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ISOLATION AND CHARACTERIZATION OF *CITROBACTER FREUNDII* FROM SHEEP AND DETECT SOME OF THEIR VIRULENCE GENE USING PCR TECHNIQUE

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Abstract:

The goal of this investigation was to see if there was a virulence gene in *Citrobacter Freundii* isolates from sheep in Baghdad. Total of (100) clinical specimens were collected as rectal swabs. From December 2020 to March 2021, these specimens were collected from sheep with diarrhoea and normal sheep. Different cultural and biochemical tests were performed, and then a Vitek2 system was used to identify *Citrobacter freundii* isolates.
In this investigation *viaB*, *hlyA*, *LT*, and *STp* genes prevalence between isolated bacteria was low. When DNA extracted from the eight (8) bacterial isolates, PCR technique was used to amplify the particular *viaB* primer; following that, gel electrophoresis revealed that only 2 (12.5 percent) of the (8) samples have the (516) bp DNA fragment (viaB gene) when compare to the allele ladder. To see if *hlyA* gene[(597) bp fragment] are found in the eight *isolate* of ***Citrobacter Freundii*** , gel electrophoresis revealed that only 4 (50 %) of the (8) samples contain the (597bp) using primers of *hlyA* When compared to an allelic ladder. For the detection the prescence of LT gene the gel lectrophoresis revealed that only three 3 (37.5%) of the (8) isolates produced the (273bp) DNA fragment. Furthermore, 5 (62.5%) isolates have the particular (166 bp) DNA fragment was shown to be the heat stable toxin gene among isolates.

Keywords: Citrobacter Freundii, sheep

INTRODUCTION:

Citrobacter freundii is a commensal bacterium that lives in the intestines of both humans and animals (Guerrant *et al.*,1976).*Citrobacter spp.* can infect animals and humans under stressful situations (Elsherief *et al.*, 2014; Oliveira *et al.*, 2017). The importance of *Citrobacter* species in animals has yet to be determined. Mammals, birds, and reptiles, such as snakes, lizards, and tortoises, are all susceptible to fecal carriage and extra intestinal diseases. (Luperchio and Schauer, 2001).
Three *C.freundii* isolates were randomly obtained from twenty-five chicken flesh samples taken from different marketplaces in Baghdad .(Hashim and AlKhafaji.,2018). Notable cases of disease of *Citrobacter spp.*, isolated from fish, animals, humans, soil, water, and food (Brenner .,1999). Antibacterial resistance mechanisms in catfish (Nawaz *et al.*,2008) and disease in rainbow trout caused by *Citrobacter freundii* (Aydn *et al.*,1997) were investigated using bacteria from the genus *Citrobacter*.
C. freundi and other *Citrobacter spp*. isolated from four floating cage farms in the Al-Hilla river by Al-Haider *et al.*,2019 are toxic and may be harmful to public health if consumed. Al-Samarraae and Mohammed(2020) isolated C.Freundii from sheep diarrhea in Baghdad.

MATERIAL AND METHODS:

Samples collection:

One hundred sheep feces samples were gathered from various locations throughout Baghdad, then packed in ice and delivered to the lab in less than two hours (Quinn *et al.*, 2011). The samples were incubated at 37°C for 24 hours after being cultivated on MacConkey and XLD agar. The size, form, and color of the colonies were noted, and the probable *Citrobacter* was identified using a biochemical test (McFadden, 2000) before being validated using the Vitek-2 Compact (Bio Mérieux, France).
HiPurA® Bacterial Genomic DNA Purification Kit (HiMedia/IndiaPellet) was used to extract DNA. For prolonged storage, Storage of purified DNA in -20°C or (-80°C) For short-term or long-term storage.

Virulence genes have been discovered.

Using the Polymerase Chain Reaction, The primers used in the PCR for the detection of the associated virulence genes *viB*, *hlyA*, *LT*, and *STp* which were purchased from (Schmidt et al., 1983; Wong et al., 1997; Bo lin et al., 2006; and Sjo ling et al., 2007)., listed in Table(1).

