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1 # Bacteriophage activity against and characterisation of avian pathogenic
2 # *Escherichia coli* isolated from colibacillosis cases in Uganda

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Abstract

A laboratory-based study aimed at establishing a stock of avian pathogenic *Escherichia coli* (APEC) lytic bacteriophages, for future development of cocktail products for controlling colibacillosis as well as minimizing use of antimicrobial drugs in the poultry production systems in Uganda. Specifically, the study determined the antibiotic susceptibility; phylogenetic categories, occurrence of selected virulence genes among *Escherichia coli* stock isolates from cases of chicken colibacillosis; and isolation of specific bacteriophages. Fifty six isolates were confirmed as *E. coli* by standard phenotypic tests. All the 56 (100%) isolates were resistant to at least one antibiotic while 50 (89.3%) isolates were resistant to at least three classes of antimicrobial drugs and were therefore designated as multi-drug resistant. Phylogenetically, APEC isolates mainly belonged to phylogroups A and D which represented 44.6% and 39.3%, respectively. Virulence genes, *ompT* and *iutA* were the most frequent with 33 (58.9%) and 32 (57.1%) isolates respectively; while *iroN* least occurred in 23 (41.1%) isolates. Of the 56 isolates, 69.6% harbored at least one virulence gene, while 50% had at least four virulence genes; hence confirmed as APEC. None of the isolates belonged to the selected serotypes O1, O2 and O78. Seven specific bacteriophages were isolated and their host range, varied from 1.8% to 17.9% (n=56 APEC isolates), while the combined lytic spectrum of all the phages was 25%. Phage stability was negatively affected by increasing temperatures with both UPEC04 and UPEC10 phages becoming undetectable at 70°C; however activity was detected between pH 2 and 12. The high occurrence of APEC isolates with resistance against the commonly used antibiotics supports the need for alternative strategies of bacterial infections control in poultry. The low host range exhibited by the phages calls for search for more candidates before more in-depth studies are done for phage characterization and application.

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42 Keywords: APEC; virulence genes; phylogenetic groups; antibiotic susceptibility; bacteriophage

43 1. Introduction

44 Avian colibacillosis refers to any localized or systemic infection caused by Avian
45 Pathogenic *Escherichia coli* (APEC) belonging to several serogroups; and remains one of the most
46 prevalent bacterial diseases affecting the poultry industry worldwide [1]. In Uganda, colibacillosis
47 is the most frequent bacterial infection among the chicken samples submitted to the Central
48 Diagnostic Laboratory with a prevalence of 14% [2]. Majalija *et al* (2010) reported 87%
49 *Escherichia coli*, isolated from broiler farms kept under the deep litter system, being resistant to
50 at least one antimicrobial agent [3].

51 *Escherichia coli* strains possess various virulence factors for extra-intestinal survival [4–
52 8]. However, capacity to cause disease in a specific host species, depends on acquisition of
53 appropriate virulence gene combination by a given *E. coli* strain [5]. The number of detected genes
54 can be used as a reliable index of their virulence; and strains typed as APEC possess five to eight
55 genes, while the non-APEC ones harbor less than four genes [5,8]. Virulence-associated genes,
56 namely, *iutA, hlyF, iss, iroN*, and *ompT* were suggested as the minimum that can be used to identify
57 an APEC strain with the highest pathogenicity [9].

58 Bacterial diseases of significance in animal production systems affect productivity, may be
59 zoonotic and some have been associated with drug resistant pathogens [6,10]. The high occurrence
60 of drug resistant organisms warrants search for alternative strategies, such as, use of
61 bacteriophages, in management of bacterial infections, like colibacillosis. Bacteriophages are
62 naturally occurring viruses in the environment that routinely control bacterial populations [11].
63 The specific action against bacteria, self-replicating and self-limiting nature; makes bacteriophages

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64 attractive alternatives to antibiotics to prevent and treat bacterial diseases. Phages have been
65 applied in control of various bacterial agents; including the drug resistant strains. Bacteriophages
66 are increasingly explored as non-antibiotic strategies for control of bacterial diseases in
67 agricultural systems for food safety and security [12–14]. In some developed countries, phages
68 have been approved and are commercially available for use, especially in management of
69 microbial contamination of plant-based foods [15]. In poultry, phages have demonstrated
70 effectiveness; and some e.g. ListShield™ from Intralytix, Inc; have been approved by the United
71 States Food and Drug Administration (FDA) for managing bacterial infections including *E. coli*
72 (EcoShield™) [12,16,17].

73 The APEC strains circulating on poultry farms in Uganda have neither been characterized
74 nor the virulence genes they harbor documented. Unlike most poultry diseases, there are no
75 vaccines for controlling colibacillosis, hence its management depends on hygienic measures as
76 well as use of antibacterial agents. Antibiotic use is associated with resistance development and
77 undesirable drug residues in the poultry products; which calls for alternative antimicrobial
78 strategies. The research aimed at establishing a stock of APEC lytic bacteriophages, for future
79 development of cocktail products for controlling colibacillosis as well as minimizing use of
80 antimicrobial drugs in the poultry production systems in Uganda. Specifically, the *E. coli* isolates
81 associated with cases of poultry colibacillosis were characterized by drug susceptibility,
82 phylogenetic group and the virulent genes harbored. The research also sought to establish presence
83 of APEC serotypes O1, O2 and O78; and stock of lytic bacteriophages that specifically target
84 APEC.

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85 # 2. Materials and Methods

86 ## 2.1 Bacterial isolates

87 Previously archived APEC isolates from post-mortem samples of colibacillosis suspect
88 chicken collected between 2017 and 2018 from poultry farms around Kampala district were used
89 for the study. The isolates had been stored at the microbiology laboratory of the College of
90 Veterinary Medicine, Animal Resources and Biosecurity, Makerere University. Identity of 56
91 *Escherichia coli* isolates was confirmed by standard bacteriological and biochemical methods.

92 ## 2.2 Antimicrobial susceptibility testing

93 Antimicrobial susceptibility testing was carried out by the Disk diffusion method [18], as
94 recommended by the Clinical and Laboratory Standards Institute [19]. Twelve antibiotics,
95 including Tetracycline 25mcg, Chloramphenicol 5mcg, Nalidixic acid 30mcg, Ampicillin 10mcg,
96 Streptomycin 10mcg, Co-trimoxazole 25mcg, Ciprofloxacin 5mcg, Penicillin G 10mcg, Cefixime
97 30mcg, Amoxicillin 30mcg, Nitrofurantoin 300mcg, and Gentamicin 30mcg (Bioanalyse®) were
98 tested on Mueller-Hinton agar. Growth-inhibition zones were recorded and interpreted as
99 susceptible (S), intermediate (I), and resistant (R). An *E. coli* reference strain (ATCC 25922) was
100 used for quality control of the test.

