LETTER TO THE EDITOR

High prevalence of the mcr-1 gene in retail chicken meat in the Netherlands in 2015

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Abstract
Recently, plasmid-mediated colistin resistance was reported in humans, animals and food. We studied the presence of mcr-1 and mcr-2 in Dutch retail chicken meat. The prevalence of mcr-1 was 24.8% (53/214), whereas mcr-2 was not found. The presence of mcr-1-positive Enterobacteriaceae was confirmed by culture in 34/53 samples (64.2%). The prevalence depended on the supermarket chain and was lower in free-range chicken samples. The unexpected high prevalence of mcr-1 in food is cause for concern.

Keywords: Colistin resistance, Chicken meat, mcr-1, Netherlands, Prevalence, PCR method, Enterobacteriaceae

Article
Recently, a plasmid-mediated colistin resistance gene, called mcr-1, was reported from China [1], which was soon followed by several reports on mcr-1 positive Enterobacteriaceae from food, animals and the environment across the world [2, 3]. This is of particular concern as colistin is currently considered as a last resort agent for treatment of infections with isolates that contain other resistance traits, like extended-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae or carbapenem-resistant bacteria [4–6].

Recent investigations using metagenomics, indicated a substantial larger environmental reservoir regarding the mcr-1 gene in the Chinese population [7]. This indicates that other approaches are needed to reveal the true reservoir of mcr-1.

In the Netherlands, mcr-1 was detected at low prevalence in E. coli isolates from livestock and meat (< 2%) and at very low frequencies in the human population [6, 8, 9]. The aim of this study was to determine the prevalence of mcr-1 and mcr-2 in a collection of poultry samples from Dutch supermarkets using a PCR-based method.

Collection and analysis of retail chicken samples
Chicken meat samples (n = 214) were bought from four supermarket chains throughout the Netherlands in 2015. The number of samples was balanced across supermarkets and one sample per production batch was included. Meat samples were enriched overnight in non-selective tryptic soy broth (TSB) and subsequently stored at -80 °C until further testing. DNA was isolated from 50 μl of the defrosted TSB using NucliSens EasyMAG (Biomérieux). Detection of mcr-1 and mcr-2 gene was performed by real-time multiplex PCR (ABI 7500 system) using the following primers and probes: mcr1-2_forward AAATGCMTRCARACCGACCAAG, mcr-1_reverse TCTCACCACCAATCACCCAC, mcr-1_probe YY-BHQ1 TTTGATGGCGCCGATTGGGCTTGATC, mcr-2 probe FAM-BHQ1 TGCAGACCACCAAGCCGAGCGAG. Control isolates that contained either mcr-1 or mcr-2 were used.

Concurrently, 100 μl of TSB was inoculated in fresh TSB and incubated at 35-37°C overnight. Subsequently, 10 μl of this overnight grown TSB was streaked onto a CLED-colistin-agar with 1.5 μg/ml colistin (Duchefa) and 10 μg/ml Daptomycin (Novartis). All colistin resistant isolates that could grow on the selective CLED-colistin-agar were confirmed by Vitek MS (Biomérieux) and non-intrinsic colistin resistant isolates found, were further tested for the presence of mcr genes by PCR. The isolates were tested by broth-micro-dilution (BMD), in cation-adjusted Mueller Hinton broth [10], for colistin susceptibility and Vitek2

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(AST N344) (Biomérieux) to determine the susceptibility for various other antibiotics.

Prevalence of MCR-1 in retail chicken samples
The prevalence of mcr-1 on retail chicken meat using PCR on TSB was 24.8% (53/214 samples positive) and no mcr-2 was detected. Using a selective culture method, the presence of mcr-1 was confirmed in 34 of these 53 (64.2%) samples with a positive result by PCR. Using this culture method, intrinsically resistant isolates had the ability to grow as well. No mcr-positive Enterobacteriaceae were found in all other samples. E. coli was identified in 32 samples, and K. pneumoniae in two samples. The median CT-value of the culture positive samples was significantly lower: culture-positive, 25.6 and culture-negative, 30.1, \( p < 0.001 \), Mann-Whitney U-test).

The prevalence of mcr-1 according to the method of farming husbandry (free range: yes/no) and supermarket chain is shown in Table 1. Using multivariable regression analysis it was shown that both variables were statistically significant and independently related to the presence of mcr-1. We also investigated the country of origin (COO), as indicated on the label (Table 1). This variable showed co-linearity with the supermarket chain and was not included in the multivariate analysis (Fig. 1).

The in vitro antimicrobial susceptibility for the 35 mcr-1 positive isolates, which were found by culture, is shown in Table 2. One sample (nr. 11) harboured two isolates which were mcr-1 positive but with different susceptibility patterns. There were high levels of resistance against ampicillin (100%), amoxicillin-clavulanic acid (89%), trimethoprim/sulfamethoxazol (69%) and ciprofloxacin (57%). Only one mcr-1 positive ESBL-producer was found (sample 34) and all isolates were susceptible to meropenem.

Discussion
In this study, a PCR-based detection method identified an unexpected high prevalence (24.8%) of mcr-1 in retail chicken meat samples and no mcr-2 was found. The majority of the PCR positive samples were confirmed by selective culture. The 19 PCR positive samples that could not be confirmed by culture were all overgrown by intrinsically colistin-resistant bacterial species (e.g. Serratia spp. and Proteus spp.), which decreases the sensitivity of the culture for mcr-1 harbouring bacteria. In addition, the higher CT-values in the culture-negative samples are indicative of a lower bacterial load, which may further explain the negative findings. Moreover, the freeze-thaw step of the samples might have played a role in the viability of the colistin-resistant bacteria.

A link between mcr-1 in humans and food has been proposed in the first report from China, in which 28% of poultry samples harboured mcr-1 [1]. In a study from south America, chicken meat was also identified as a reservoir for mcr-1-harboring E.coli isolates (19.5%) based on a selective culture approach. It should be realized that Brazil is the third-largest chicken meat producer and the largest exporter of this product [11]. Subsequent studies confirmed the presence of mcr-1 in isolates from poultry and other meat products from Europe, but at much lower rates [2]. A recent study form Germany, including 580 E. coli isolates from chicken meat, found a decreasing prevalence of mcr-1,

| Determinant         | Samples | mcr-1 PCR positive n (%) | OR (95% CI) | Adjusted OR (95% CI) |
|---------------------|---------|--------------------------|-------------|----------------------|
| Labelling as free-range |         |                          |             |                      |
| Yes                 | 70      | 10 (14.3)                | reference   | reference            |
| No                  | 144     | 43 (29.8)                | 2.6 (1.2-5.5) | 3.0 (1.3-6.6)        |
| Supermarket chain   |         |                          |             |                      |
| A                   | 53      | 1 (1.9)                  | reference   | reference            |
| B                   | 53      | 10 (18.9)                | 12.1 (1.5-98.3) | 12.5 (1.5-101.8) |
| C                   | 54      | 21 (38.9)                | 33.1 (4.2-257.8) | 34.6 (4.4-272.0) |
| D                   | 54      | 21 (38.9)                | 33.1 (4.2-257.8) | 37.5 (4.8-295.3) |
| Country of origin a |         |                          |             |                      |
| NL                  | 67      | 21 (30.3)                | 2.9 (1.3-6.5) |
| GER                 | 44      | 19 (43.2)                | 4.8 (2.0-11.4) |
| DEN                 | 9       | 1 (11.1)                 | 0.8 (0.1-6.9)  |
| NL/GER              | 80      | 11 (13.8)                | reference   |                      |
| NL/GER/BE           | 12      | 0 (0.0)                  | not applicable |                      |
| Unknown             | 2       | 1 (50.0)                 | 6.3 (0.4-107.8) |

*a NL The Netherlands, GER Germany, DEN Denmark, BE Belgium*
from 8.1% in 2011 to 0.5% in 2014, however, this was based on isolate screening [12].