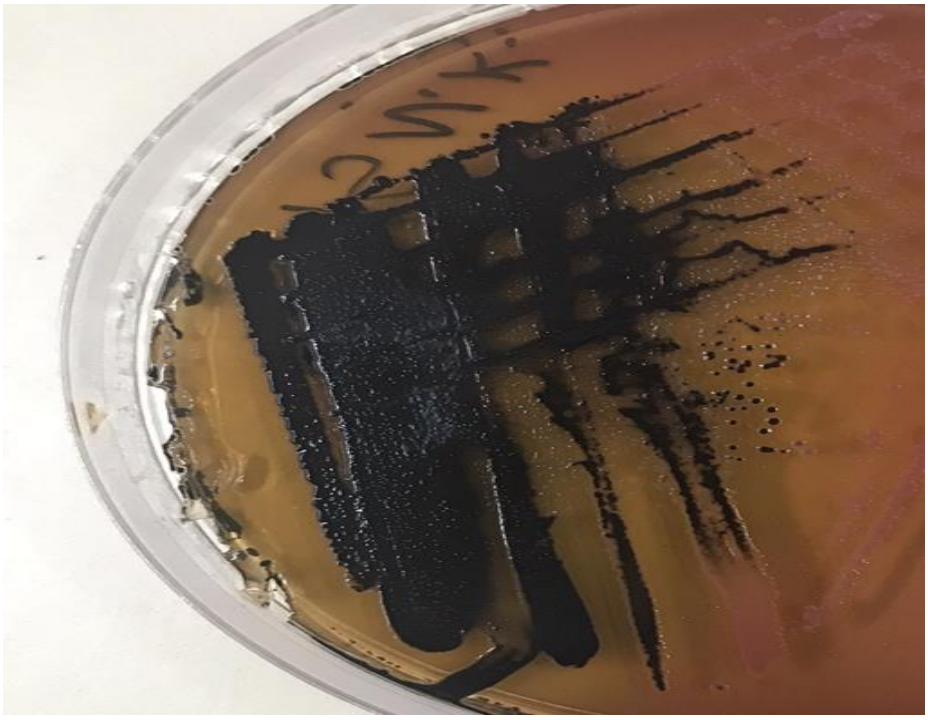
0.5 µLiters Taq polymerase, 10 µL Taq buffer, 2µL liters dNTP, 5.0 µL tuning buffer, 1.0 µL template, 12 µL dissolved water, and 1 µL from each of the primer pair made up the PCR mixture. 35 cycles of 30 seconds at 94°C, 0.5 minute annealing at temperature 62°C (for the hlyA gene), 2 minute at 72°C for the extension , 1 minute at 72°C final extension were used in the amplification strategy. 2 percent agarose gels were used for separation of PCR products and observed under UV light. For amplification of the *via B* gene, *LT*, and *STp* The thermal cycle was consist of 5 minutes initial denaturation at 95°C, 0.5 min. anneal at temperature 62°C, 60 seconds of elongation at a temperature 72°C, and 10 minutes of final extension at 72°C. On an agarose gel, ethidium bromide stained the PCR results. under ultraviolet light PCR bands were examined then photographed (Bunyan.,2020).

Table (1) : DNA Genes Specific Primers which are used in PCR for Detection of Bacterial Isolates

primers	Sequence of nucleotide	fragments size (bp)	References
viaB	F-TGTCGAGCAGATGGATGAGCAT R-ACGGCTGAAGGTTACGGACCGA	516	Schmidt *et al.*, 1983
hylA	F-GGC CGG TGG CCC GAA GAT ACG GG R-GGC GGC GCC GGA CGA GAC GGG	597	Wong *et al.*,1997
LT	F-ACGGCGTTACTATCCTCTC R-TGGTCTCGGTCAGATATGTG	273	Sjoling *et al.*, 2007
STp	F-TCTTTCCCCTCTTTTAGTCAG R-ACAGGCAGGATTACAACAAAG	166	Bo lin *et al* .,2006

RESULTS: -

C. freundii isolates were acquired from one hundred fecal samples (normal feces and diarrhea) and eight isolates were recovered out.*C. freundii* was identified by examining colonial morphology on Salmonella Shiglla agar(SS), MacConkey agar (M.A) and **Xylose Lysine Deoxycholate agar** (XLD). *Citrobactor Freundii* colony appeared on SS agar as small or large pale flat with black centers because H2S producing ability after 24 hours on S.S agar. Figure (1).



