DETECTION OF PLASMID MEDIATED COLISTIN RESISTANT (*MCR-1*) GENE IN *SALMONELLA* SPP. ISOLATED FROM CHICKEN

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ABSTRACT. The emergence of multidrug resistant (MDR) microbes is a major threat worldwide, and it has become worse with the emergence of resistance towards colistin antibiotic. Presence of plasmid mediated colistin resistant gene (*mcr-1*) in bacteria contributes much to the spread of colistin resistant among Enterobacteriaceae such as *Escherichia.coli* and *Salmonella* spp. Since the first description of *mcr-1* gene in *E. coli* from livestock in 2015, numerous reports had revealed a worldwide spread of *mcr-1* gene among foodborne pathogen. In Malaysia, there is a lack of data on detection of *mcr-1* gene among *Salmonella* strains in food producing animals. Hence, this paper focuses on the detection of *mcr-1* gene in *Salmonella* spp. isolated from chickens submitted to the Veterinary Research Institute (VRI) using published primers. A total of 284 salmonella isolates retrieved from monitoring and surveillance cases submitted to VRI from year 2016 and 2017 were used in this study. 3.17% (9/284) of the isolates were found to carry the *mcr-1* gene. This finding supports the existence of *mcr-1* gene in *Salmonella* spp. from chickens in the country. Antimicrobial resistance problems become complex when the bacterial strain carrying *mcr-1* gene become highly resistant to colistin, the last choice of antibiotic mainly used for the treatment against Gram-negative bacterial infection both in human and animals. Inter-agency collaboration to gather baseline data on colistin usage is crucial to assess the issue and help draw guidelines to reduce the impact of colistin resistance in Malaysia.

Keywords: Salmonella, *mcr-1* gene, chicken.

INTRODUCTION

The continuous rise of antimicrobial resistance is a worldwide concern, and the emergence of multidrug resistant bacteria has led to the revival of colistin usage in both human and animal health. The antibiotic Colistin, which is previously known as polymyxin E, is reserved as the last resort for treating infections caused by carbapenem resistant Enterobacteriacae (Wang *et al*., 2018). Colistin is a cationic antimicrobial polypeptide cylic, which has been introduced for clinical use in human medicine as early as 1950s. However, its use has been diminished since 1980s due to its nephrotoxic and neurotoxic effects (Schwarz and Johnson, 2016). In veterinary medicine, colistin has been extensively used as a prophylactic additive in livestock feed (Ohsaki *et al*., 2017). Besides that, colistin is the drug of choice for treating Gram-negative enteric infections including colibacillosis, septicaemia, salmonellosis and urinary infections in various animal species (OIE, 2014). Over time, increasing reports of resistance in Gram-negative bacteria and rise in infections caused by multidrug resistant Enterobacteriacea including *Escherichia coli*, *Klebsiella pneumonia*, *Acinetobacter baumannii* and *Pseudomonas aeuroginosa* has led to the resuscitation of older class

and less user-friendly antibiotics including polymyxins as a treatment option (Olaitan *et al.,* 2014).

Widespread dissemination of polymyxin resistance among Enterobactericea in animals was speculated due to the extensive use of polymyxins (Cui *et al*., 2017). Previously, the resistance to polymyxins was commonly associated with modification of lipopolysaccharide (LPS) outer membrane of the bacteria (Anjum *et al*., 2016). However, recent discovery of mobile colistin resistant gene (*mcr-1*) further complicates the scenario of colistin resistance (Zhang *et al*., 2018). This transferable plasmid mediated resistance gene encodes the lipid A modification enzyme, phosphoethanolamine transferase can interact with lipopolysaccharide and phospholipids at the outer membrane of Gram-negative bacteria, thus preventing colistin from binding to the bacterial cell wall impairing colistin function (Liu *et al*., 2016). This novel mechanism of polymyxin resistance became a centre of issues highlighted in combatting antimicrobial resistance because previous well-known polymyxin resistance developments were associated with chromosomal mutation (Cannatelli *et al*., 2018)