101 ## 2.3 DNA extraction

102 Template DNA was extracted using the boiling method as described by Wang *et al* [20].
103 Briefly, bacteria DNA was prepared by suspending one colony of the isolate in 100µL of distilled
104 water. The suspension was rapidly boiled in a water bath at 95℃ for 10 minutes and then cooled
105 to room temperature. The cool suspension was then centrifuged (Eppendorf centrifuge 5424R,

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106 Germany) for 3 minutes at 12000rpm to remove cell debris; and the supernatant stored at -20℃
107 formed the stock from which aliquots of template DNA were obtained for use in PCR.

108 2.4 Detection of the virulence genes and determination of 109 phylogroups and serogroups of APEC using PCR

110 Amplification of the selected *E. coli* virulence genes was carried out following a method
111 described by Johnson *et al* [9]. The positive controls used in PCR assays were *E. coli* strains
112 BEN2268, BEN2908 which were kindly provided by Dr. Catherine Schouler.

113 A triplex PCR was carried out following a method described by Clermont *et al* [21] to
114 determine the phylogenetic groups of the APEC isolates; where four major phylogenetic groups
115 (A, B1, B2 and D) were targeted. The *E. coli* K-12 (phylogroup A), STEC O111 (phylogroup B1),
116 and O157:H7 (phylogroup D) were used as positive controls.

117 Serogroup identification was done using an allele-specific PCR assay with primers
118 designed for the most common serotypes (O1, O2 and O78) as described by Wang *et al* [22]. *E.*
119 *coli* strains BEN2268 and BEN2908 were used as positive controls with nuclease free water used
120 as the negative control. The primers and the PCR conditions used are listed in the supplementary
121 files (S1-S3 Appendices).

122 2.5 Isolation of bacteriophages

123 *Escherichia coli* specific phages were isolated through enrichment, from effluent and
124 chicken droppings that were obtained from three selected chicken houses and slaughter places
125 around Kampala district. The phage isolation process followed the procedure described by Oliveira
126 *et al* [23] with slight modifications including use of Tryptic soy broth (TSB) (Condalab, Madrid,

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127 Spain) instead of Luria Bertani broth (LB). Briefly, 50g of the faecal samples were homogenized
128 in 50 ml of Tryptic soy broth (TSB). The effluent (50 ml) and the homogenised samples were
129 centrifuged at 10,000 ×g for 10 min. The supernatant was filtered through a 0.45µm membrane
130 (ADVANTEC®, USA) and 10 ml of the filtrate was added to 10 ml of double strength TSB
131 containing 40µL of 1M Calcium Chloride ($CaCl_2$). Then 100µL of overnight *E. coli* ATCC 25922
132 broth culture was added for enrichment. The mixture was incubated at 30°C for up to 48 hours on
133 a shaker (New Brunswick™ Innova® 40, Germany) at 120 rev/min; after which it was centrifuged
134 at 7000 rpm (Hermle Z32K, Germany) for 5 mins at 4°C. The supernatant was then filtered through
135 0.45µm syringe filters. Presence of phages was determined using the spot assay method.

136 2.6 Spot assay method

137 A spot assay was carried out as described by Mirzaei & Nilsson [24] with slight
138 modifications. Briefly, the soft agar overlay was prepared by mixing 100 µL of an overnight *E.*
139 *coli* broth culture with 5mL of TSB containing 0.7% agar maintained in the molten form in a water
140 bath at 45°C. The agar overlay was poured on to base plates containing 20–30mL of Tryptic Soy
141 Agar (TSA) (Condalab, Madrid, Spain) with 1.5% agar and then swirled to allow uniform spread.
142 On solidifying, 10 µL of the phage filtrate was spotted on top of the soft agar and allowed to dry.
143 The plates were examined for lysis or plaque formation after overnight incubation at 37°C. A clear
144 zone indicated presence of phage.

145 2.7 Purification of bacteriophages

146 Phage purification was done using the agar overlay technique as described by Oliveira *et*
147 *al* [23], with some modifications. The method employed base plates, containing 20–30mL of TSA
148 with 1.5% agar and soft agar overlays composed of TSB with 0.7% agar. Ten-fold serial dilutions

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149 (10^0 - 10^{-9}) of the above filtered phages were prepared using the phage SM buffer (0.05M Tris,
150 0.1M NaCl, 0.008M $MgSO_4$, 0.01% w/v gelatin, pH 7.5). Equal volumes (100 µL) of the diluted
151 phage and of overnight host *E. coli* were mixed with 5mL of soft agar overlay, spread onto TSA
152 plates and incubated overnight at 37°C. Phages were purified by successive single plaque isolation,
153 from the higher dilutions plates where plaques were distinct. A single plaque was picked from the
154 bacteria lawn, suspended into an overnight host *E. coli* culture, incubated overnight at 37°C and
155 the lysate plated as described above. After repeating the cycle three more times, lysates from single
156 plaques were centrifuged at 5000 g for 5 min. The phages were recovered from the supernatant by
157 filtering through a 0.45 µm membrane. Purified phages were stored in SM buffer at 4°C for
158 working stock, while for long term-storage, phage stocks were stored in 1 ml aliquots at -80°C in
159 7% Dimethyl Sulfoxide (DMSO).

160 2.8 Determination of phage titres by agar overlay method

161 Phage concentration (titre) was determined using a method described by Carey-Smith *et al*
162 [25] with some modifications. Tryptic Soy broth instead of Luria Bertani broth was used as the
163 culture medium. Ten-fold serial dilutions (10^0 – 10^{-8}) of the purified phages were prepared using
164 SM buffer. Overlays (5ml) were inoculated with 100 µL of overnight host *E. coli* and poured on a
165 base plate previously marked in a grid to allow identification of each phage dilution. Once the
166 overlay was gelled and dried, 10 µL of each phage dilution was spotted. The plates were incubated
167 at 37°C, and examined for plaques after 24 hours. Distinct plaques obtained from the lowest
168 dilution were counted and used to calculate the phage titre. The titres were expressed as plaque
169 forming units (PFU) per ml.

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170 ## 2.9 Bacteriophage host range determination

171 Bacteriophage activity was tested on 56 APEC isolates using the spot assay method as
172 described above. Presence of clear zones indicated sensitivity of a given APEC isolate to the lytic
173 activity of the phage. Out of the seven phages, two phages with the broadest host ranges were
174 selected for pH and thermal stability testing.

