Susceptibility of *Campylobacter* Strains to Selected Natural Products and Frontline Antibiotics

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1. Introduction

❑ **Why testing *Campylobacter* species against frontline antibiotics?**

- Campylobacter species causes diarrhea, different sequelae (GBS, RA, IBD), & have developed resistance to existing antibiotics.
- The resistance to drugs of choice (Quinolones & Macrolides) and alternative drugs is of concern.
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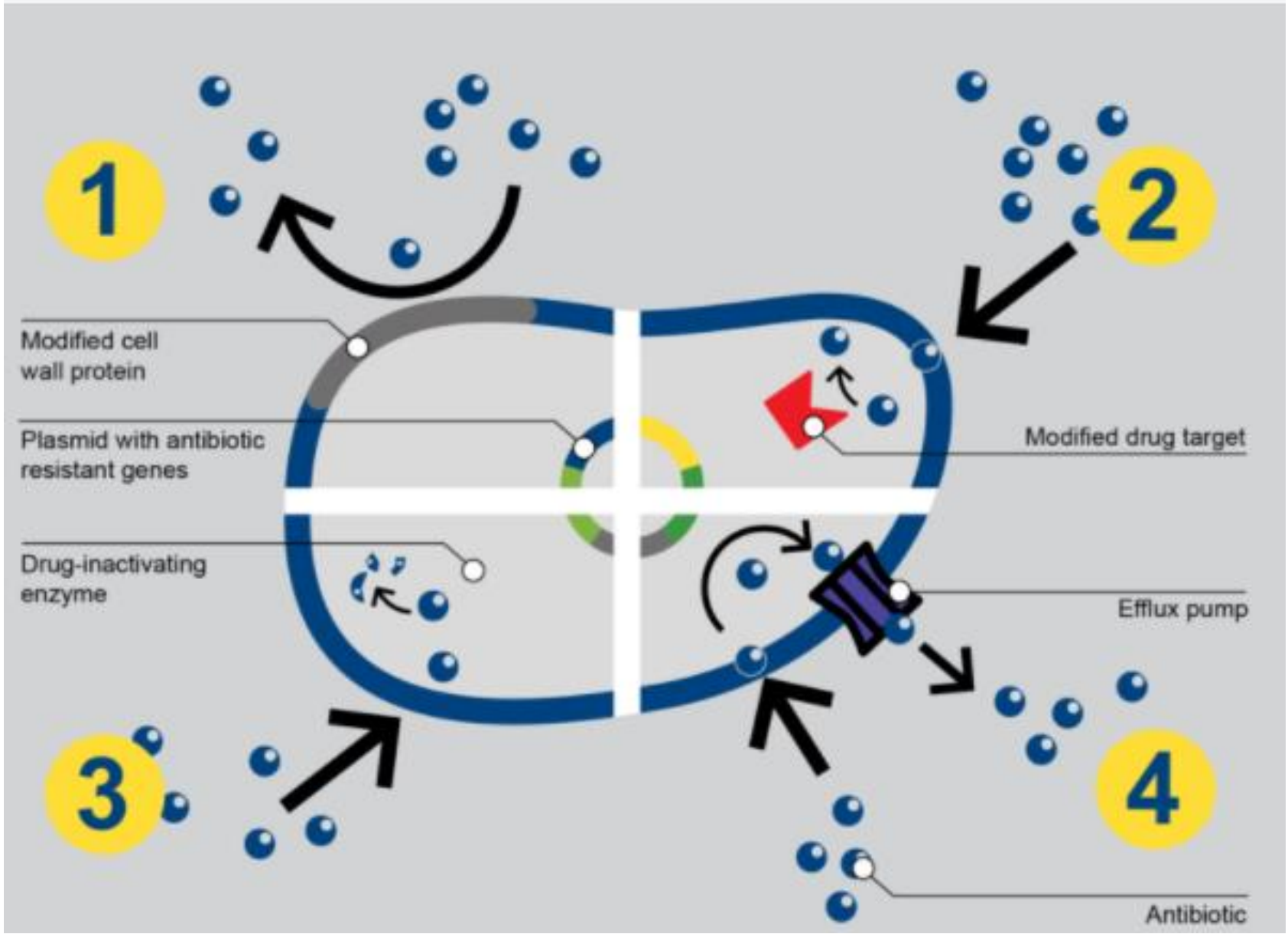
- The over/misuse of antibiotics in human and veterinary medicine contribute to increasing resistance to antibiotics.
- Mutations in specific genes & acquiring efflux pumps