Figure(1) *Citrobactor freundii* on SS agar

Because of the lactose fermenter, *Citrobacter* isolates exhibited as pink colonies on MacConkey agar, Figure 2:

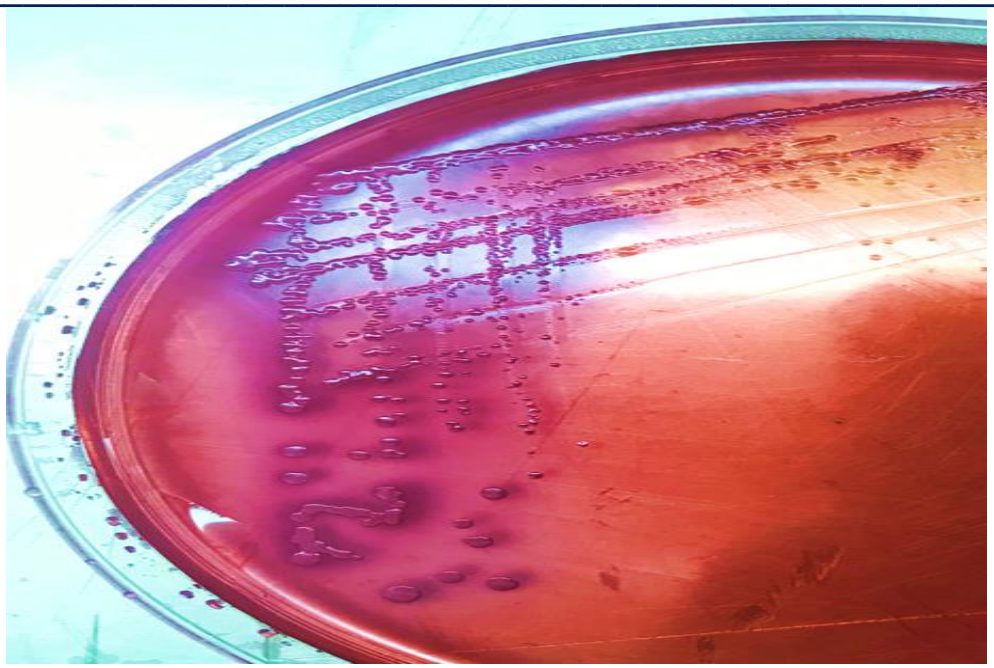


Figure 2: *Citrobacter freundii* on MacConkey agar

Citrobacter showed up as yellow colonies on XLD figure (3)

