plasmid replicons of mcr-1.1 bearing Escherichia coli. Abbreviation: Indian maternal rectal sample (IN-MR), resistant (R), sequence type (ST), incompatibility (Inc), kilobase (kb), plasmid 1 (P1), plasmid 2 (P2).

Figure 3: Alignment of mcr-1.1-bearing IncHI2 plasmid sequences of the study isolates. (a) ~216 kb: IN-MR750R1 and IN-MR750R4 were identical, hence in this figure only IN-MR750R1 has been included. b) ~240 kb: Due poor assembly issue, IN-MR364R1 was
excluded from this analysis. Yellow ochre-colored arrows: different genes, green arrow: mcr-1.1. Shaded regions: percentage similarities.

Figure 4: Genetic environment of mcr-1.1 found among study isolates. Genes and their corresponding transcription orientations are indicated by horizontal arrows. Grey shaded region: homology, light yellow shaded region: inversion.
Figure 5: Transmission events of \textit{mcr-1.1} gene occurring within and in different maternal samples. Abbreviation: India-Maternal rectal specimen (IN-MR), resistant (R), susceptible (S), not found (NF), incompatibility (Inc).

<table>
<thead>
<tr>
<th>Mother Id (IN-MR)</th>
<th>Date of enrolment</th>
<th>Sequence type (ST)</th>
<th>Phylogroup</th>
<th>Phanid type (size in kb) with \textit{mcr-1.1}</th>
<th>Genetic background \textit{mcr-1.1}</th>
<th>Inter/intra-mother transmission</th>
<th>Identical mcr-negative strain</th>
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</thead>
<tbody>
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<td>ST1795</td>
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<td>IncHSV1 (-244kb)</td>
<td>Intra-mother</td>
<td>IN-MR56993</td>
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<tr>
<td>680R1</td>
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<td>Intra-mother</td>
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<td></td>
</tr>
<tr>
<td>683R1</td>
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<td>ST1795</td>
<td>A</td>
<td>IncHSV1 (-244kb)</td>
<td>Intra-mother</td>
<td></td>
<td></td>
</tr>
<tr>
<td>364R1</td>
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<td>IncHSV2 (-248kb)</td>
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<td>IN-MR56993</td>
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<tr>
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<td></td>
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<tr>
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<tr>
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<td>October, 2017</td>
<td>ST9423</td>
<td>B1</td>
<td>IncHSV2 (-216kb)</td>
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<tr>
<td>727R1</td>
<td>October, 2017</td>
<td>ST1286</td>
<td>A</td>
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<td>Intra-mother</td>
<td>NF</td>
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</tr>
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<td>727R2</td>
<td>October, 2017</td>
<td>ST373</td>
<td>B1</td>
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<td>759R1</td>
<td>October, 2017</td>
<td>ST394</td>
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<td>759R2</td>
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<td>ST394</td>
<td>D</td>
<td>IncHSV2 (-218kb)</td>
<td>Intra-mother</td>
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</tr>
</tbody>
</table>

When transmission occurred by plasmid, the plasmid is shown in blue. When transmission occurred by integration, the integration is shown in red.
Figure 6: A core genome phylogenetic tree summarising the isolates with short-read WGS available from this study. Due to contamination and poor assembly issue, IN-MR364R1 and IN-MR683R1 were excluded from this analysis.

Figure 7: Core genome phylogenetic tree of *mcr-1* E. coli collected from published studies in Southeast Asia. Sequence type (ST), green shading: study isolates. IN-MR364R1 and IN-MR683R1 were excluded due to contamination and poor assembly.
Table 1: Colistin susceptibility and genotypic characterization of \textit{mcr-1.1}-positive \textit{Escherichia coli}, their transconjugants (TCs) and other susceptible \textit{Escherichia coli} from the same rectal specimen.

<table>
<thead>
<tr>
<th>Strain Id</th>
<th>Colony Id</th>
<th>Phylo group</th>
<th>Sequence type (ST)</th>
<th>AMR phenotype</th>
<th>Colistin MIC (mg/L)</th>
<th>\textit{mcr} genes</th>
<th>Resistance determinants present/transfered</th>
<th>Plasmid types</th>
<th>Plasmid ST (pST) of \textit{mcr-1.1}-bearing plasmid</th>
<th>Virulence genes</th>
<th>Other bacterial species detected in the same rectal specimen</th>
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<td>\textit{mdf(A)}</td>
<td>\textit{bla\textit{TEM-1}}, \textit{bla\textit{EC-15}}, \textit{aadA1}, \textit{aadA2}, \textit{aph(3'\prime)}\textit{-Ib}, \textit{aph(3')}\textit{-Ia}, \textit{aph(6)\textit{-Id}}, \textit{cmlA1}, \textit{dfrA14}, \textit{floR}, \textit{mef(B)}, \textit{mph(A)}, \textit{suI3}, \textit{tet(34)}, \textit{tet(A)}</td>
<td>IncFIA (H11), IncFIB (K)</td>
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<td>\textit{gad}, ter\textit{C}</td>
<td>No</td>
</tr>
<tr>
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<td>181</td>
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<td></td>
<td>IncHI2 (~240 kb)</td>
<td>IncFIA (H11), IncFIB (K), IncH12, IncH2 A</td>
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<td>NA</td>
<td>No</td>
</tr>
<tr>
<td>T C</td>
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<td>ND</td>
<td>AMP</td>
<td>16 \textit{mcr-1.1}</td>
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<td></td>
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<td>NA</td>
<td>Absent</td>
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<td>R1.T C1</td>
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<td>gad, lpfA, terC</td>
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</table>