- Sequelae:
  GBS: Guillin- Barré Syndrome
  RA: Reactis Arthritis
  IBD: Inflammatory Bowel Disease
- *Quinolones:*
  - *Ciprofloxacin*
  - *Nalidixic acid*
- *Macrolides:*
  - *Erythromycin*
  - *Azithromycin*

Growth promotion, prophylaxis, therapy

*gyr*A, *tet*(O), *cme*ABC



Mechanisms of resistance

1. Introduction cont'd…

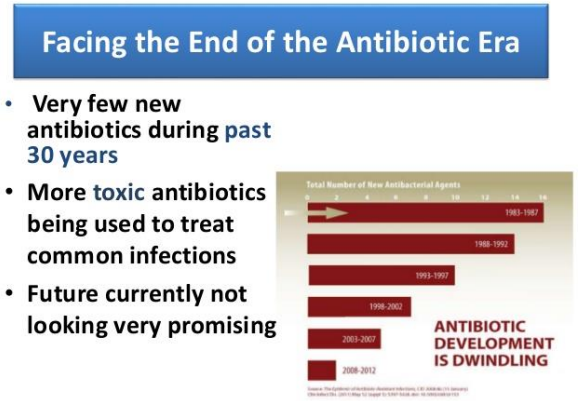
- **Why testing *Campylobacter* species from chicken against natural products?**

- Fluoroquinolone-resistant *Campylobacter* species in on WHO list of pathogens requiring urgent alternative treatment.
- By 2050, if no adequate actions are taken, the annual death rate due to AMR would reach 10 M people worldwide and cost USD 100 trillion.
- Poultry is at the top of major reservoirs of fluoroquinolone-resistant *Campylobacte*r strains.
- End of antibiotic era.
- Herbal medicines are safe, cheap, and 87% of citizens of LMICs rely on medicinal plants for primary healthcare.

LIMCs: Low and middle income countries

- natural products:
  - Plant extracts
  - Essential oils
  - Phytochemicals

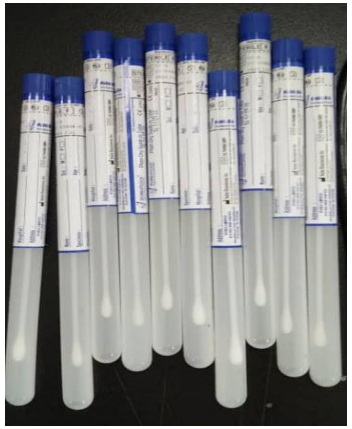
- *WHO: World Health Organization*
- *AMR: Antimicrobial resistance*



2. Materials & methods

Sample collection → Swabs put on ice → ATCC cultures

Two poultry farms in Korea



Transported to the laboratory within 1hr

Campylobacter jejuni subsp. *jejuni*
ATCC® 33560™

Campylobacter coli (Doyle) Veron and Chatelain
ATCC® 33559™

➢Isolation & antimicrobial susceptibility testing (AST)

❑Isolation

➢ **Inoculation**

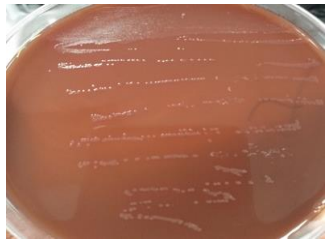
- Selective media (mCCDA + SR155E)