175 ## 2.10 pH and thermal stability test

176 pH stability and thermal stability tests were carried out for the two phages with the broadest
177 host ranges as described by Jung *et al* and Yu *et al* [26,27]. Briefly, the phages (10^8 PFU/ml) were
178 incubated at different temperatures (20℃ to 70℃) for 30 mins. This range of temperatures was
179 selected because it encompasses both the room temperature and body temperature of chicken
180 among other temperatures. Afterwards, the phage suspensions were immediately placed in an ice
181 bath.

182 The pH stability of the phages was evaluated using SM buffer solution adjusted to the
183 required pH using concentrated hydrochloric acid (HCl) or Sodium hydroxide (NaOH). The
184 phages (10^8 PFU/ml) were subjected to different ranges of pH from 2 to 12 for 30 mins at 25℃
185 and at 40℃. The two temperatures were selected to represent the room temperature and the body
186 temperature of chicken respectively; while pH was studied because it affects phage adsorption
187 onto the bacteria and its subsequent propagation. Afterwards, the phage suspensions were
188 immediately diluted with the SM buffer to limit further exposure. After both the heat and pH
189 treatment, viable phages were quantified using the agar overlay method as described above. All
190 assays were performed in duplicates.

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191 ## 2.11 Research approval

192 The study was endorsed by the Higher Degrees Research Committee of the College of
193 Veterinary Medicine, Animal Resources and Biosecurity of Makerere University.

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214 # 3. Results

215 ## 3.1 Antimicrobial susceptibility testing

216 All the 56 (100%) isolates exhibited resistance to at least one antibiotic. Fig 1 presents the
217 proportion of resistant isolates for each of the tested antibiotic. High frequency of resistance was
218 encountered for the antibiotics: Penicillin G (100%), Sulphamethoxazole/Trimethoprim (87.5%),
219 Tetracycline (83.9%), Ampicillin (80.4%), Amoxicillin (69.6%), Streptomycin (67.9%) and
220 Nalidixic acid (60.7%). Average frequency of resistance was found in case of Chloramphenicol
221 (35.7%). Low frequency of resistance was revealed in case of Gentamicin (10.7%) and
222 Nitrofurantoin (8.9%); while all the 56 (100%) isolates were susceptible to Cefixime. Resistance
223 to at least three antimicrobial drug classes; and hence multi-drug resistance (MDR), was
224 encountered in 50 (89.3%) isolates.

225 **Fig 1. Antimicrobial susceptibility test results for Avian Pathogenic *E. coli*.** The bars represent
226 the percentages of the 56 APEC isolates that were resistant, intermediate or susceptible to the 12
227 antibiotics as determined by the Disk diffusion method.

228 CIP – Ciprofloxacin, P- Penicillin G, CFM – Cefixime, SXT - Sulphamethoxazole/Trimethoprim,
229 C- Chloramphenicol, AX – Amoxillin, F – Nitrofurantoin; S – Streptomycin, NA - Nalidixic acid,
230 AM - Ampicillin, TE- Tetracycline, CN – Gentamicin

231 ## 3.2 Phylogenetic groups of the APEC isolates

232 The multiplex PCR amplification targeting the *ChuA, yjaA* and TspE4.C2 genes
233 categorized the 56 APEC isolates into phylogenetic groups A, B1, B2 and D with 25 (44.6%),

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234 eight (14.3%), one (1.8%) and 22 (39.3%) isolates, respectively. Table 1 presents the genes and/or
235 their combinations, the phylogenetic group and proportion of the *E. coli* isolates in each category.

236 **Table 1. Phylogenetic groups of the APEC suspect isolates**

ChuA	***yjaA***	**TSPE4.C2**	**Phylogroup assignment**	**Frequency n = 56 (100%)**
-	+/-	-	A	25 (44.6)
-	-	+	B1	8 (14.3)
+	+	+/-	B2	1 (1.8)
+	-	+/-	D	22 (39.3)

237 (+) Presence of gene; (-) Absence of gene

238 3.3 Frequency of APEC virulence genes

239 Table 2 presents the frequency of the APEC isolates harboring the selected virulence genes,
240 that is, *iroN, ompT, hlyF, iss,* and *iutA.* Out of the 56 isolates, 39 (69.6%) had at least one virulence
241 gene. The virulence genes *ompT* and *iutA* had the highest prevalence at 33 (58.9%) and 32 (57.1%)
242 respectively with *iroN* having the lowest prevalence at 23 (41.1%). Of the 56 isolates, only 28
243 (50%) harboured four or more virulence genes and thus confirmed as APEC.

244 **Table 2. Frequency of the selected virulence genes among the APEC suspect isolates**

Gene	**Description**	**Frequency n = 56 (100%)**
iutA	Aerobactin siderophore receptor gene	32 (57.1)
iss	Episomal increased serum survival gene	31 (55.4)

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hlyF	Putative avian hemolysin	31 (55.4)
ompT	Episomal outer membrane protease gene	33 (58.9)
iroN	Salmochelin siderophore receptor gene	23 (41.1)

245

246 With respect to presence of at least one virulence gene in relation to the phylogroup; 14
247 out of 25 in group A, 5 out of 8 in group B and 19 out of 22 in group D, had virulence genes (Fig
248 2).

249 **Fig 2. Virulence gene content of APEC isolates within each phylogenetic group.** The dark bars
250 indicate the proportion of APEC isolates within a phylogenetic group that had virulence genes
251 while the white ones indicate those without virulence genes.

252 3.4 Serological genotyping

253 Of the 56 isolates, none generated amplicons of sizes expected for the O1, O2 and O78
254 serogroups (Fig 3).

255 **Fig 3. Agarose gel showing PCR amplicons from selected APEC isolates for Serogroup**
256 **O78**. Lane M: DNA marker (100bp DNA ladder, ThermoFisher Scientific); Lanes 1-10: APEC
257 isolates; Lane 11: APEC strain BEN2268 (positive control)**.**

258 3.5 Phage isolates and their host range

259 A total of 10 crude phage isolates were obtained but seven were successfully purified. The
260 purified phages were code-named as UPEC01, UPEC03, UPEC04, UPEC06, UPEC08, UPEC09
261 and UPEC10. The phage host range, as exhibited by lytic activity against 56 APEC isolates varied
262 from one (1.8%) to 10 (17.9%). Phage UPEC04 had the broadest host range, inhibiting 10 (17.9%)

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263 APEC isolates followed by UPEC06 and UPEC10 at 6 (10.7%) isolates each, then UPEC03 at 5
264 (8.9%) isolates, UPEC01 and UPEC08 at 4 (7.1%) isolates each; while UPEC09 had the narrowest
265 host range of 1 (1.8%) isolate. Only 14 (25%) APEC isolates out of the 56 were sensitive to any
266 one phage and the combined lytic spectrum of UPEC04 and UPEC10 phages includes all the total
267 APEC isolates that were sensitive. Therefore UPEC04 and UPEC10 phages were selected for
268 further analysis. Out of the 14 APEC isolates sensitive to the phages, 11 were multi drug resistant.
269 The phage sensitivity pattern of the seven phages on the 14 APEC isolates is presented in S1 Table.

