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Title: Transmission of Multidrug Resistant *Campylobacter jejuni* to Children from Different Sources in Pakistan

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1 **Transmission of Multidrug Resistant *Campylobacter jejuni* to Children**
2 **from Different Sources in Pakistan**

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24 **Abstract**

25 **Objectives**: *Campylobacter jejuni* has been classified as a member of priority pathogens
26 group due to the rapid emergence of multidrug resistant isolates. In the present study we
27 planned to determine the prevalence, antibiotic resistance patterns and source tracking of
28 clinical *C. jejuni* isolates from pediatric diarrheal patients in Pakistan.

29 **Methods:** A total of 150 stool samples from children were processed for the presence of
30 *Campylobacter jejuni* using culture, biochemical tests and by species specific PCR.
31 Antibiotic susceptibility of the isolates was determined by disc diffusion method and
32 metallo-beta-lactamase (MBL) producers were detected using gene specific PCR. Source
33 tracking was done using source predictive PCR.

34 **Results:** *Campylobacter jejuni* was present in 54.6% of the processed samples. More
35 than 80% of the isolated strains were resistant to 7 out of 12 antibiotics tested. High level
36 of susceptibility was observed against imipenem (12.2%) and tigecycline (9.7%). Six
37 isolates (7.3%) were metallo-beta-lactamase producers and were positive for at least one
38 of the five metallo-beta-lactamase genes. Source tracking showed that 57.3% of the
39 isolates belonged to livestock associated cluster (C1 to C6) and 42.8% were assigned to
40 non-livestock /environmental clusters (C7-C9). Isolates belonging to livestock cluster had
41 high Multiple Antibiotic Resistance (MAR) index (p value<0.001) as compared to non-
42 livestock.

43 **Conclusion:** High prevalence of multidrug resistant *C. jejuni* among pediatric diarrheal
44 patients was observed. Moreover, association of these isolates to livestock clades suggest
45 transmission to human population via food chain and presence of imipenem resistant
46 MBL producing *C. jejuni* can lead to serious public health concerns.

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47 **Keywords:** *Campylobacter jejuni*; Antibiotic resistance; Source attribution; Metallo-β-
48 lactamase; Imipenem.

49

50 ## 1. Introduction

51 Diarrhea, despite being preventable and treatable, is still the second leading cause of
52 mortality among children less than five years of age, worldwide [1]. Diarrheal illness can
53 be caused by both viral and bacterial infections. *Campylobacter jejuni* has been listed as
54 one of the most frequent bacterial cause of diarrhea in recent years [2,3]. *C. jejuni* is a
55 foodborne pathogen that is transmitted to humans via handling or consumption of
56 undercooked meat (poultry, cattle and pigs), raw milk and untreated water [4,5].
57 Campylobacteriosis is generally a self-limiting disease but antibiotics like macrolides
58 (azithromycin and erythromycin) and fluoroquinolones (ciprofloxacin) are recommended
59 in some cases, for instance in treatment of immunocompromised patients [6,7]. Over the
60 past two decades the emergence of multidrug resistant *C. jejuni* especially against
61 erythromycin, fluoroquinolones and tetracycline have been reported from both
62 developing and developed countries [7,8]. *C. jejuni,* being a zoonotic pathogen acquires
63 antimicrobial resistance mainly through use of these antibiotics as prophylaxis in animal
64 farming [9, 10,11]. Disproportionate use of antibiotics in livestock industry results in
65 development of more resistant isolates either by the introduction of mutations or
66 acquisition of antibiotic resistance genes through horizontal gene transfer from other
67 bacteria [12,13]. Nevertheless, sources other than livestock such as wild birds and pets
68 have been recently reported to contribute to the campylobacteriosis burden [10,14,15].
69 Over the past years many outbreaks of campylobacteriosis have been reported due to