The first report on plasmid mediated colistin resistance mechanism revealed the presence of *mcr-1* gene circulating in commensal *Escherichia coli* isolated from pigs and poultry in China, and occurrence of the *mcr-1* gene in Enterobacteriacea in many parts of the world increased rapidly due to a great deal of discoveries since the first publication (Anjum *et al*., 2016). Worldwide reports revealed the detection of *mcr-1* gene in a range of Enterobacteriaceae found in both healthy and diseased human and animal hosts (Doumith *et al*., 2016; Barbeiri *et al*., 2017). On the other hand, detection of *mcr-1* gene among *E. coli* culture collection from animal samples indicates that the *mcr-1* gene existed in Malaysia (Liu *et al*., 2016).

Data on distribution of *mcr-1* gene in foodborne pathogen including *Salmonella* sp. in the country is still lacking. Evidence of positive detection of *mcr-1* gene in *Salmonella* sp. may be a triggering alarm signs to public health risks as they can spread rapidly by horizontal transfer between animals and the human population. Therefore, this study is conducted to investigate the presence of *mcr-1* gene in S*almonella* sp. isolated from chicken, as the poultry industry is a well sustained livestock business in Malaysia. Findings from this study may hasten the implementation of proper guidelines to reduce and control the problem of antimicrobial resistance in animal health in Malaysia.

MATERIALS AND METHOD

Isolation of *Salmonella* sp.

In this study, a total of 284 *Salmonella* sp. isolates from the top five predominant serotypes identified in the Bacteriology Laboratory, Veterinary Research Institute (VRI) in years 2016 and 2017, were screened for presence of the *mcr-1* gene. The isolates were obtained from chicken meat and cloacal swab samples collected from processing plants and poultry farms all over Malaysia for monitoring or surveillance of salmonellosis. Isolation and identification

of *Salmonella* isolates were conducted by five Veterinary Regional Laboratories and at VRI, according to standard protocols (VRI, 2015). The isolates were submitted to VRI in nutrient agar slant for further serotype identification. The isolates were reconfirmed as *Salmonella* sp. according to standard biochemical tests as described in protocols (VRI, 2015) and further tested with commercial Salmonella antisera Poly A-S (Staten Serum Institute, Denmark).

Serotyping of *Salmonella* sp.

The determination of *Salmonella* serotypes was conducted using slide agglutination and Schworm agar method using the commercial somatic and flagellar salmonella antisera (Staten Serum Institute, Denmark) according to Kauffmann-White Scheme (Grimont and Weill, 2007). The top five most frequently identified *Salmonella* serotypes in chicken for years 2016 and 2017 were selected for this study.

Determination of 'O' antigen using slide agglutination method

A loopful of normal saline was placed on a glass slide. *Salmonella* grown on nutrient agar slant was mixed into the first saline drop on the slide, and the step was repeated with the second drop for negative control test to ensure a smooth, opaque suspension in both drops. Any positive agglutination with normal saline was reported as auto-agglutination and no further serotyping was conducted.

One drop of salmonella polyvalent 'O' (A-S/42-67) antisera was added to the first drop on glass slide, and the glass slide was rocked gently for 1 minute and observed for agglutination. If positive agglutination of the polyvalent 'O' (A-S/42-67) antisera was observed, the sample need to be further tested with each single group of antisera (OMA, OMB, OMC,OMD, OME, OMF and OMG) on glass slides with a loopful of bacterial suspension. Positive agglutination for each group was recorded and further tested with specific monovalent 'O' antisera for the positive 'O' group (Figure 1).

Determination of Phase 1 'H' antigen using Schworm agar method

Schworm agar for 'H' antigen test was prepared following manufacture protocols. *Salmonella* culture from nutrient agar slant was transferred to solidified Schworm agar (SSI) by spot inoculation at the centre and incubated at 37 °C for 18 hours. After that, a loopful of growth from edges of the motility zone on Schworm agar was removed and mixed into the drop of normal saline on a glass slide. A drop pf polyvalent 'H' antisera was mixed to the suspension and rocked gently for 2 minutes to observe for agglutination. If positive agglutination was observed, the sample needs to be further tested with each single group antisera (HMA, HMB, HMC, HMD, HME and HMF) on a glass slide with a loopful of bacterial suspension.

Determination of Phase 2 'H' antigen using Schworm agar method.

A drop of concentrated antisera against the detected 'H' antigen in Phase 1 'H' step was added and mixed well to a small

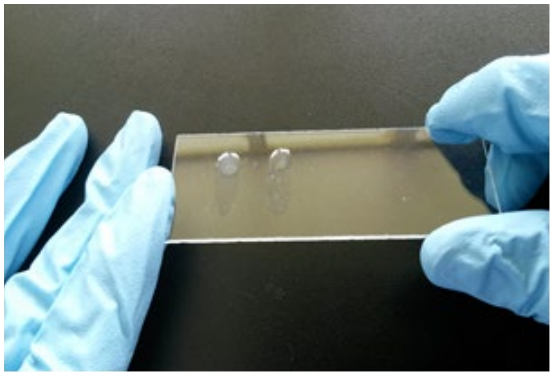

Figure 1. Determination of 'O' antigen using slide agglutination method. Positive result show agglutination (right). Negative result show no agglutination (left).

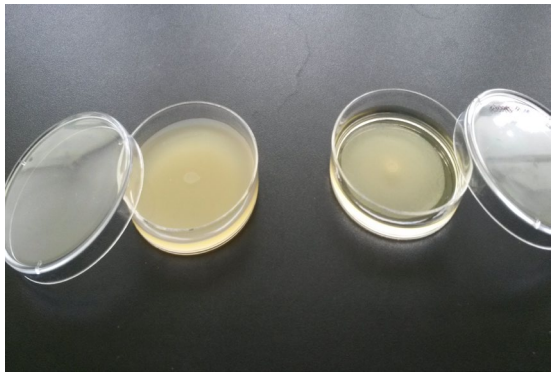

Figure 2. Determination of 'H' antigen using Schworm agar method. Positive result show swarming on the agar (right). Negative result show no swarming on the agar (left).

petri dish with 5 ml melted Schworm agar (55-60 °C). The Schworm agar was allowed to solidify at room temperature. After that, *Salmonella* culture from phase 1 'H' step was inoculated in one spot at the centre of the agar and incubated at 37 °C for 18 hours. The Phase 2 'H' antigen was detected using the same procedure as described for phase 1 'H' antigen. Identification of the specific serovar in the Kauffmann–White Scheme depends on the combination of both 'O' and 'H' reactions. Necessary biochemical test is needed if other than *Salmonella enterica* subsp. *enterica* is suspected in the scheme (Figure 2).

DNA extraction

DNA extraction of *Salmonella* strain was by the boiling method. A loopful of *Salmonella* culture was suspended in 100 µl of sterile distilled water. The suspension was boiled for 15 minutes at 95 °C. Then, the suspension was centrifuged for 3 minutes at 13,000 rpm and the supernatant was used as the DNA template.

Polymerase Chain Reaction (PCR)

All isolates were screened for the presence of *mcr-1* gene using published primers CLR-F (5′-CGGTCAGTCCGTTTGTTC-3′) and CLR-R (5′-CTTGGTCGGTCTGTAGGG-3′) as described in the laboratory protocol by Statens Serum Institute (Cavaco and Hendriksen, 2015). A 25 µl PCR reaction was carried out with the following amplification conditions 94 °C for 15 min; 25 cycles of 94 °C for 30 sec; 58 °C for 90 sec; 72 °C for 60 sec with a final extension of 72 °C for 10 min. PCR generated amplicons were run on a 1.5% agarose gel at 100V for 45 minute and visualised for 309 bp of PCR product using gel documentation system (Major Sciences, USA).