270 3.6 Thermal and pH stability of UPEC04 and UPEC10 phages

271 Phages UPEC04 and UPEC10 were selected for further investigation because the combined
272 lytic activity of the two yielded the maximum host range of 14 out of the 56 tested APEC isolates.
273 Therefore, the heat sensitivity of these two phages was determined for temperatures ranging from
274 20°C - 70°C (Fig 4). The phages were stable to heat with only slight reductions in titers up to 50°C,
275 followed by a steep decline up to 70°C; beyond which they were undetectable. The highest titers
276 were obtained between 20°C – 50°C making this the range of temperature at which the two phages
277 are most stable.

278 **Fig 4. Effect of temperature on UPEC04 and UPEC10 phage viability**. Phage viability was
279 determined by obtaining the phage titers at the different temperatures using the agar overlay
280 method. Values are an average for duplicate tests.

281 3.7 Effect of pH on phage titer

282 The stability of UPEC04 and UPEC10 to pH ranges from 2 to 12 at both 25°C and 40°C is
283 presented in Fig 5. The phages retained viability across the different pH values with the lowest
284 titers registered at the extremes of pH (2 and 12), while the highest titers were registered between

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285 pH 4 and 8. The changes in the titers followed a similar pattern at the two temperatures, though
286 the titers were consistently higher at 25℃ compared to 40℃.

287 **Fig 5. Effect of pH on phage viability at 25℃ and 40℃.** A) Phage UPEC04. B) Phage UPEC10.
288 Phage viability was determined by obtaining the phage titers at the different pH using the agar
289 overlay method. Values are an average for duplicate tests.

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298 4. Discussion

299 The APEC isolates showed high resistance to commonly used antibiotics in poultry like
300 tetracycline, ampicillin and cotrimoxazole. This high level resistance has been reported in *E. coli*
301 from poultry and other sources [3,28]. This is likely to be as a result of irrational drug use,
302 especially among the poultry farmers and use of antibiotic supplemented feeds. Indeed, in Uganda,
303 Bashahun & Odoch (2015) reported that 96.7% of the poultry farmers frequently used antibiotics
304 for prevention and control of infectious diseases while 33.3% used the antibiotics to promote

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305 growth and enhance feed efficiency [29]. Resistance to antibiotics not commonly used in animal
306 production systems, such as chloramphenicol was unexpected but the ease of access without a
307 valid prescription to human drugs over the counter in pharmacies results in their misuse in animals
308 [30]. The latter is likely to be the explanation for the average frequency of resistance that was
309 encountered in case of Chloramphenicol (35.7%). Low frequency of resistance was revealed in
310 case of Gentamicin (10.7%) and Nitrofurantoin (8.9%); while all the 56 (100%) isolates were
311 susceptible to Cefixime. Susceptibility of all the isolates to Cefixime, could be due to the fact that
312 this is a recently introduced antibiotic, quite expensive and not readily available to the farmers.
313 This is in agreement with a study done by Dou *et al* (2015) who found out that there was low
314 resistance towards newly developed drugs [31]. A high rate of multidrug resistance has also been
315 reported elsewhere [10,31,32].The high level of antimicrobial resistance of APEC demonstrated
316 in this study calls for stringent regulations on antibiotic use on poultry farms. Additionally, due to
317 the challenges of developing new antibiotics, the high resistance rates reiterates the need to
318 introduce alternatives to drug use, such as the bio-control agents, like the bacteriophages.

319 Phylogenetic typing determines the genetic background or ancestry of an organism as well
320 as differentiating between the pathogenic *E. coli* strains (B2 and D) from commensals (A and B1)
321 [33,34]. Overall, phylogenetic analysis of APEC strains in this study revealed that majority
322 belonged to Phylogenetic groups A and D. This is in agreement with several studies done
323 elsewhere [20,28,35,36]. Johnson *et al* (2008) found out that majority of the APEC isolates
324 characterized belonged to A, B1 and D phylogenetic groups [9]. The 11 isolates from group A and
325 the three isolates from group B1 that lacked the virulence genes but were isolated from
326 colibacillosis suspect birds probably harbored other virulence genes that were not tested for during
327 the current study or they were just opportunistic. This is in agreement with Picard *et al* (1999) who

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328 found out that some strains of *E. coli* belonging to Phylogenetic groups A and B1 exhibiting
329 commensal characteristics would cause disease [37]. The 14 and five isolates from group A and
330 B1, respectively; that possessed virulence genes could have acquired them by horizontal gene
331 transfer from the pathogenic strains [31,38]. The three isolates from phylogroup D that lacked the
332 tested virulence genes probably caused colibacillosis by possessing other virulence genes not
333 screened for in this study. The above findings agree with other studies that demonstrated diversity
334 of Phylogenetic groups among APEC [39,40].

335 The selected virulence genes occurred in 69.6% of the *E. coli* isolates with varying
336 frequencies; indicating that they were potentially pathogenic. However, only 50% of the isolates
337 that had four or more genes can be categorized as APEC according to Johnson *et al* [9]. The
338 findings are supported by Kuhnert *et al* who concluded that pathogenicity of a given *E. coli* strain
339 is mainly determined by specific virulence factors which include adhesins, invasins, toxins and
340 capsule [41]. Seventeen isolates (30.4%) did not exhibit a single virulence gene. These isolates
341 could have been commensals that had become opportunistic due to host-dependent factors like
342 other infections, environmental stress, poor nutrition and hygiene [37,39,42]. Alternatively, these
343 isolates could be harboring other virulence genes that were not screened for in the present study
344 [9,32]. Several studies show that it is rare for all the virulence genes to be present in the same
345 isolate [20,31,43]. For instance, Delicato *et al* reported that 27.5% of the colibacillosis-derived
346 isolates did not possess any of the virulence-associated genes investigated [44].

347 The Episomal outer membrane protease gene (*ompT*) showed the highest prevalence at
348 58.9%. This gene encodes a protease that cleaves colicin, an inhibitory protein produced by other
349 *E. coli* [45]. The *ompT* gene is located on the ColV plasmid alongside other virulence genes like

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350 *iss*, *hlyF* and *iroN* [46]. A relatively high number of isolates harbored the *ompT* gene for protection
351 against colicin produced by other *E. coli*.

352 The lowest frequency was shown by Salmochelin siderophore receptor gene (*iroN*) at
353 41.1%. Like the *ompT* gene, *iroN* is located on the ColV plasmid and is one of the genes
354 responsible for iron acquisition [45,46]. Presence of virulence genes distinguishes APEC from
355 commensals and as a result these can be used as molecular markers for detection of colibacillosis
356 in combination with other diagnostic tools [47]. However, there is need to determine whether the
357 various isolates from this study are capable of establishing an infection in order to confirm their
358 pathogenicity.

359 Out of the 56 APEC isolates, none belonged to the serogroups O1, O2 and O78 which were
360 reported to be the most common [48]. This means that the above serogroups are not common
361 among APEC infecting chicken around Kampala. This can be explained by the fact that
362 distribution of serogroups varies from one region to another and that the APEC serogroups O1, O2
363 and O78 may not be as common as indicated in other countries like China [20,31]. Indeed, Riaz *et*
364 *al* reported occurrence of serogroups O1 and O2 but not O78 [49]. Ewers *et al* also demonstrated
365 that colibacillosis can be associated with serogroups other than O1, O2 and O78 [38]. Over 100
366 APEC serogroups have been reported and most of the previous research was carried out in Europe,
367 Asia and some in Brazil, which are geographically distant from Uganda [20,31,48]. The difference
368 in the prevalent serogroups is not unexpected and infers that vaccines against avian colibacillosis
369 developed elsewhere may not offer protection to chicken in Uganda.

370 From the findings regarding host range, no single phage was able to lyse all the studied
371 APEC strains. The maximum number that could be lysed was 14 out of 56 (25%). This is because
372 phages are highly specific towards their hosts [50]. This is in agreement with other studies that

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373 demonstrated that phages usually have a limited host range [25,51]. Having a relatively broad host
374 range is one of the desirable properties for selection of candidates for phage therapy [52]. The two
375 phages, UPEC04 and UPEC10, which had a combined lytic activity against 14 APEC isolates are
376 better candidates for formulation of cocktails for therapeutic intervention compared to the others.
377 However, there is need to obtain more phages with a wider host range by using either a mix of
378 multiple host strains of the same species for phage isolation or growth on multiple hosts
379 sequentially, that is, one host at a time [52]. Lysis of the eleven multi-drug resistant APEC isolates
380 by the phages demonstrates the potential of phages in controlling infections caused by multi drug
381 resistant bacteria.

382 The main physical factors affecting phage adsorption and growth include pH and
383 temperature [53]. The different pH and temperature ranges in this study were selected to mimic
384 those that would be encountered during the handling and application of these phages as therapeutic
385 or sanitizing bio-control agents on poultry farms. Both UPEC04 and UPEC10 were stable to heat
386 up to 60°C. At 70°C, the phages were inactivated which is in agreement with Lu *et al* (2003) and
387 Shende *et al* (2017) who reported that phages get inactivated at 70°C and above [51,53].

388 The effect of pH on phage viability at 25°C and at 40°C represented activity at room
389 temperature and body temperature of chicken, respectively. The two phages were tolerant to a
390 broad range of pH similar to what was observed in previous studies [26]. The tolerance to a broad
391 range of temperature and pH coupled with a wide host range, makes the two phages suitable
392 potential candidates for a cocktail product that can be used as an alternative to antibiotics in the
393 control of APEC infections [27].

394

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402 # 5. Conclusion

403 None of the APEC isolates analysed belonged to the most common serotypes O1, O2 and
404 O78 as reported elsewhere. Over 80% of the strains exhibited multi drug resistance against the
405 most commonly used antimicrobials. The *E. coli* isolates belonged to various phylogenetic groups,
406 with the majority belonging to phylogroup A and the minority to phylogroup B2. The selected five
407 virulence genes were present in 69.6% of the APEC isolates at varying frequencies. Of the seven
408 phages that were isolated, two had the highest combined host range of 25% and exhibited lytic
409 activity under a wide range of temperatures and pH, making them potential candidates for a
410 therapeutic cocktail product.

411 ## Recommendations

412 Future studies can be carried out to determine other virulence genes responsible for the
413 pathogenicity of APEC. There is need to establish the circulating APEC serotypes in Uganda,
414 hence comprehensive screening for other serotypes is necessary. Further investigations are needed

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415 to determine the characteristics of the bacteriophages such as, growth rates, latent times, burst
416 sizes, morphology and genome sequences before they can be applied as therapeutic agents in the
417 control of colibacillosis.

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422 for phage isolation were obtained; are greatly acknowledged.

423 Authors Contributions

424 GK – Molecular analyses, bacteriophage evaluation; PK – Bacteria and bacteriophage isolation;
425 RA – Antimicrobial sensitivity testing, bacteriophage evaluation; SA – bacteriophage evaluation;
426 AN – Molecular analyses; JLN – Conceptualized the research, data analysis. All authors read and
427 contributed to the drafting of the manuscript.

428 Availability of data and materials

429 Relevant data generated or analyzed during this study are included in this article and supporting
430 information files.

431 Ethics approval and consent to participate

432 Not applicable.

433 Consent for publication

434 Not applicable.

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603

604 # Supporting information

605 **S1 Table. Host Range. Sensitivity patterns of the seven phages on the 14 APEC isolates**