➢ **Incubation**

- 37°C for 48 h
- Microaerophilic conditions

➢ **Subculture**

- Mueller Hinton Agar (MHA) + 5% Horse Blood



- **Species confirmation: PCR & sequencing**

❑Antimicrobial susceptibility testing

➢ **Antibiotics**

- Quinolones (ciprofloxacin, nalidixic acid)
- Macrolide (erythromycin)
- Tetracyclines (tetracycline)
- Aminoglycosides (gentamicin)

mCCDA: modified charcoal cefoperazone deoxycholate agar

Target gene	Primer name	Sequence (5' -3')	Amplicon size	Annealing T°
16S rRNA	C412F	GGATGACACTTTTCGGAGC	816	55
	C1228R	CATTGTAGCACGTGTGTC		
cj0414	C1F	CAAATAAAGTTAGAGGTAGAATGT	161	
	C3R	CCATAAGCACTAGCTAGCTGAT		
ask	CC18F	GGTATGATTTCTACAAAGCGAG	502	
	CC519R	ATAAAAGACTATCGTCGCGTG		
tet(O)	*tet*(O)F	GCGTTTTGTTTATGTGCG	559	
	tet(O)R	ATGGACAACCCGACAGAAG		
*cjgyr*A	QRDRF	GCCTGACGCAAGAGATGGTTTA	454	
	QRDRR	TATGAGGCGGGATGTTTGTCG		
*cme*B	*cme*BF	TCCTAGCAGCACAATATG	241	
	*cme*BR	AGCTTCGATAGCTGCATC		

Target genes, primer sequences, & amplification conditions

Information on used Natural products

➢ Plant extracts

Library code	Family	Scientific name	Common name	Collection site	Collection date	Part of plant	Extraction solvent
BE0005B1	Lamiaceae	*Meehania urticifolia* (Miq.) Makino	Nettle-leaf mint	Gangneung, Gangwon	2016	Aerial part	Ethanol
BE0165A1	Lamiaceae	*Scutellaria baicalensis* Georgi	Skullcap	Yeosu, Jeonnam	2017	Root	Ethanol
BE0167A1	Lamiaceae	*Mentha canadensis* L.	Wild mintt	Andong, Gyeongbuk	2017	Aerial part	Ethanol
BE1192A1	Lamiaceae	*Salvia plebeia* R.Br.	Common sage	Paju, Gyeonggi	2015	Whole plant	Ethanol
BEA585A1	Lauraceae	*Cinnamomum cassia* (L.) J.Presl	Cinnamon	Gyeongdong Seoul	2015	Bark	Ethyl acetate

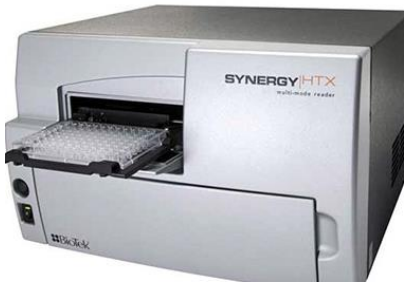
➢ Essential oils

- Clove oil
- Cinnamon oil

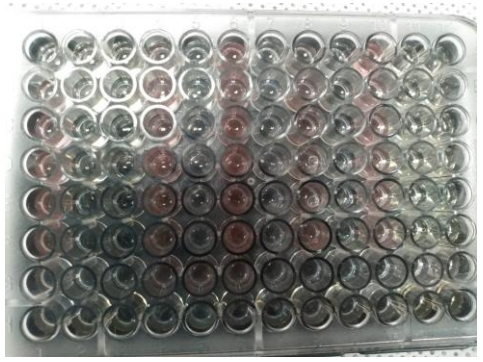
➢ Phytochemicals

- Eugenol
- (E)-Cinnamaldehyde
- Baicalein
- Kuraridin
- Emodin

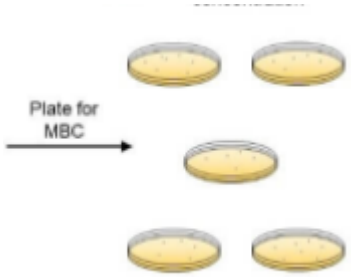
- MIC: minimal inhibitory concentration
- MBC: minimal bactericidal concentration
- EUCAST cut-off values