RESULTS

Distribution of the top five *Salmonella* serotypes in chicken samples received by VRI in year 2016 and 2017 based on diagnostic cases showed that the most predominant *Salmonella* serotypes identified in chicken were *S*. Brancaster (40.5%; 115/284), followed by *S*. Enteritidis (22.5%; 64/284), *S*. Corvallis (16.2%; 46/284) *S*. Albany (15.5%; 44/284) and *S*. Typhimurium (5.3%; 15/284) (Figure 3).

The plasmid mediated colistin resistance gene (*mcr-1*) was detected in 3.17% (9/284) of *Salmonella* isolates tested. Positive *mcr-1* gene was frequently detected in 4.3% of both *S*. Brancaster (5/115 isolates) and *S*. Corvallis (2/46 isolates). The gene was also found in one isolate of *S*. Albany and *S*. Typhimurium. However, *mcr- 1* gene was not detected in *S*. Enteritidis tested in this study (Table 1). The *S*. Brancaster harbouring *mcr-1* isolated from cloacal swab, while *S*. Corvallis,

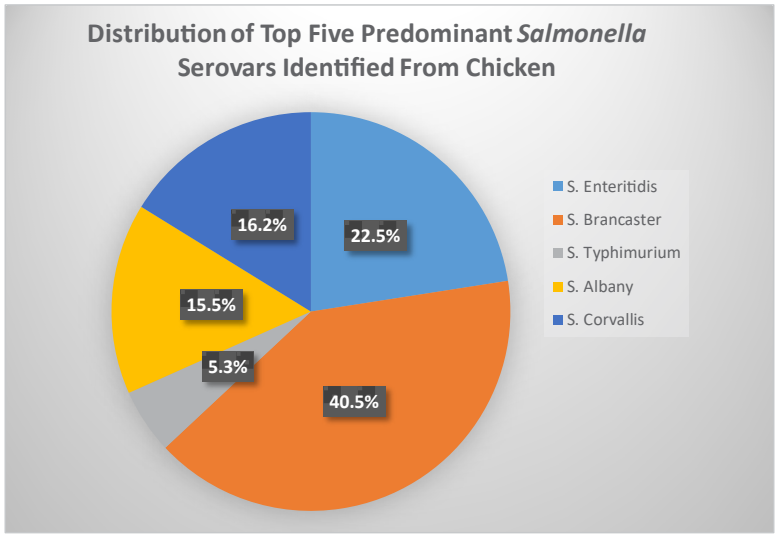

Figure 3. Distribution of top five most common identified *Salmonella* serovars from chicken in year 2016 and 2017.

Table 1. Detection of *mcr-1* gene among selected *Salmonella* serovars isolated from Chicken.

Serotypes	Total of isolates	Positive *mcr-1* gene
S. Brancaster	115	5
S. Enteritidis	64	0
S. Corvallis	46	2
S. Albany	44	1
S. Typhimurium	15	1
Total	284	9

S. Albany and *S*. Typhimurium detected with *mcr-1* were isolated from chicken meat.

In Malaysia, the first detection of *mcr-1* gene was found in *E. coli* cultures in a collection of chicken and pig samples (Liu *et al.,* 2016). Since then, several studies on retrospective detection of this gene in *E. coli* have been reported. Retrospective studies on more than 900 bacteria isolates retrieved from different sources and locations in the country have been conducted (Yu *et al.,* 2016), which revealed the presence of *mcr-1* gene in *E. coli* isolated from chicken visceral organ and feed. A high frequency of *mcr-1* gene was also found in *E. coli* isolated from raw chicken meat and chicken samples (Aklilu *et al*., 2016; Roseliza *et al*., 2016). However, there has been no report on *mcr-1* gene detection in other food-borne pathogens such as *Salmonella* sp. in the country. Thus, for the first time, this study reports positive detection of *mcr-1* gene of commonly identified salmonella serovars isolated from chickens.