606 **S1 Appendix. PCR protocol for detecting the virulence genes**

607 **S2 Appendix. PCR protocol for determining phylogenetic groups**

608 **S3 Appendix. PCR protocol for detection of APEC serotypes**

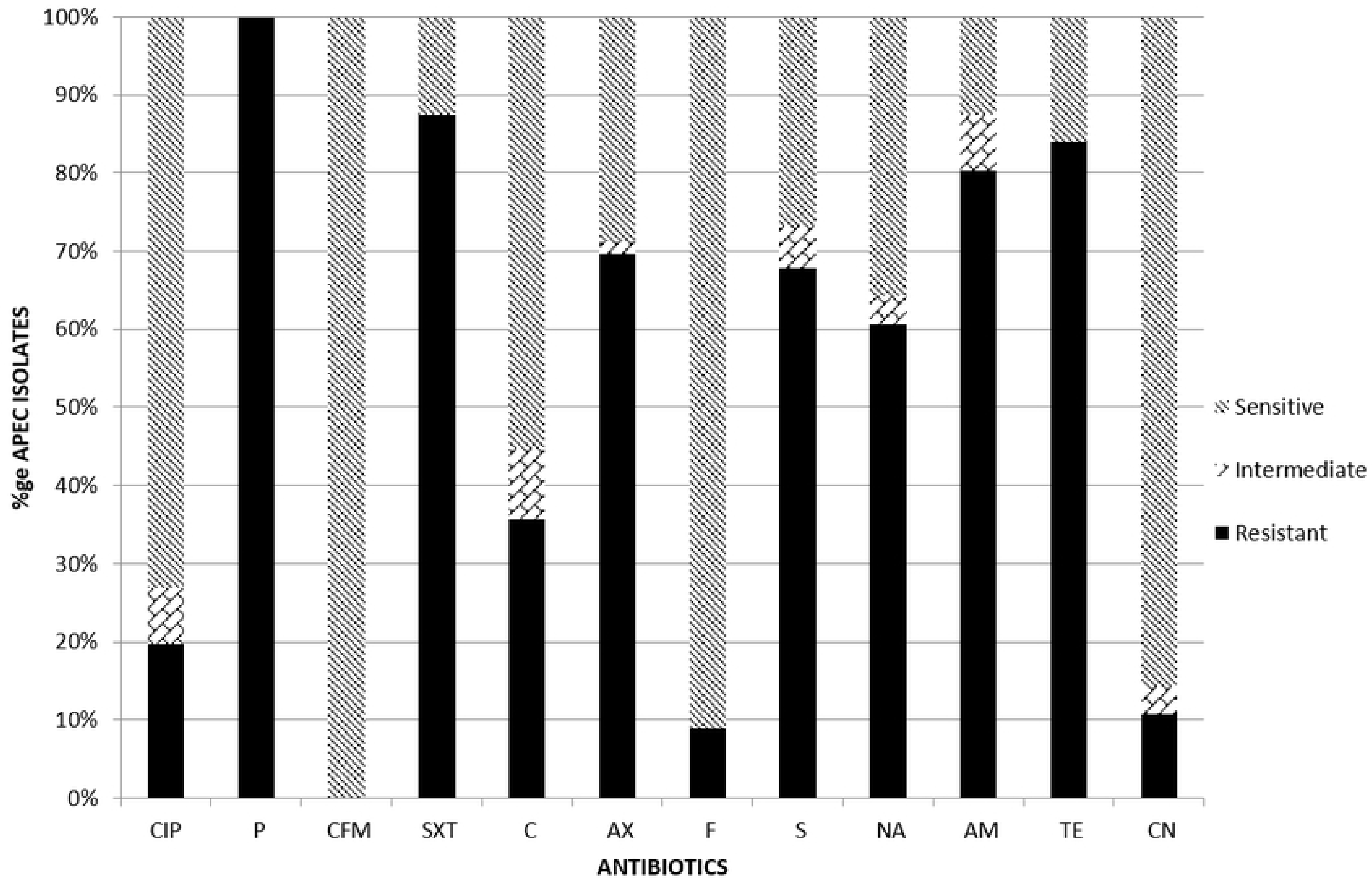


Fig. 1 Antimicrobial susceptibility test results for Avian Pathogen

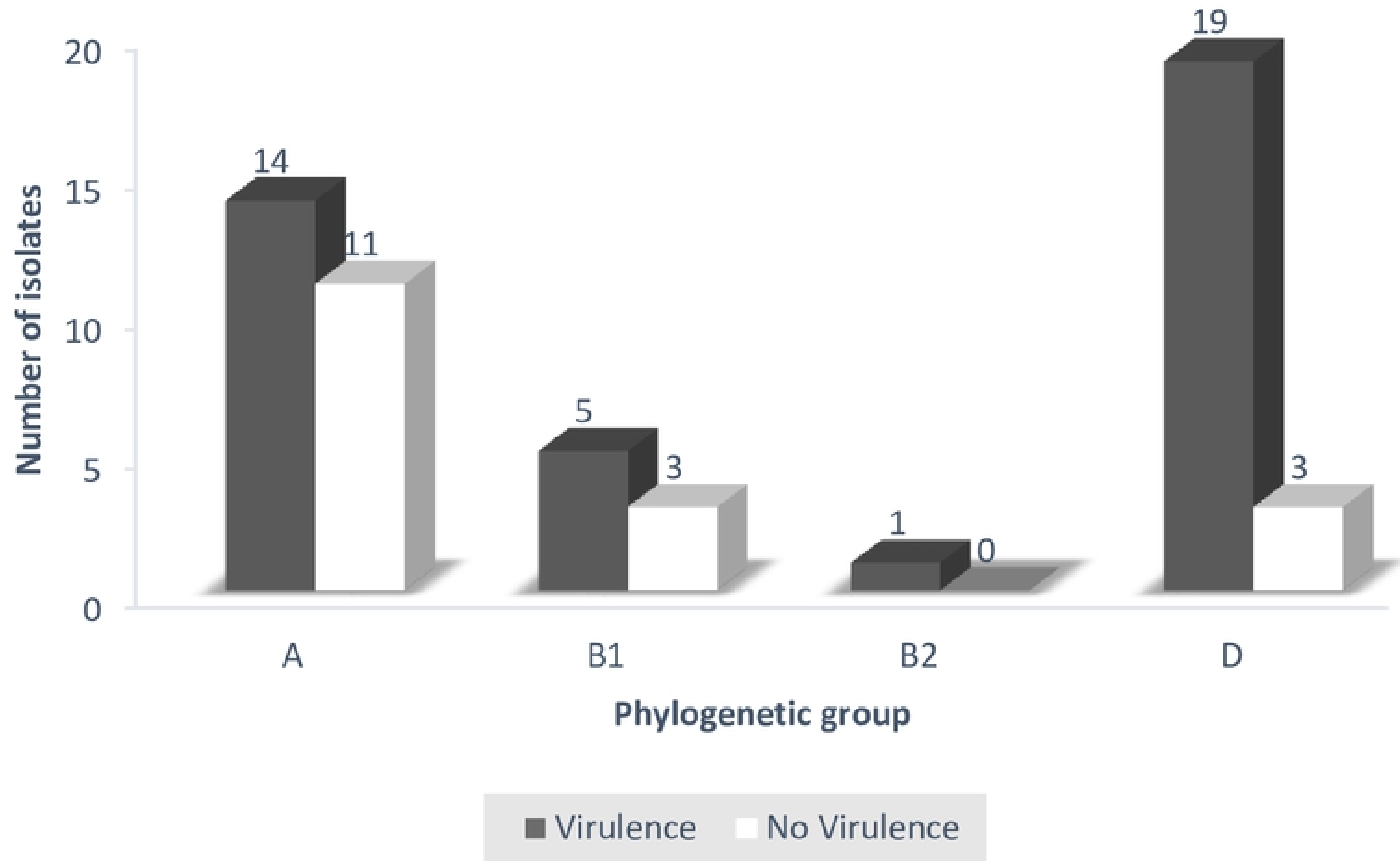


Fig 2. Virulence gene content of APEC isolates within each phylog

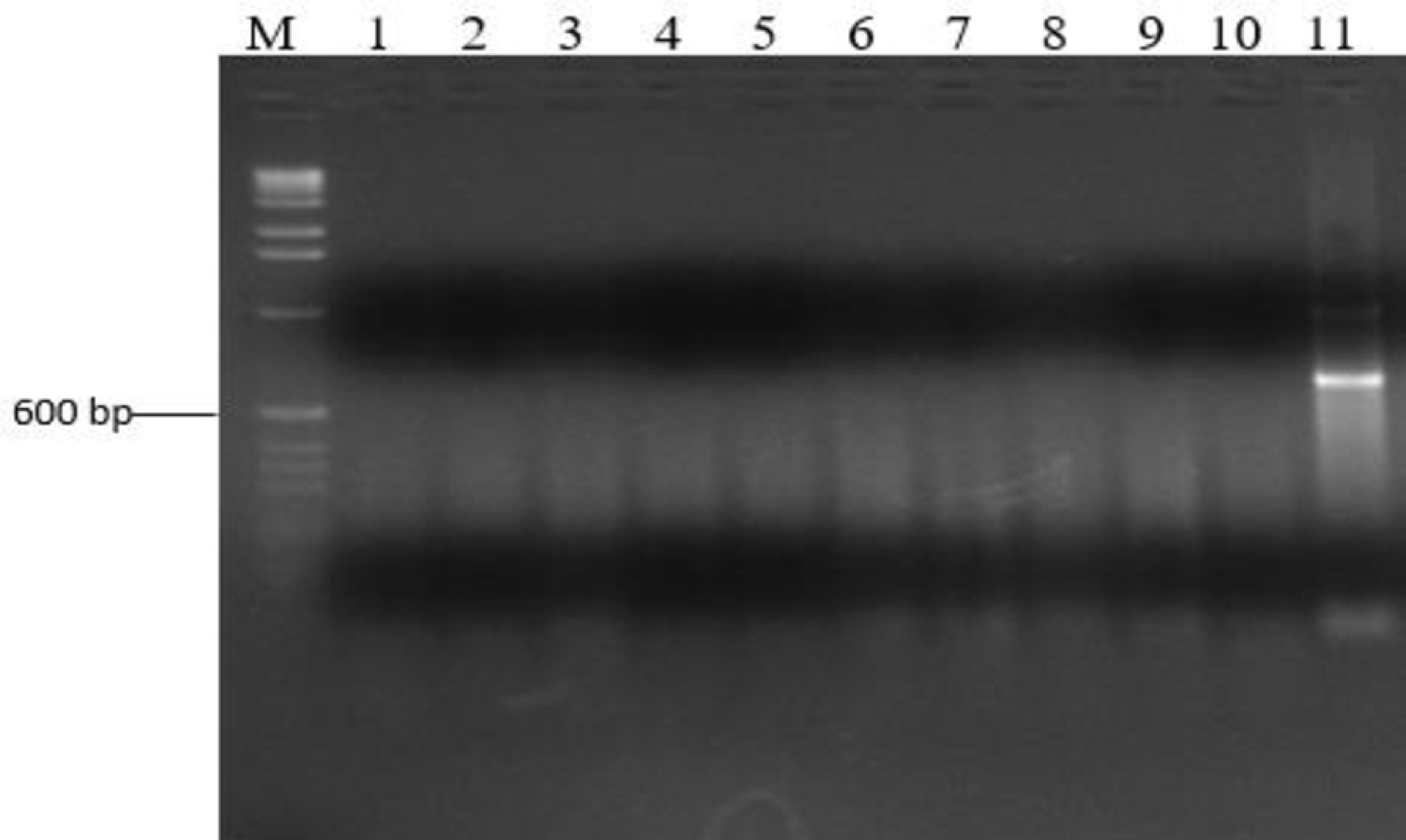


Fig 3. Agarose gel showing PCR amplicons from selected APEC is

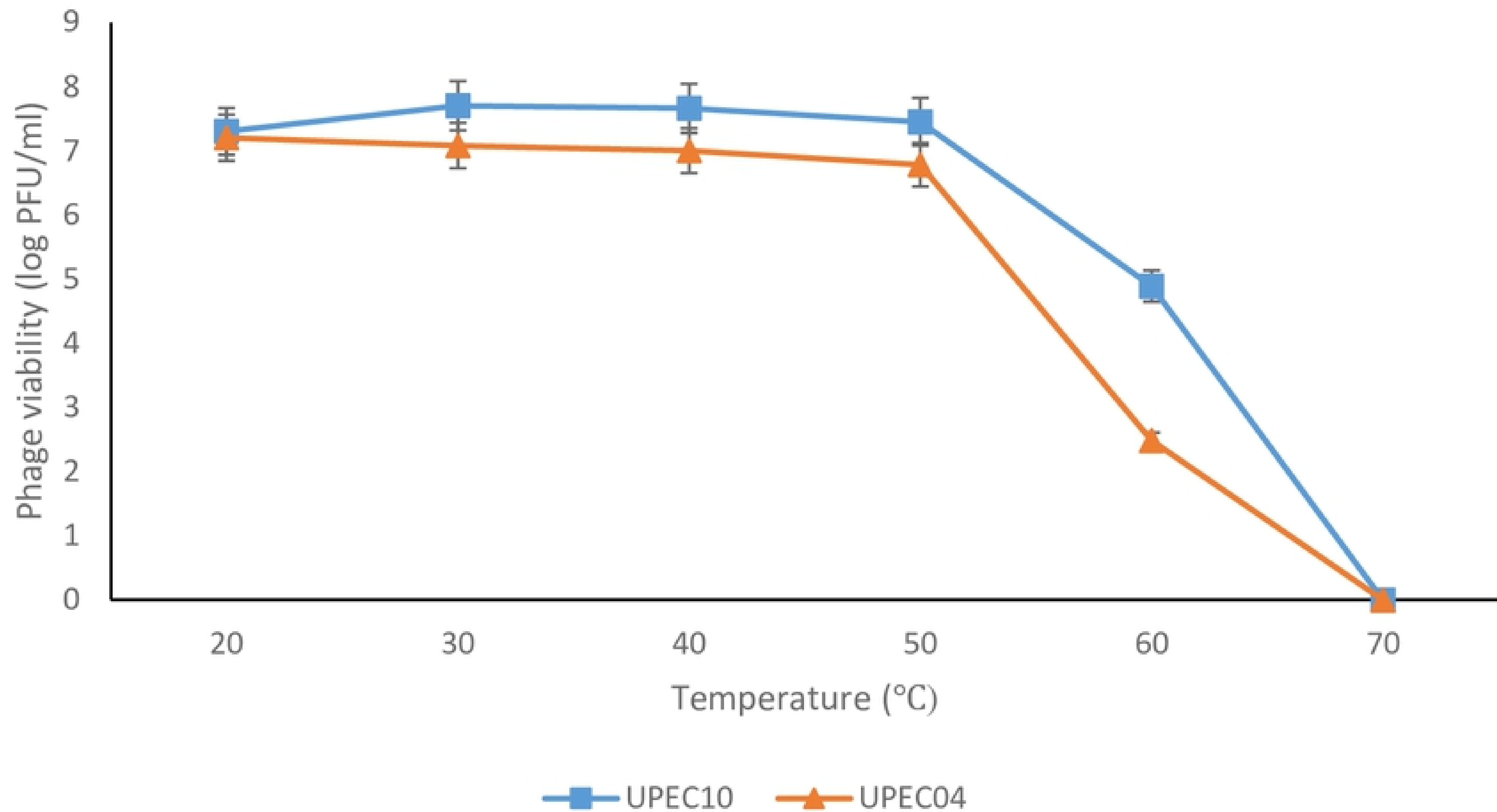
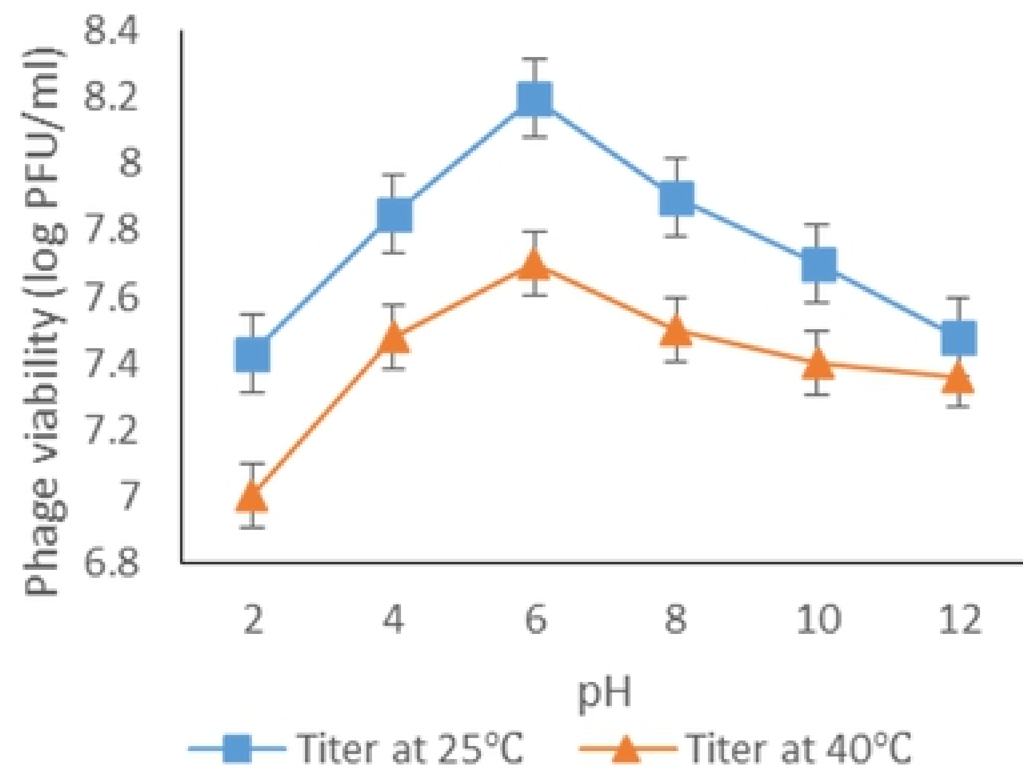


Fig 4. Effect of temperature on UPEC04 and UPEC10 phage viabi



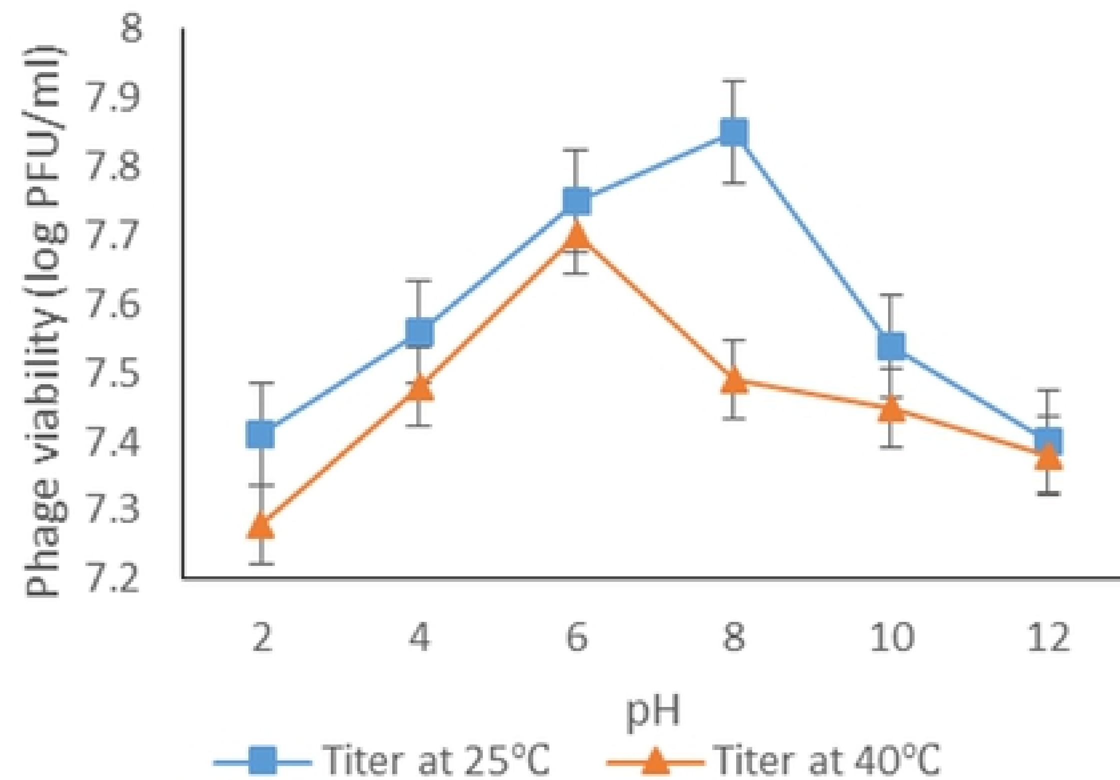


Fig 5. Effect of pH on phage viability at 25 and 40