OD at 600nm



Iodo nitro tetrazolium chloride (INT addition)



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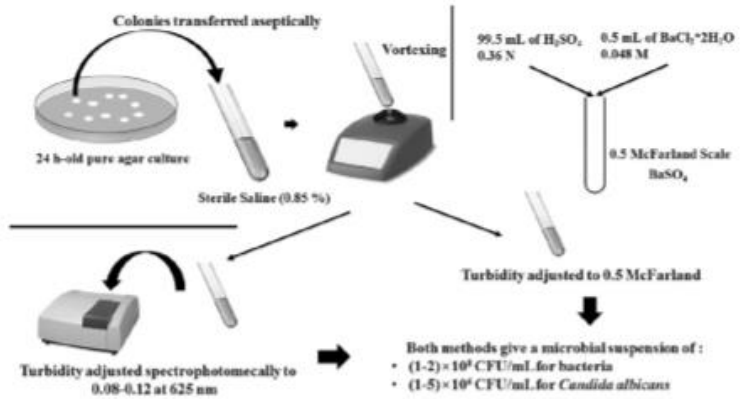


Fig. 3. 0.5 McFarland microbial inoculum preparation by the direct colony suspension as recommended by CLSI guidelines.

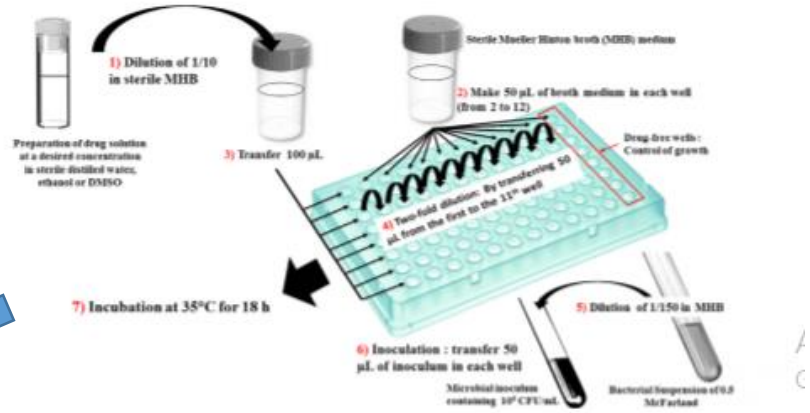
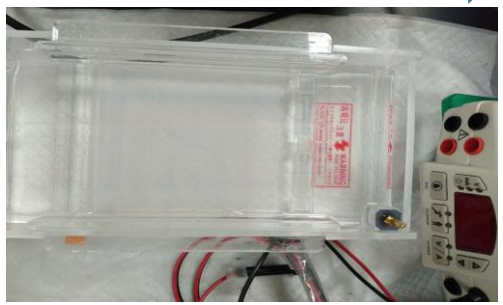
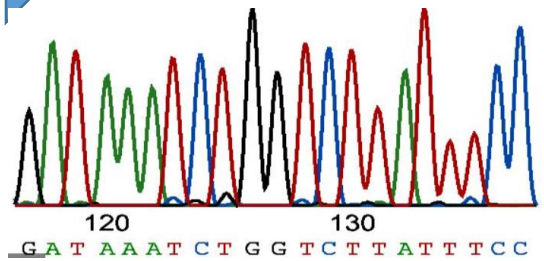


Fig. 4. Broth microdilution for antibacterial testing as recommended by CLSI protocol.