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70 consumption of water contaminated with domestic and wild animal fecal material as well
71 as sewage discharge [4, 16, 17, 18]. Source attribution can not only help to identify the
72 origin of *C. jejuni* infection but also help solve the puzzle by linking extremely drug
73 resistant pathogens circulating in human populations especially among vulnerable
74 children to infection source. Genotyping of *C. jejuni* isolates by MLST has been
75 commonly employed for the clonal clustering of strains on the basis of potential source;
76 however, it does not take into account genetics adaptation to specific environment/niche.
77 A source predictive Multiplex PCR, based on adaptive genotypes identified by
78 microarray analysis, has been developed by Stabler *et al.* (2013) to cluster *C. jejuni*
79 isolates into livestock and non-livestock groups [19, 20, 21, 22, 23]. This source
80 predictive PCR method has been shown earlier to provide comparable results in source
81 tracking of human isolates to MLST [19]. In developing countries like Pakistan, this
82 source predictive PCR method provides a reliable and cheap alternate for genotyping of
83 human isolates to predict their origin [30]. The present study was aimed to investigate the
84 prevalence and antibiotic resistance profile of *C. jejuni* among paediatric diarrheal
85 patients; possible linkage of human infections to livestock or non-livestock sources
86 (water and wildlife) using source predictive markers and linking antibiotic resistance
87 potential of the isolates to a particular source.
88

89 **2. Materials and Methods**

90 **2.1 Sampling Collection and Identification of *Campylobacter jejuni***

91 A total of one hundred and fifty human diarrheal stool samples (including both non-
92 bloody and bloody diarrheal cases) were collected from hospitalized pediatric patients

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93 from December 2014 - September 2015. Samples were collected from hospitals of major
94 cities of Pakistan (Supplementary table 1). Children's age varied from 2 months to 5
95 years. All the cases were domestic in origin. The samples were streaked onto modified
96 charcoal cefoperazone deoxycho-late agar (mCCDA) (Oxoid, Basingstoke, UK) and
97 incubated for 48-72 hrs at 42°C under microaerophilic conditions. Preliminary
98 identification of *Campylobacter jejuni* colonies was performed by biochemical tests
99 including oxidase, catalase, indoxyl acetate and hippurate hydrolysis. PCR based
100 detection was done using primers specific for *hip*O gene (hippurate hydrolysis gene) [24].
101 (supplementary table 2)

102 **2.2 Antimicrobial Resistance profiling**

103 Antibiotic resistance profiling of the identified *C. jejuni* isolates was carried out using the
104 following antibiotics: ceftriaxone (CTX)(30 µg) (Oxoid, UK), ampicillin (AMP)(10 µg)
105 (Oxoid, UK), chloramphenicol (C)(30 µg) (Oxoid, UK), tetracycline (TE) (30 µg)
106 (Oxoid, UK), streptomycin (S) (10 µg) (Oxoid, UK), ciprofloxacin (CIP) (5 µg) (Oxoid,
107 UK), nalidixic acid (NA) (30 µg) (Oxoid, UK), erythromycin (E) (30 µg) (Oxoid, UK),
108 gentamycin (CN) (10 µg) (Oxoid, UK), sulphomethoxazole + trimethoprim (SXT) (25
109 µg) (Oxoid, UK), tigecycline (TGC)(15 µg) (Oxoid, UK) andimipenem (IMP) (10 µg)
110 (Oxoid, UK) [25, 26]. This panel of antibiotics was chosen in accordance with Clinical &
111 Laboratory Standards Institute (CLSI) guidelines and previously reported emerging
112 resistance reports in *C. jejuni* [25]. Analysis of zone diameter for resistance profiling of
113 erythromycin, ciprofloxacin, tetracycline, ampicillin and nalidixic acid was done
114 according to the Clinical & Laboratory Standards Institute (CLSI) (2016) for
115 Campylobacter and rest of the antibiotics guidelines for enterobacteriaceae were used.

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116 Multiple antibiotic resistance (MAR) was calculated by using the formula a/b where 'a' is
117 the number of antibiotics to which a isolate is resistant and 'b' is the total number of
118 antibiotics tested [27].

119 **2.3 Detection of Metallo-β-lactamase**

120 Phenotypic identification of MBL enzyme in imipenem resistant isolates was performed
121 using the combined disk method (CDT) [28]. Briefly, overnight cultures of the test strains
122 were exposed to one imipenem and another imipenem disk amended with ethylene
123 diamine tetra-acetic acid (EDTA) placed 25 mm apart on Muller Hinton agar plates. An
124 increase of ≥7 mm in the diameter of the imipenem inhibitory zone compared to
125 imipenem-EDTA disk after 24 hours of incubation confirmed presence of metallo-β-
126 lactamase positive organism. To verify the presence MBL genes at molecular level a
127 multiplex PCR was used. Five primer pairs, specific for each family of acquired MBLs,
128 were used for the detection of bla_{IMP}, bla_{VIM}, bla_{SPM}, bla_{GIM} and bla_{SIM} genes [29]. The
129 primers and amplification conditions used are listed in supplementary table 2.

130 **2.4 Source Attribution**

131 To predict the possible origin of *C. jejuni* in human samples source predictive markers
132 were used as described by Stabler et al., [21]. Six source discriminatory genes i.e.,
133 Cj0056c (hypothetical protein), Cj0485 (putative oxidoreductase), Cj1139c (beta- 1,3
134 galactosyltransferase), Cj1324 (hypothetical protein) Cj1422c (putative sugar
135 transferase), and Cj1720 (hypothetical protein) were amplified in two sets of multiplex
136 PCRs (M1 and M2). The strain Cj255 from chicken was used as a positive control with
137 all target genes present, and nuclease free water with no DNA template was used as a
138 negative control [30]. Primers and the thermal amplification condition used in the two

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139 multiplex PCRs are mentioned in supplementary table 2. The results of PCR were
140 converted into binary data (based on presence or absence of amplified product) and
141 analyzed according to the binary code provided by Stabler et al. [21]. Dendrogram based
142 on this binary data was constructed using PAST3.16 Software [31].

143 **2.5 Statistical analysis**

144 Statistical analysis was performed using the Student's t-test. Data were considered
145 significant at a p value of ≤ 0.05

146

147 **3. Results and Discussion**

148 Diarrhoeal disease, world-wide, ranks as the fourth most frequent cause of death. The
149 estimated diarrheol related disability-adjusted life-years (DALYs) burden is
150 underestimated due inconsideration of the long-term sequelae as a consequence of
151 undernutrition, increased risk of infectious disease and increased prevalence of protein
152 energy malnutrition associated with the disease in children under 5[32] . While the
153 disease affects most of the worlds population, more than half of the total number of
154 reported cases and 80% of the child mortalities occur in South Asia and Africa [2].
155 *Campylobacter jejuni* has been classified by WHO as "High priority pathogens" due to
156 emergence of multidrug resistance. Therefore, routine surveillance is necessary for the
157 control of disease. The prevalence of multidrug resistant isolates of *C. jejuni* has been
158 under reported in developing countries like Pakistan, compared to developed countries,
159 due to lack of proper surveillance programs. In the present study the prevalence of
160 multidrug resistant *C. jejuni*, and their sources of transmission in human clinical cases
161 was investigated . A total of 150 stool samples from children suffering from diarrhea,

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162 predominantly belonging to low income group (LIG), were collected and cultured on
163 mCCDA agar (Supplementary table 1). Biochemically identified *C. jejuni* strains were
164 further confirmed by PCR using primers against the *hipO* gene (hippurate hydrolysis
165 gene). *C. jejuni* was found to be present in 82 samples indicating a relatively high
166 isolation rate of 54.6% compared to previous reportsfrom Pakistan indicating11.3-29.5%
167 prevalence rates during the years 1993-2013 [26, 32, 33, 34]. This increase in prevalence
168 of *C. jejuni* could be attributed to many reasons including the increase in antibiotic
169 resistant strains in the population in the past few years. To validate this hypothesis, we
170 compared the antibiotic resistance profile of *C. jejuni* to that of previously reported
171 isolates from our laboratory in year 2011-2012 [26].

172 Antibiotic susceptibility testing of the isolates showed that most of the isolates, ranging
173 from 9.8-98% were resistant to tested antibiotics.. More than 80% of the isolates were
174 found to be resistant to the following antibiotics: ampicillin (98%), erythromycin (98%),
175 streptomycin (94%) and tetracycline (89%), trimethoprim/sulfamethoxazole (88%),
176 cefotaxime (83%), gentamycin (80%), nalidixic acid (82%), ciprofloxacin (73%).
177 Whereas, a moderate percentage of isolates were resistant to chloramphenicol (40%) .
178 Isolates were more sensitive to imipenem (12% resistant) and tigecycline (9% resistant).
179 Overall 75% of isolates were resistant to more than eight of the tested antibiotics.
180 Comparison of resistance profiles of *C. jejuni* isolates from 2011-2012 indicated an
181 increased rate of resistance to all the tested antibiotics. The percentage of resistant
182 isolates to ampicillin increased from 40% to 98%, streptomycin from 53% to 94%,
183 ciprofloxacin 20% to 73%, erythromycin 27% to 98%, tetracycline 27% to 89%,
184 sulphomethoxazole + trimethoprin 40% to 88%, gentamycin from 7% to 80%,

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185 chloramphenicol 20% to 40% and nalidixic acid 13% to 82% [26]. Poultry related *C.*
186 *jejuni* isolates have earlier been reported to show higher resistance to antibiotics due to
187 their indiscriminate use as a growth promoter such as streptomycin and erythromycin in
188 the feed which may lead to cross transmission in humans [35, 36]. The high resistance
189 profile observed in this study is similar to those reported in poultry isolates previously,
190 compared to clinical isolates, suggesting the possible transmission from poultry to
191 humans [26, 37, 38, 39].

192 Antibiotics belonging to the class β-lactams have been used as first line of therapy due to
193 their high efficacy and low toxicity to humans. The choice of β-lactams is reduced to
194 carbapenems when infections are caused by extended-spectrum β-lactamase (ESBL)
195 producing organisms. Such ESBL producing isolates have also been reported in *C. jejuni*
196 but studies so far have not reported any resistance to carbapenem. In the present study 10
197 *C. jejuni* isolates were found to show resistance to imipenem (member of carbapenem
198 family) by the disc diffusion method. MBL enzyme detection through CDT showed that
199 10% (n=8) of the isolates were MBL positive. PCR amplification of bla_{IMP}, bla_{VIM},
200 bla_{SPM}, bla_{GIM} and bla_{SIM}, genes showed that 7.3% (n=6) of isolates were positive for
201 MBL genes. Three isolates carried both bla_{IMP} and bla_{VIM} genes, three isolates carried
202 either bla_{VIM}, bla_{IMP} or bla_{SIM} gene. None of the isolates were positive for GIM or SPM
203 (Fig. 1). Two isolates out of 8 that showed phenotypic MBL production were PCR
204 negative for the screened MBL genes. This is the first report on emergence of imipenem
205 resistant isolates among *C. jejuni* in clinical samples and hence may affect the treatment
206 and subsequent recovery among patients (Table 1).

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207 In order to determine the source of transmission of resistant strains, all the *C. jejuni*
208 isolates were assigned to strain clusters using the binary code based on the presence or
209 absence of amplified products of six source predictive genes sequences (Fig. 2,3A). The
210 results showed that isolates were well distributed among all groups i.e., C1/C2/C3
211 (14.6%), C4/C6 (26.8%), C5 (15.8%), C7/C8 (24.3%) and C9 (18.2%). Overall 57.3% of
212 the isolates belonged to C1 to C6 clusters which have been previously described as
213 livestock-associated (Fig. 4B) whereas 42.8% of isolates belonged to C7 to C9 clusters
214 predicted to be of non-livestock /environmental origin [20,21]. The relationship of
215 livestock and non-livestock associated strains along with MAR Index is shown in
216 phenogram [31] (Fig. 2). The MAR index is a health risk assessment tool which coupled
217 with source prediction may be used to determine origin of antibiotic resistant strains
218 which may be linked to sources exposed to high antibiotic use. High values indicate
219 'higher-risk' source of isolate [40]. Interestingly, in the present study isolates belonging
220 to livestock cluster had high MAR index ($p<0.001$) compared to non-livestock suggesting
221 presence of more multidrug resistance among isolates of livestock origin (Fig. 4B, 4C).
222 Excessive use of antibiotics in livestock industry exerts a selective pressure on bacterial
223 pathogens resulting in development of more resistant isolates by either mutation or
224 acquisition of antibiotic resistance genes through horizontal gene transfer from other
225 bacteria [12,13].
226 Pakistan is a livestock raising country with approximately 180 million head of livestock
227 and 1640 million of commercial poultry and a few millions of poultry in backyard
228 practices. The poultry industry contributes 26.8% to total meat production in Pakistan.
229 However, with current farming practices, use of antimicrobials to cure and prevent

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230 disease as well as promote growth have been extensive leading to a rise in multi-drug
231 resistance in associated microbiota including *C. jejuni.* The association of more resistant
232 *C. jejuni* to livestock clades in the present study depicts the ability for such strains to
233 persist and spread, through the food chain, from animals to humans.. High prevalence of
234 antibiotic resistance and linkage of isolates, with high MAR index, to livestock clade
235 observed in the present study provide evidence of possible transmission of *C. jejuni* from
236 animals to human, thus posing serious health concern.
237

238 **4. Conclusion**

239 In the present study, a high prevalence of *C. jejuni* among paediatric diarrheal patients
240 were recorded which were linked to both livestock and non-agricultural/non-
241 domesticated sources. Association of significant number of isolates with high MAR with
242 livestock indicate possible selection and transmission of these isolates from animals to
243 humans, thus stressing the need to the control of the indiscriminate use of antimicrobials
244 in animal farming.
245

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246 **Declarations**

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249 **Competing Interests:** No

250 **Ethical Approval:** Not required

251

252

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410 Table 1: Detail of imipenem resistant *C. jejuni* strains.

Strain ID	Imipenem (Zone of Inhibition mm)	Phenotypic Identification of MBL	MBL Gene	MAR index	Source attribution cluster
BH14	R (11)	+	bla_{IMP} , bla_{VIM}	0.75	C1/C2/C3
SH56	R (14)	+	bla_{IMP}	0.83	C1/C2/C3
PH14	R (16)	+	bla_{SIM}	0.75	C7/C8
PH2	R (15)	+	bla_{VIM}	0.83	C5
PH8	R (14)	+	bla_{IMP} , bla_{VIM}	0.67	C5
PH15	R (16)	+	bla_{IMP} , bla_{VIM}	0.75	C1/C2/C3
AS51	R (13)	+	-	0.75	C1/C2/C3
PH29	R (17)	-	-	0.83	C4/C6

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SH58	R (15)	+	-	0.839	C4/C6
PH11	R (12)	-	-	0.830.71	C1/C2/C3

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416 **Figure Legends**

417 Figure 1 Detection of metallo-b-lactamases in *C. jejuni* using multiplex PCR assay
418 for MBL encoding genes i.e., bla_{IMP} (188bp), bla_{SIM} (390bp), and
419 bla_{VIM}(570bp)

420 Figure 2 Dendrogram displaying source attribution clusters of *C. jejuni* strains,
421 based on binary data of PCR profiles by using UPGMA analysis
422 (PAST3.16 Software); Green strain labels MAR=0.35-0.5, Blue strain
423 labels- MAR=0.5-0.75, Red strain labels=0.8-0.9; *** indicate isolates
424 which are MBL producers.

425 Figure 3 A) Source predictive multiplex PCR: lane 1, 100-bp ladder; lane 2, strain
426 BH20 (mPCR 1); lane 3, strain BH20 (mPCR 2); lane 4, strain SH11
427 (mPCR 1); lane 5, strain SH11 (mPCR 2). mPCR 1 involved amplification
428 of genes Cj1422, Cj1139 and Cj0056. mPCR 2 involved amplification of
429 genes Cj1324, Cj1720 and Cj0485. B) Percentage of isolates and average
430 MAR index of isolates attributed to livestock and non-livestock clusters.
431 C) Percentage of resistant isolates belong to livestock and non-livestock

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432 cluster to ceftriaxone (CTX), ampicillin (AMP), chloramphenicol C,
433 tetracycline (TE), streptomycin (S), ciprofloxacin (CIP), nalidixic acid
434 (NA), erythromycin (E), gentamycin (CN), sulphomethoxazole +
435 trimethoprim (SXT), tigecycline (TGC), imipenem (IMP)
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Highlights

- High prevalence of among diarrheal paediatric patients.
- Majority of C. jejuni (>90%) isolates were multidrug resistant.
- 7.3% of isolates were metallo-beta lactamase producers.
- Livestock associated Isolates had high MAR index as compared to non-livestock.

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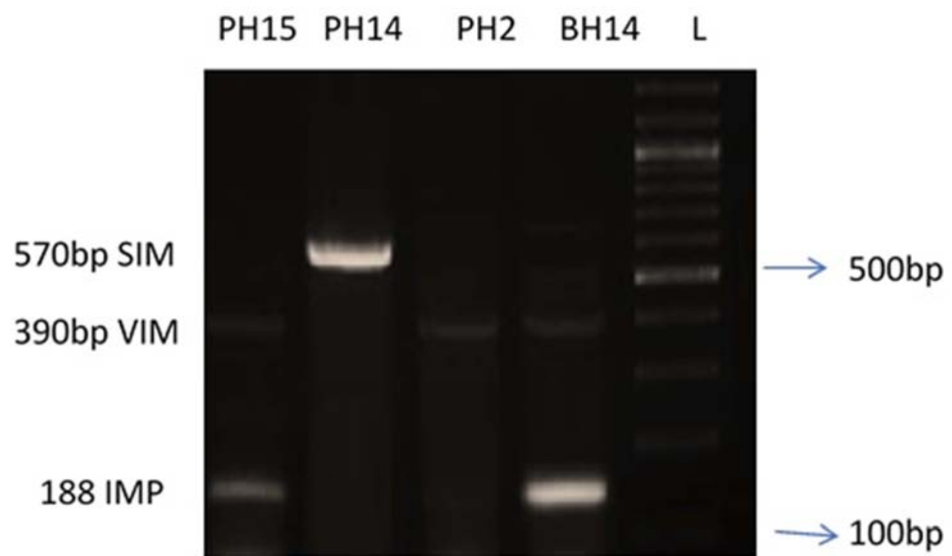

Figure 1.

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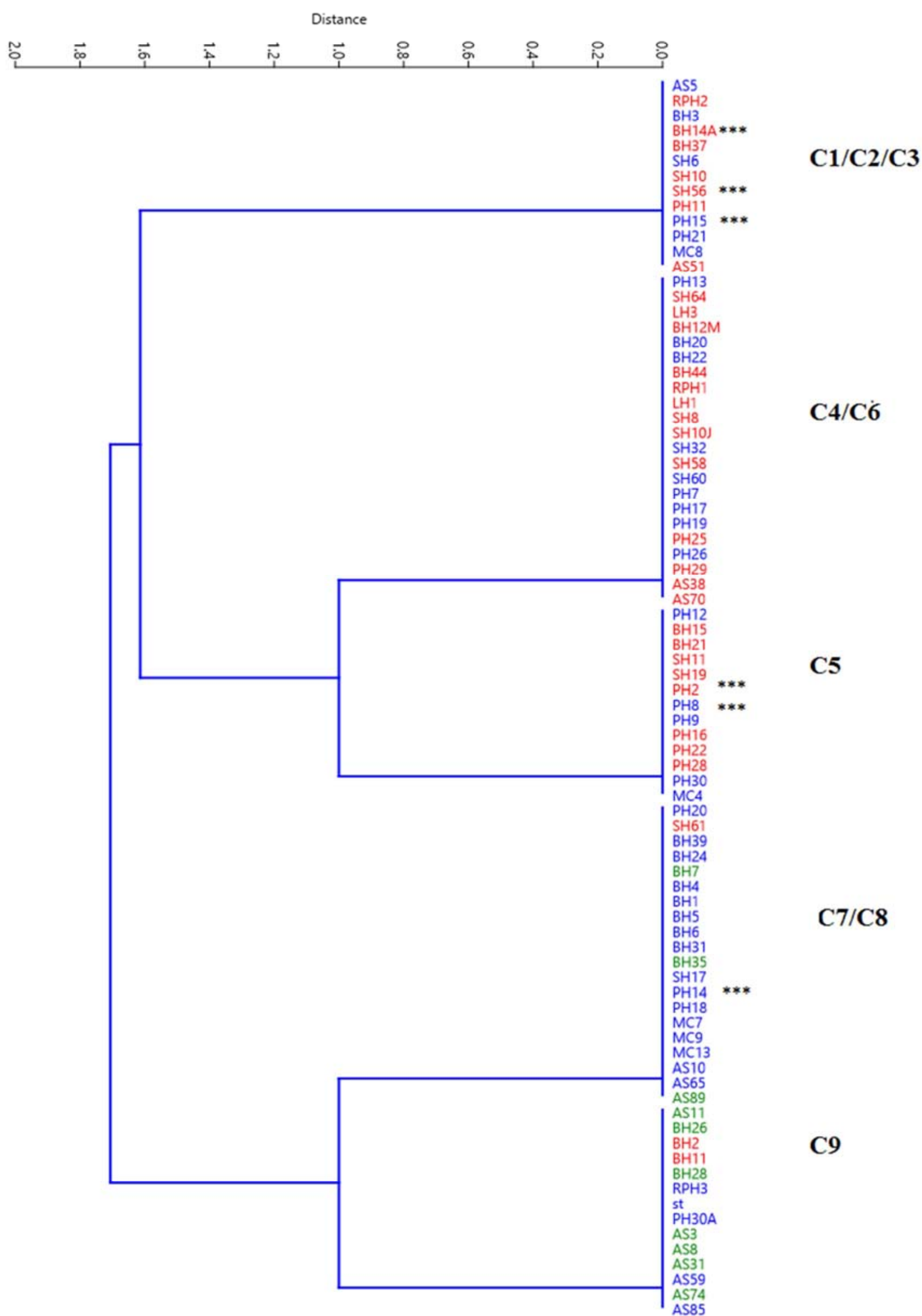