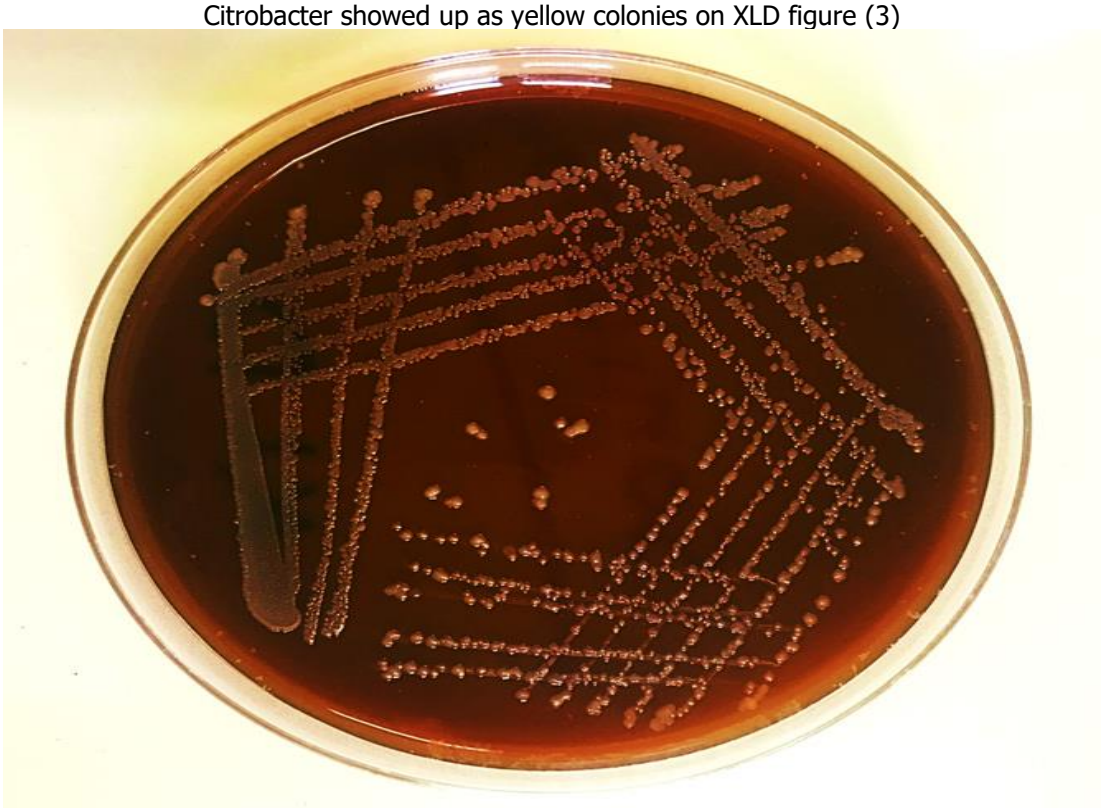


Figure 3: *Citrobacter freundii* on MacConkey agar

To ensure that the identification on previous agars was correct Gram stain used to observe characteristics of Gram negative bacteria under microscope. To confirm *Citerobacter spp.* identification. Biochemical analyses were performed on all isolates and the standard strain (table 3).

Tests	**Results**
Growing on MacConkey agar	Pink colonies
S.S agar	Black center
XLD agar	Yellowish colonies
Gram stain reaction	G-
Urease	-
Catalase	+
Oxidase	-
Gelatinase	-

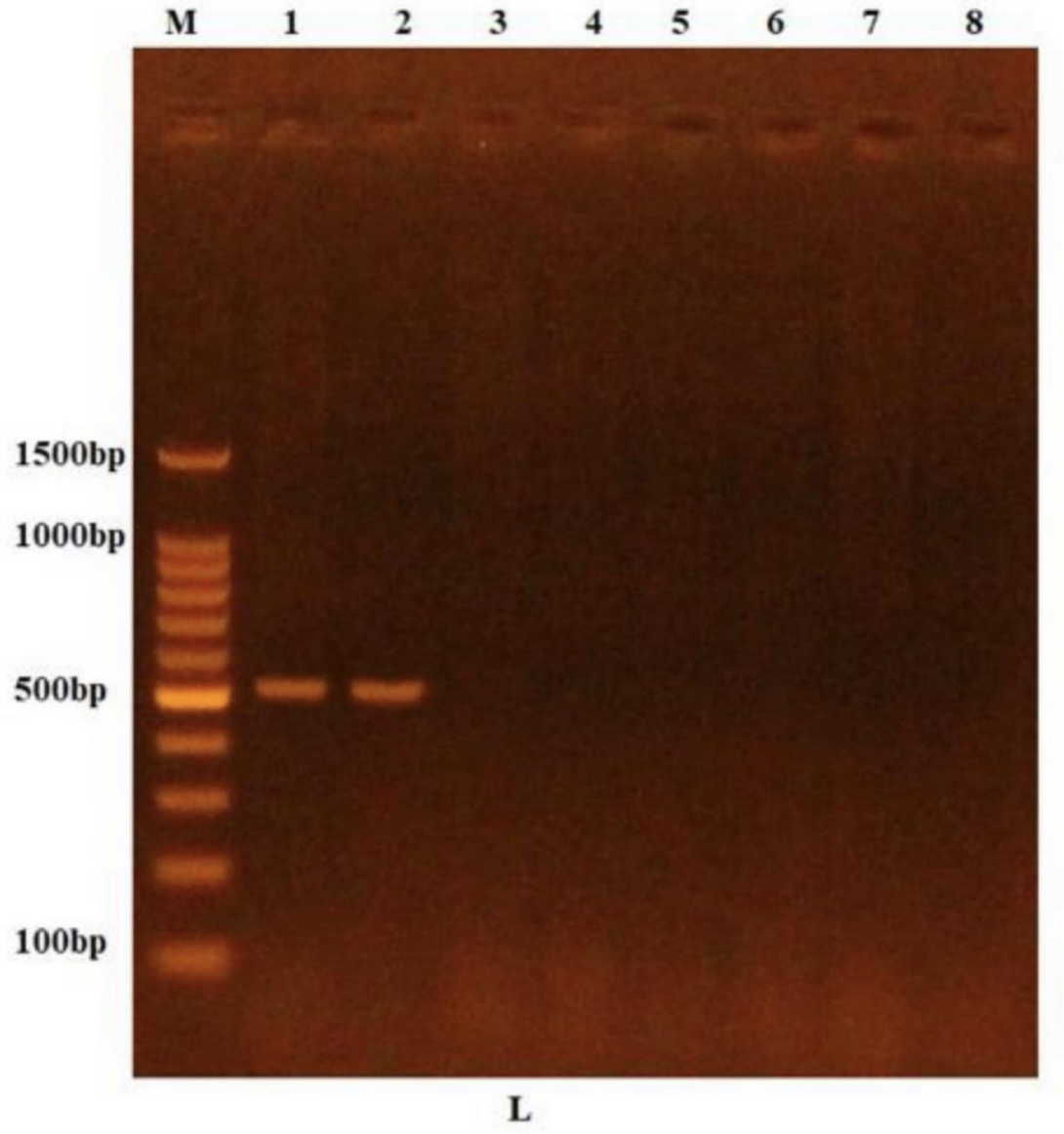
The *Citerobacter spp.* identification was confirmed using the Vitek 2 compact system. *Citrobacter freundii* was shown to be the cause of the eight bacteria recovered in this study.

Citrobacter spp. are nosocomial bacteria that can infect the urinary tract, hematologic, or neonatal systems (e.g., meningitis, sepsis, general bacteremia), intra-abdominal sepsis, brain abscesses, or pneumonia. (Ryan et al., 2004; Raphael-Riley, 2017). One hundred fecal samples (normal feces and diarrhea) of female and male animals yielded positive findings for *C. freundii* isolation (25 percent). These findings corroborated previous findings of Al-Muslemaw, 2007) In Baghdad, eight *C. freundii* isolates were identified from 250 clinical samples such as feces and urine, and *C. freundii* was the most frequent type, accounting for 75% of clinical isolates. These findings are also in line with (Hashim- AlKhafaji, 2018). Which identified (3) *C. freundii* from a sample of 25 chicken meats from a local market in Baghdad.

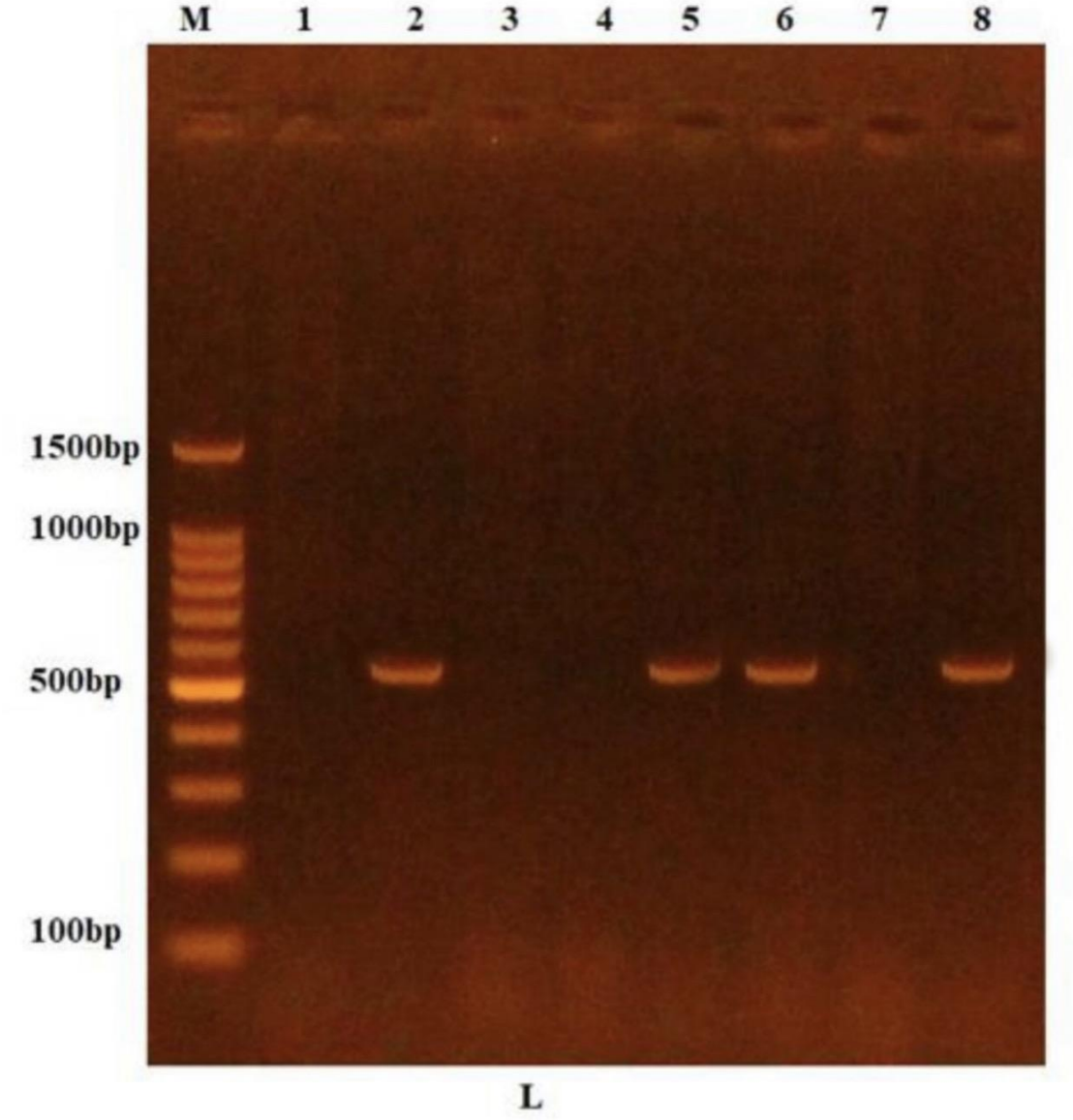
MOLECULAR RESEARCH

Between the C.F isolate found in this study presence of via B and hlyA , LT, and STp related genes were found to be different in this study. The DNA were extracted fron all (8) isolates, and standard PCR technique used to amplify particular *viaB* primers utilizing these DNA samples, according to the sequences in Table 1. When compared to an allelic ladder, gel electrophoresis revealed that only two (12.5 %) of the eight samples have the particular DNA fragment (516 bp) Figure (4). When tested with a specific primer, these findings agreed with (Hossain *et al*. 2017). The viaB gene permits the evading of the bacteria the host's immune system, allowing infection to last longer (Rondini et al., 2017).

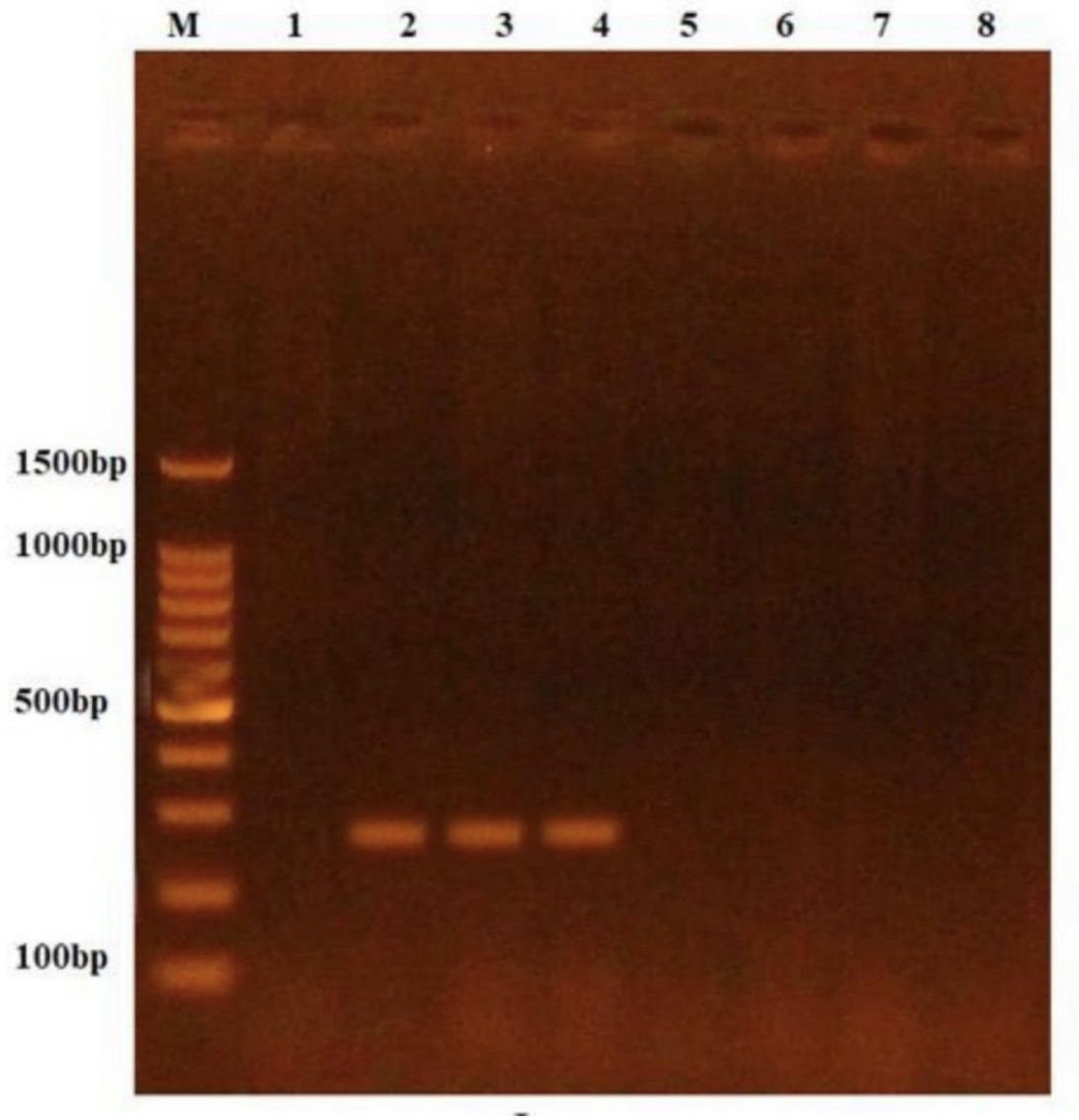
For the presence of hlyA gene in the (8) *Citrobacter freundii* a PCR was done using hlyA primers to investigate if a fragment (597) bp which represents the hlyA gene. When compared to an allelic ladder, gel electrophoresis revealed that only 4 (50%) of the (8) samples produced the specified (597bp) DNA fragment figure(6). In a study of *Citrobacter freundii* isolates, For the hlyA gene, Rondini *et al*., (2017) found no positive results. Several strains of *C.freundii* have been shown to have heat-stable toxins, Shiga toxins, , and the(cfxAB) cholera toxin homologs according to earlier reports. (Guarino *et al*., 1987; Tschape, 1995 and Karasawa *et al*., 2002). We were able to detect the genes of Shiga toxin, as well as heat labile and heat stable enterotoxins, using previously described primer. When compared to an allelic ladder, gel electrophoresis to detect LT gene revealed that 3 (37.5%) of the (8) samples produced the required (273bp) DNA fragment, as shown in Figure (7). Furthermore, as shown in Figure(8), In a prior investigation, 4 (50%) isolates have the DNA fragment (166 bp) (heat stable toxin) while heat lable gene were found in (3) isolate olny . According to studies, the Shiga toxin (LT and ST) genes were found in *C. freundii* (Schmidt *et al*., 1983 and Herold *et al*., 2004). Overall, *C. freundii* isolates with virulent features identified using conventional PCR had the possiblity to be animal pathogens.



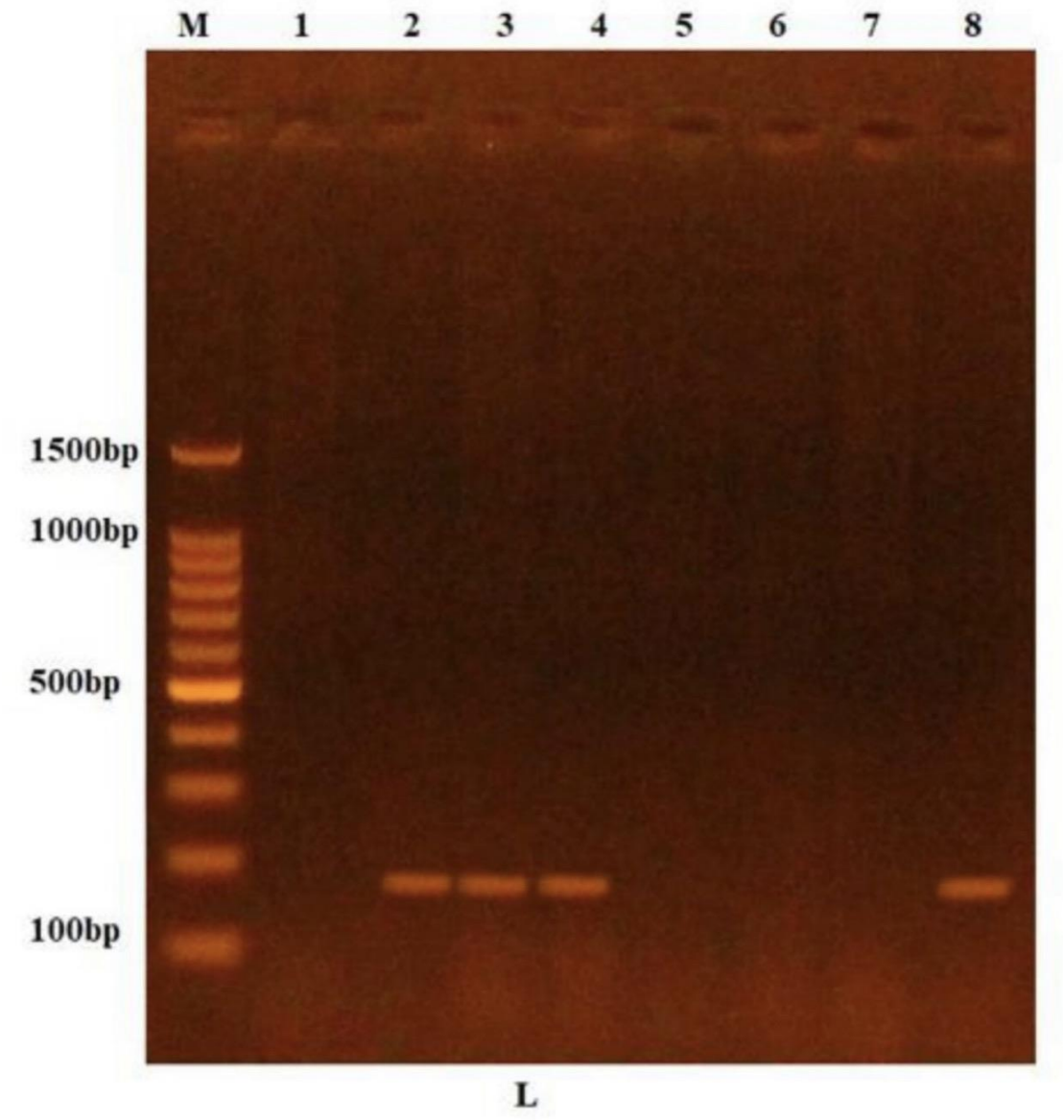
Figure(5) gel electrophoresis of the eight samples to the particular (516 bp) DNA fragment (viaB gene).



Figure(6): gel electrophoresis of the eight samples to the particular (567 bp) DNA fragment (hlaA gene).



Figure(7):): gel electrophoresis of the eight samples to the particular (273 bp) DNA fragment (Lt gene).



Figure(8): gel electrophoresis of the eight samples to the particular (166 bp) DNA fragment (St gene).

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