➢DNA Extraction and Sequencing

DNA extraction → PCR → Gel Electrophoresis → Recording image → Sequencing





- PCR was done for species identification and antimicrobial resistance genes confirmation

➢Data Analysis

Chromatogram analysis → BLAST → Multiple sequence alignment → Translation → Submission

- Editing
- Aligning
- Analyzing

BioEdit version 7.2.6.1

- Compare our sequence to those from database

NCBI BLAST

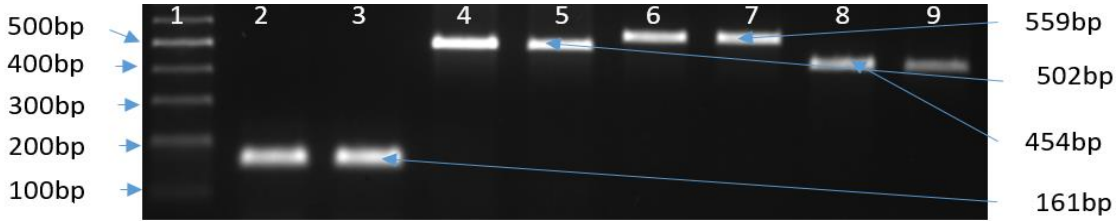
Clustal Omega

ExPASyTranslate tool

ExPASy Bioinformatics Resource Portal

- Sequences were submitted to:
  - ❖ GenBank to get accession numbers

3. Results



Bands of *C. jejuni*, *C. coli*, *tet*(O), and *gyr*A. 1: marker; 2: CJ-RS; 3: CJ-CI; 4: CC-RS; 5: CC-CI; 6–7: *tet*(O) gene (559 bp), and 8–9: *gyr*A gene (454 bp) from antibiotic-resistant strains.

MIC & MBC for plant extracts

NP/antibiotic	CJ–ATCC		CC–ATCC		CJ–Chicken		CC–chicken	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
M. urticifolia	400	800	400	800	400	800	400	800
S. baicalensis	400	800	400	800	400	800	400	800
M. canadensis	400	800	400	800	800	1600	400	800
S. plebeia	400	800	400	800	800	1600	400	800
C. cassia	200	400	200	400	200	400	200	400

MIC: minimal inhibitory concentration (µg/mL)
MBC: minimal bactericidal concentration
CJ: *C. jejuni*; CC: *C. coli*

MIC & MBC for essential oils & phytochemicals

NP/antibiotic	CJ–RS		CC–RS		CJ–CI		CC–CI	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Clove oil	50	100	100	400	50	100	200	400
Cinnamon oil	25	25	50	100	25	50	50	100
Eugenol	50	100	100	200	50	100	100	200
(E)-Cinnamaldehyde	25	25	50	50	25	50	50	50
Baicalein	32	64	64	64	32	64	64	64
Kuraridin	48	ND	48	ND	48	ND	48	ND
Emodin	50	ND	200	ND	50	ND	200	ND
Ciprofloxacin	0.125	1	0.5	1	32	64	64	128
Erythromycin	0.5	1	1	4	0.5	1	2	4
Gentamicin	2	8	2	8	1	2	1	8
Tetracycline	1	4	1	4	256	512	64	128
Nalidixic acid	16	32	8	32	128	256	64	128

- **Mutations in *gyr*A sequences of *C. jejuni* (A) and *C. coli* (B).**

- Mutation (Thr86Ile) is caused by change from ACA to ATA (*C. jejuni*) & ACT to ATT (*C. coli*).
- Silent mutations in *gyr*A are also depicted. Mutations are bolded and underlined.
- L04566.1 & U63413.1 are standard strains (without mutation), while KX982339.1 & MT176401.1 are resistant strains.
- MT947448 and MT947449 are chicken isolates of this study.

Tet(O) genes of

- *C. jejuni* (MT967269) &
- *C. coli* (MT967270)

100% similarity

tet (O)-resistance genes sequences in Genbank.

4. Discussion

- The objective of this study was to evaluate the susceptibility of *Campylobacter* strains to various NPs and frontline antibiotics.

- Cinnamon extract, oil, & (E)-Cinnamaldehyde had the lowest MIC values (25-200 µg/mL), which concurs with previous results (46.8–600 µg/mL) (Hossan et al., 2018).

- Exception: MIC for cinnamon oil <1000 µg/mL reported in Egypt (Hassan et al., 2019).
- Clove oil & eugenol had MICs (50–100 µg/mL) > 20 µg/mL for clove oil (Kovács et al., 2016) but <500 µg/mL previously reported for eugenol(Hassan et al., 2019)

- Essential oils are given to broilers to control *Campylobacter.*

- **Why differences in MIC values?**
  - The method used (agar-based vs broth microdilution).
  - The location and extraction procedures
  - The presence of biofilm, virulence, and antibiotic-resistance genes

4. Discussion Cont'd

- Except for cinnamon, other extracts had MIC values of 400-800 µg/mL.
- The susceptibility of screened extracts was found to be **moderate to weak** according to the classification of Kuete (2010):
- Significant (MIC < 100), moderate (100 < MIC < 625), & weak (MIC > 625µg/mL)
- There is lack of information on the biological activity of *M. urticifolia* but it is expected to have antimicrobial activities (phenolic compounds & phenylpropanoids).
- *Scutellaria baicalensis* is used in the treatment of *H. pylori & S. aureus* infections, & it is advocated to be a source of new drugs against *H. pylori.*
- The MIC for Baicalein from *S. baicalensis* was 32–64 µg/mL which concurs with the previous report on *S. aureus* (Chan et al.,2011).
- *Mentha canadensis* L. *(*antidiarrheic and anti-dysentery plant) has been reported to inhibit *H. pylori* and *C. jejuni* (Thawkar et al., 2016).
- The antimicrobial activities of *S. plebeia* on different pathogens have been extensively reported (Liang et al., 2020).

4. Discussion Cont'd

- The MIC of Kuradin against all isolates was 48 µg/mL which is more or less similar to the value of 50 µg/mL reported for *S. aureus* but >20 µg/mL previously reported.
- Kuraridin, from *Sophora flavescens*, has been reported as a potential antimicrobial compound.
- The MIC of emodin was 50 µg/mL <70–90 µg/mL reported for *P. aeruginosa* &*S. aureus*.
- The literature on both kuraridin and emodin is scanty, and there are no previous findings against *Campylobacter* species. Further studies on *Campylobacter* strains from different sources are needed to confirm the effectiveness of both kuraridin and emodin.

- Irrational use of antibiotics like quinolones in animal husbandry

Chicken isolates → High resistance → Quinolones (CIP & NAL)

- mutation in *gyr*A (Thr86Ile)
- Ciprofloxacin is used in treating diarrhea cases of unknown etiology
- Resistance to fluoroquinolones can be maintained in populations even after being banned in animal production

- *tet*(O) genes:
  - MT967269
  - MT967270

→ High similarity →

- Genbank strains
  - 81-176,
  - NG_048260.1,
  - CP044175.1

- Tet (O) gene is found:
  - Either on plasmid or bacterial chromosome

- The Thr86Ile substitution was found in *Campylobacter* species isolated from chicken (this study), which is in agreement with the broth microdilution and PCR results.

• 3.4. Discussion Cont'd

- The screened NPs may be candidates for in-vivo studies.

- The *C. jejuni* isolated from chicken (MT947450) showed 100% similarity with *C. jejuni* (CP047481.1) isolated from patients with gastroenteritis in Chile suggesting possible transmission of *Campylobacter* from poultry to humans and several reports confirmed this association.

- Control measures & adherence to hygienic practices are required to reduce the transmission of *Campylobacter* from animals to humans.

- We also recommend studies on the synergistic activities of both NPs & existing antibiotics aimed at reducing the MIC values of drugs of choice &, thus, slow down AMR and extend the effectiveness of existing antibiotics.

5. Conclusion

- The isolates from chicken were sensitive to erythromycin and gentamicin, but they were resistant to quinolones (ciprofloxacin & nalidixic acid) and tetracycline.

- The mutations in *gyr*A and *tet*(O) were confirmed by PCR & sequencing.

- The tested NPs were active against both antibiotic-sensitive and antibiotic-resistant *Campylobacter* strains.

- Effective NPs can be exploited by the food processing industry and poultry farms to control foodborne pathogens.

- We also recommend studies on the synergistic activities of both NPs and existing antibiotics aimed at reducing the MIC values of drugs of choice &, thus, slow down AMR & extend the effectiveness of existing antibiotics.

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