Figure 2

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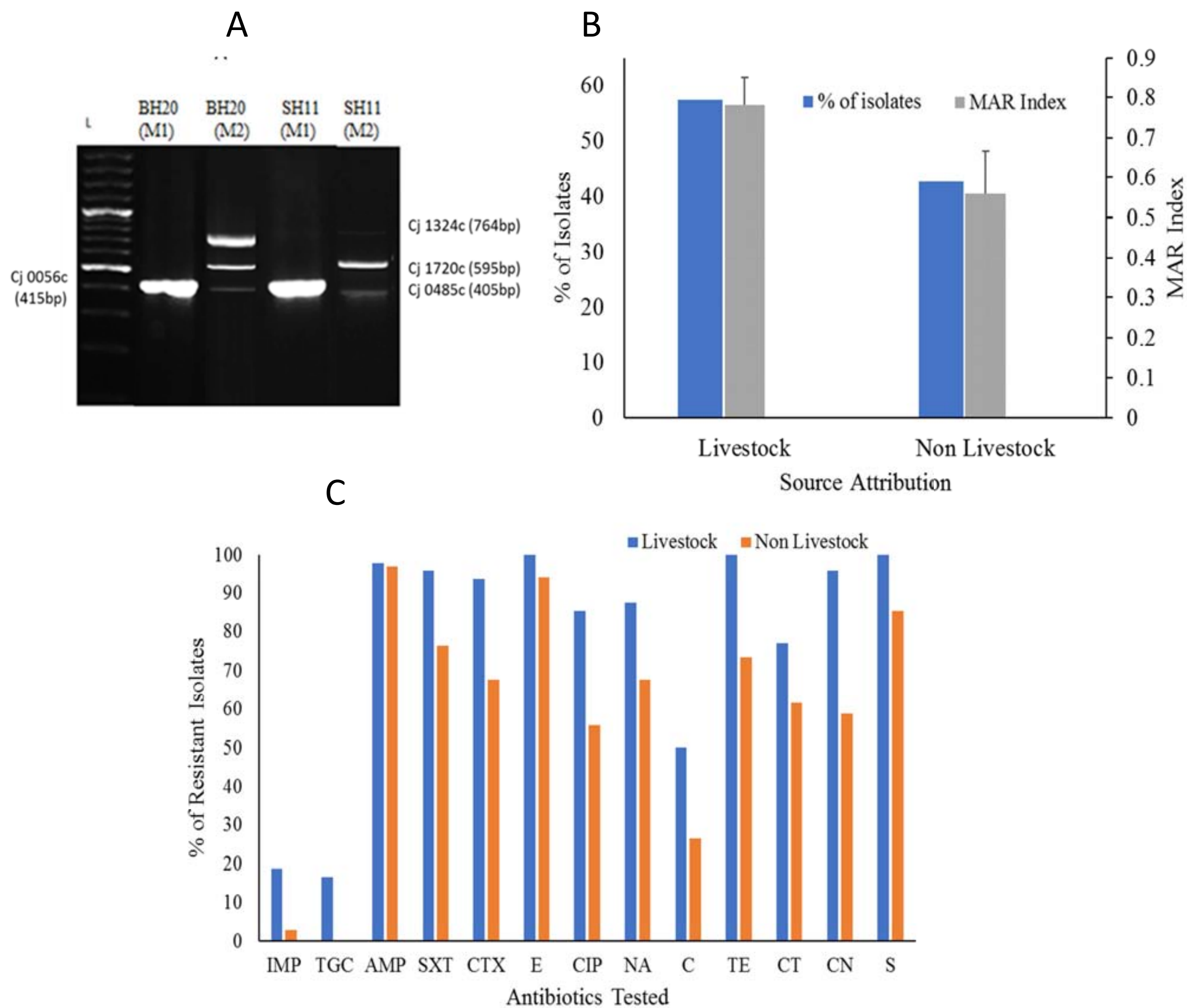

Figure 3.