The first report on the detection of *mcr-1* gene in *Salmonella* sp. was through analysis of whole genome sequencing (WGS) that is available in GenBank, which identified *mcr-1* bearing plasmid in clinical *S. enterica* isolates including *S*. Typhimurium, *S*. Paratyphi B var Java and *S*. Virchow (Cui *et al.,* 2017). Subsequently, the *mcr-1* gene has been detected in *Salmonella* strains isolated from food and animal specimens in many countries including Europe, US and China (Doumith *et al*., 2016). The prevalence of *mcr-1* gene in *Salmonella* species in this study was low (2.8%), in agreement with several reports in Europe and other Asian countries (Sakdinun *et al*., 2017; Garch *et al*., 2017, Cui *et al*., 2017). Sakdinun *et al.* (2017) reported that *mcr-1* gene was found in *Salmonella* enterica Give, the most predominant serovar isolated from broilers in Thailand. A recent study in Taiwan also revealed that *mcr-1* gene was detected in *S*. Typhimurium isolated from both chicken and pigs which were derived from multiple origins using pulse field gel electrophoresis (PFGE) profiles (Chiou *et al*., 2017). Carfora *et al.* (2018) demonstrated evidence of *mcr-1* in *Salmonella* enterica Infantis, the most frequently identified serovar in broiler and among the top five serovar involved in human infection in EU countries. All the *mcr-1* positive *S*. Infantis isolates also exhibited a multidrug resistant profile including colistin. These findings support the evidence of *mcr-1* in different *Salmonella* serovars, and it was frequently found in predominant serovars isolated in different geographical locations.

Cui *et al.* (2017) discovered that the existence of *mcr-1* gene among different *Salmonella* serovars suggested that there was a strong association with the *mcr-1* bearing plasmid that was harbored by specific serotypes such as *S*. Typhimurium (Cui *et al*., 2017). Briefly, *mcr-1* gene was not detected in *S*. Enteritidis, the most predominant salmonella serotypes isolated from chicken. However, *mcr-1* gene was frequently detected in *S*. Brancaster, which is found significantly increasing in chickens since 2016. Hence, it is highly suggested that *Salmonella* resistance to colistin also depends on their LPS structure although they found to harbour *mcr-1* plasmid mediated gene (Agerso *et al*., 2012). The presence of *mcr-1* gene is suggested to facilitate in the development of colistin resistant among the

Salmonella isolates, by inducing permeability of LPS to colistin. On the other hand, Carfora *et al*. (2018) demonstrated that certain *Salmonella* serovar such as *Salmonella enterica* Typhimurium acquires specific plasmid encoding for Multiple Type II toxin/ Antitoxin module which plays an important role in sustaining plasmid or genomic islands in bacteria, thus enhancing their fitness inside eukaryotic cells. Therefore, it further supports the findings why *mcr-1* was likely to be detected in certain *Salmonella* serovar. Based on this finding, *mcr-1* gene was frequently detected among non-host adaptive *Salmonella* serovar, which is capable of infecting and colonising a wide range of hosts, without showing any clinical signs. They are also considered as asymptomatic excretor in healthy animals, and the main reservoir of *mcr-1* gene among *Salmonella* strain in animals causing ultimate challenge in the animal industry causing significant financial losses as it can persist in *Salmonella* strains with low levels of resistance to colistin (Lima *et al*., 2019).

The presence of *mcr-1* in *Salmonella* isolated from chicken poses a great concern, because *mcr-1* was found on conjugative plasmid, which is transferable at rather high frequencies to both humans as well as animals. Numerous studies have revealed that many *mcr*- harbouring plasmid also carry other resistant genes such as extended spectrum beta lactamase (ESBL) and carbapenems, which can facilitate and promote the co-transfer of those genes on the same conjugative plasmid (Schwarz and Johnson, 2016).

CONCLUSION

In conclusion, the finding from this study prompts a great concern on the potential transmission of the *mcr-1* gene in Enterobacteriacea in the country. Therefore, immediate action such as through the implementation of prudent selection and usage of colistin in livestock industries is crucial to minimise the transmission of this resistant gene.

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