At present, \textit{mcr-1} is only sporadically found in humans in the Netherlands [6, 8, 9]. This is in a situation where colistin and other polymyxins are used at very low levels. In 2014, polymyxins constituted less than 0.1% (0.01 defined daily dose (DDD)/1000 inhabitant-days) of all systemic antimicrobials used in primary care and 0.3% (0.2 DDD/100 patient-days) in the hospital setting [13]. Therefore, the selective pressure is currently low. Also, it should be taken into account that more selective approaches are necessary to reveal the true presence of \textit{mcr-1} in humans. Both the current study and the study by Wang et al. show that direct molecular techniques, molecular techniques after enrichment steps and selective culture techniques result in much higher prevalences compared to studies using non-targeted methods [7]. Considering the low selective pressure in humans and the lack of data on the resistome in humans it is not evident what the implications of these findings are for public health on the short or long term.

The culture approach showed that the majority of the \textit{mcr-1} positive isolates were susceptible to cephalosporins, carbapenems and aminoglycosides. Apparently, the \textit{mcr-1} gene is frequently present in isolates that are

\begin{figure}
\centering
\includegraphics[width=\textwidth]{distribution.png}
\caption{Distribution of the \textit{mcr-1} positive and negative chicken meat samples across supermarket chains and country of origin of poultry (n = 214)}
\end{figure}

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\textbf{Antimicrobial susceptibility of \textit{mcr-1} positive Enterobacteriaceae isolated from Dutch retail chicken meat, 2015} & & & & & & \\
\hline
\textbf{Antimicrobial} & \textbf{Antibiotic} & \textbf{BMD} & \textbf{Vitek2} & \textbf{Phenotypic} & \textbf{Phenotypic} \\
\hline
\textbf{Cephalosporin} & & & & & \\
\hline
\textbf{Carbapenem} & & & & & \\
\hline
\textbf{Aminoglycoside} & & & & & \\
\hline
\textbf{Selective culture techniques} & & & & & \\
\hline
\textbf{Enrichment steps} & & & & & \\
\hline
\textbf{Non-targeted methods} & & & & & \\
\hline
\textbf{Red in the table represents resistant, yellow intermediate and green susceptible for the given antibiotics according to EUCAST guidelines} & & & & & \\
\hline
\end{tabular}
\end{table}
susceptible to most classes of antibiotics. This might explain the relative low prevalence of mcr-1 in studies that have primarily focused on isolates with other resistance traits [6, 14–17].

The differences in mcr-1 prevalence between supermarket chains are remarkable, with the two chains with the highest prevalence (C and D) having an odds ratio that is approximately 35 times higher compared to the supermarket chain with the lowest prevalence, after adjusting for free-range rearing of the animals. We attempted to extend the multivariable regression analysis to study the reservoir of the mcr-1 gene to COO. However, in most cases, multiple countries are named on one sample without further specification. In addition, there was co-linearity with the supermarket chain (Fig. 1), prohibiting to include these variables in the regression model. Further details on the production process could not be studied as the label on the package does not provide further information. The conclusion is that there are large differences in the prevalence of mcr-1 between supermarkets which we cannot explain with the available information. As shown in Table 2, there were variable susceptibility patterns to other antibiotics, which showed a tendency to cluster within supermarket chains. We cannot draw conclusions based on these data. This would require additional research. It would be important to extend the investigations into the different chains of production of chicken meat to identify the determinants of the presence of mcr-1.

In conclusion, we have shown a high prevalence of mcr-1 in chicken meat with a large and unexplained variation between supermarket chains. The approach, specifically targeting the presence of mcr-1, resulted in a much higher prevalence than previous studies that did not specifically target colistin resistance. These findings warrant further studies to elucidate the underlying mechanisms of spread and the genetic location of the mcr-1 gene. Moreover, continued monitoring of the potential reservoirs for this plasmid-mediated colistin resistance is of utmost importance.

Abbreviations

COO: Country of origin; DDD: Defined daily dose; ESBL: Extended-spectrum beta-lactamase

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Availability of data and materials

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

Authors’ contributions

The study was planned and designed by ES, JK, MK, NS, CV and PH. PH collected the samples. NS and CV conducted the experiments. The interpretation of the results was done by ES, JK, MK and PH. The manuscript was prepared by ES and JK. All authors contributed to and commented on the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The author(s) declare(s) that they have no competing interests.

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