**IN-MR-569**

*Klebsiella pneumoniae*

**IN-MR-674**

*Pseudomonas aeruginosa, Acinetobacter baumannii*

**IN-MR-680**

*Klebsiella pneumoniae*
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<td>IncHI2, IncH2 A, IncN, IncX1, p0111</td>
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<td>C</td>
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<td>NA, gad, terC</td>
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**Acinetobacter baumannii**
Abbreviations: IN-MR (IN-India, MR-maternal rectal specimen), resistant (R), susceptible (S), minimum inhibitory concentration (MIC), Ampicillin (AMP), Cefuroxime (axetil or sodium) (CXM), Ceftriaxone (CRO), Cefoperazone (CFP)/Sulbactam (SUL), Cefepime (FEP), Nalidixicacid (NAL), Ciprofloxacin (CIP), Trimethoprim-sulfamethoxazole (SXT), Transconjugants (TC), kilobase pairs (kb), not applicable (NA), not done (ND), not found (NF). Shaded rows indicate mcr-1.1 bearing wild-type strains. Bold faced STs are novel to this study.

**Supplementary methods:**

**Processing of rectal swabs/ blood cultures, and detection of transmissible colistin-resistant genes, mcr:**

Rectal swabs were plated on chrome agar (BD BBL, MD, USA) supplemented vancomycin (10 mg/L) (MP Biomedicals, California, USA) and incubated at 37°C for 18-24 hours. Cultures from primary inoculum of each plates were screened for mcr genes by polymerase chain reaction (PCR) as described previously [1]. Likewise, blood samples obtained from neonates with suspected sepsis were cultured on blood agar plates (BD BBL, MD, USA) and screened for mcr genes by PCR. Samples positive for mcr genes were further enriched in Enterobacteriaceae Enrichment (EE) Mossel broth (18-24 hours at 37°C) (BD BBL, MD, USA)
and plated onto chrome agar supplemented with/without colistin (2 mg/L) (MP Biomedicals, California, USA). Enrichment not only prevented loss of any culturable organism from the specimens, but also helped to retrieve maximum number of organism from both types of plates. This further facilitated assessment of all culturable isolates within the specimen of same patient (mother or neonate) (intra-patient) or among different patients (inter-patient). Different colored colonies were picked from plates with/without colistin, and again screened for mcr genes. Amplified PCR products were processed for Sanger sequencing with primers as described by Ye et al. [2]. All possible PCR-positive colonies were glycerol stocked for further use. Any mcr-negative colonies with similar color as the mcr-positive ones were collected from chrome agar plates (without colistin) and glycerol stocked to compare with mcr-positive isolates.

**Whole genome sequencing (WGS) of mcr-positive/mcr-negative isolates:**

Distinct isolated colonies of same color with/without the mcr gene were cultured onto chromogenic UTI agar (Sigma Aldrich, St. Louis, MO, USA) supplemented with colistin (2 mg/L). DNA extraction and WGS was performed in Cardiff University and genomic DNA (gDNA) was extracted using the QIAamp DNA mini kit using the QIAcube (Qiagen, Germany) with an additional RNAse step. gDNA was quantified using the dsDNA BR assay kit on a Qubit fluorometer 3.0. For short-read sequencing, genomic libraries were prepared using Nextera XT V2 (Illumina, USA), with bead-based normalization. Paired-end WGS was performed on an Illumina MiSeq. Selected isolates (based on gDNA quantity) were subject to additional long-read sequencing utilizing the same gDNA extract which was concentrated and purified at a 1:1 ratio using SPRI beads (Mag-Bind TotalPure, Omega). Genomic libraries were prepared using the Rapid Barcoding Kit (SQK-RBK004; ONT), sequenced on a FLO-MIN106 R9.4 flow cell using a MinION (Oxford Nanopore Technology, UK) and base-called locally with Guppy within MinKnow. Genome assembly and annotation analysis of short-read data
has been described fully elsewhere [3]. Briefly, paired end fastq reads were trimmed with the following parameter -phred33 -q 25 --nextera --e 0.2 and reads were assembled using shovill (v0.9.0; --R1, --R2 otherwise default parameters). Genomes either 10% greater than or less than expected genome size for *E. coli* were excluded. Similarly, genomes with >500 contigs (short read only assemblies) were excluded and repeated where possible. *In silico* multilocus sequence typing (MLST) was determined using mlst (v2.17.6) (https://github.com/tseemann/mlst). ABRicate (v0.9.7) (https://github.com/tseemann/abricate) was used to screen contigs for ARGs using the Resfinder database with a >98% coverage and nucleotide identity applied [4]. For isolates with available long-read data, Unicycler (v0.4.7; -1, -2, -l --mode [normal] parameters applied) was used to generate a hybrid consensus assembly with the corresponding short-reads generated from Illumina. Isolates with novel ST allele profiles were submitted for evaluation using Enterobase (https://enterobase.warwick.ac.uk/species/ecoli/allele_st_search).

References:

