

## Phenotypic and Genotypic Detection of UV-Induced Bacteriocin Production In Enteric Gram-Negative Rods and Their Activity Against *Escherichia coli* O157:H7

### ABSTRACT

Comment [H1]: Aim was absent.

**Background:** Virulence and antibiotic resistance of bacteria pathogens is one of the most universal threats to public health care.

**Materials:** In this study, a total of two hundred and fifty seven (257) enteric bacteria isolated from human rectal swabs and cattle dung were screened for production of low-toxic, high target-specific bactericidal macromolecule peptides (Bacteriocin) against strains of *E. coli* O157:H7 using agar well diffusion method. The producer isolates were exposed to ultra-violet (UV) rays (UV 302nm and 254nm) for induction.

**Results:** Out of the 257 isolates, 21 (8.1%) were active against *E. coli* O157:H7 due to their constitutive bacteriocin, however, on induction by UV ray ; only 7 (2.1%) retained their potency for bacteriocin production. Three (42.9%) of seven isolates were randomly selected for genotypic screening (Col 28b (L) and Col K genes) because of their increased zone of inhibition (bioactive ability) after exposure to UV ray; the isolates also showed resistance to  $\leq 2$  classes of antibiotics and exhibited Gamma/alpha haemolysis.

**Conclusion:** This study revealed *Serratia marcescens* in this work to possessed an antimicrobial molecule of bacteriocinogenic type with possession of Col 28b gene

**Keywords:** bacteriocin production, enteric bacteria, Gram negative rods, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

### INTRODUCTION

Comment [H2]: Aim of this research was absent in the introduction section.

Bacteriocins are small size relatively narrow killing spectrum proteins of different types which are produced by both Gram positive and Gram negative bacteria and is plasmid-encoded antibacterial protein produced during times of stress (nutrient- limited environment and high temperature), which are generally active against strains that lack the respective encoding plasmid and closely related to the producer.<sup>1</sup> They are different to classical antibiotics: they are ribosomal synthesized and have a narrow killing spectrum in term of its specificity to bacteria that lack their particular plasmid.<sup>2</sup> Its family includes a variety of proteins in terms of different molecular sizes, mode of action, microbial targets, release to targeted microbes, and their immunity mechanisms.<sup>2</sup>

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Bacteriocins antimicrobial capabilities are achieved through diverse mechanisms which include; inhibition of cell wall synthesis, permeabilization of the target cell membrane, or inhibition of RNase or DNase activity.<sup>2,3</sup> Bacteriocins work better against their target bacteria than antibiotics at lower concentrations. Each bacteriocins has specific immunity gene to confer immunity to its producer, bacteriocin has an unequal attribute for its mechanism of target cell resistance or sensitivity when comparing to antibiotics.<sup>4</sup> Bacteria which are bacteriogenic in nature form complex in the gastrointestinal tract of human and domestic animals to prevent the accumulation of pathogenic bacteria in them.<sup>5</sup> Each bacteriocin possesses immunity protein which provides resistance to microorganism that produced it, in order to protect the producing bacteria from the toxic effect of both endogenous and exogenous bacteriocins. This immunity protein is specific to each one bacteriocin type.<sup>5</sup>

Shiga toxin producing *E coli* O157: H7 is the most dangerous bacteria strain causing severe enteric diseases and death in humans and animals.<sup>6</sup> Haemorrhagic colitis in humans are cause by several *E. coli* strains however, the most frequent outbreak of this disease has been reported in relation to O157:H7, symptoms such as watery diarrhoea, vomiting and abdominal cramps are experience by

the infected person, which become worsen if treatment is not urgent and complications could set in and result to bloody diarrhoea, kidney failure eventually leading to Human Uremic Syndrome (HUS), especially in children and aged.<sup>7,8,9</sup> The severe infection caused by *E. coli* O157:H7 can be terminated by the administration of bacteriocin produced by commensally non-pathogenic bacteria.<sup>1</sup>

## MATERIALS AND METHODS

### Collection of Human Rectal Swab and Cow Dung Samples

Sample collection was done from July 2015 to January 2016, sterile swabs stick was used to collect rectal sample, inserted into 5ml sterile nutrient broth and transported on ice to the laboratory. The medium was incubated at 37 °C for 24 hours. Cow dung samples were obtained from selected cattle rearing settlement located within Osogbo metropolis. Freshly dropped dung from 62 apparently healthy adult cattles from the cattle rearing settlement were aseptically collected using sterile cotton swabs and aseptically put into 5 ml sterile universal sampling bottles containing sterile nutrient broth and analyzed within six hours of collection.

### Isolation of Gram Negative Rod Bacteria from the Samples

Gram negative rod bacteria were isolated from the human rectal swab (human origin) and cow dung (animal origin) samples using streaking method.<sup>10</sup> A loopful of the samples from inoculated transport medium was streaked on Eosin-Methylene Blue agar (EMB) and MacConkey agar plates, incubated at 37°C for 24hours. Discrete colonies were sub-cultured and purified on nutrient agar plates to develop a pure isolate. A total of 257 morphologically distinct Gram negative rod bacteria isolate colonies from both human rectal and cow dung samples were sub cultured and purified on nutrient agar plates for the development of pure isolates, stored on nutrient agar slants at 4°C for further characterization and identification tests.<sup>11</sup>

Comment [H5]: Which tests ???

### **Bacteriocin Production Potential of the Gram Negative Rod Bacterial Isolates:**

*E. coli* 0157:H7 (ATCC 35150) which is the indicator isolate was collected from Molecular Laboratory of the Department of Medical Laboratory Science, of Ladoke Akintola university Ogbomoso. The strain was sub-cultured on MacConkey and EMB agar plates to refresh the culture before use. The screening was done using agar well diffusion method. A total of 257 Gram negative rods bacteria isolates were sub-cultured into 5% glycerol nutrient broth incubated at 37°C for 24 hours on constant shaking. The culture was centrifuge to separate supernatant from packed cell at 4000 rpm for 30minutes. Indicator organism *E. coli* 0157:H7 (ATCC 35150) was standardized to 0.5 McFarland standard, by inoculating it into Physiological saline solution (0.85% NaCl) and adjusting to the standard (using 0.5 ml of 0.048 M of BaCl<sub>2</sub> added to 99.5 ml of 0.18 M H<sub>2</sub>SO<sub>4</sub> as standard for comparison). One hundred microlitres of solution was inoculated using spread plate method on to Mueller Hinton agar plates, sterile cork-borer was used to make 8 mm holes at proper distance on the plate while the wells were filled with 20 µl of both raw culture and centrifuged supernatant separately. The plates were left for 1 hour before incubated at 37°C for 24 hours. The antibacterial activity of the producers' strain appeared in the form of zone of inhibition form around the well, measured and recorded in mm.<sup>11</sup>

### **Induction of Positive Bacteriocin Producing Bacteria:**

Colony from pure cultures of bacteriocin producing Bacteria strains were inoculated in 5% Glycerol Nutrient Broth, incubated at 37°C for 24 hours. Induction methods described by <sup>10,11,12,13</sup> were modified. Portions of 5 ml, of 5% Glycerol Nutrient Broth culture were irradiated in test tubes at mid log phase of growth, to induce bacteriocin production using a UV type A and C lamp of 302 nm and 254 nm wavelength 30-w GE germicidal lamp for 5minutes respectively and incubated at 39 °C for 5 hr. The cells were removed by centrifugation at 4000 rpm for 30 minutes, standardized

indicator organism was swab on Mueller Hinton agar plates and chloroform treated supernatant (20 µl) was poured in to 8 mm wells bored at proper distance on plates. The plates were left to stand for 1 hour at room temperature, and then incubated for 24 hours at 37°C. The clear zone indicate the activity of bacteriocin positive isolates, they were subsequently selected for further studies and stored in glycerol broth at -20°C

### **Preliminary Pathogenicity and Antibiotic Susceptibility Screening of Bioactive Gram negative Isolates**

For evaluation of haemolysin production, each pure strain of the bacteriocin-producing isolate was streaked on 5% blood agar incubated at 37°C for 24 hours.<sup>11</sup> Antibiotic susceptibility of the bacteriocin-producing isolates to various antibiotics including Ampicillin (10 µg), Amoxicilin/Clavulanic acid (30µg), Chloramphenicol (30µg), Tetracyclin (30µg), Nalidixic acid (30µg), Gentamicin (10µg), Sulphamethoxazole/Trimetroprim (25µg) and Nitrofuranton (300µg) were determined on Muller Hinton media using disc diffusion method for the standard procedure of the Clinical and Laboratory Standards Institute.<sup>14</sup>

### **Identification Procedure using MICROBACT 12E**

Identification of active isolates against *E. coli* O157:H7 was carried out by using standard MICROBACT GNB 12E microplate format (Oxoid product) for the identification.

### **Protein Assay and Molecular Detection of bioactive isolates**

The Bradford assay is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins. Method described previously was adopted.<sup>15</sup> 100 µL of each of the diluted isolate was added to the serial standard in a separate test tube. 5.0 mL of Coomassie Blue was added to each tube and mix, the visible spectrophotometer was adjusted to a wavelength

of 595 nm, and distilled water was used as blank. Standards and samples were read at 595 nm wavelength after 5 minutes.

Determination of protein molecular weight was carried out using SDS-PAGE screening method. A 10 µl aliquot was applied to a gel as well as the standard marker been loaded in the first gel lane. 100 µl of the supernatant centrifuge isolates was suspended in electrophoresis loading buffer and heated for 5 min at 100°C. The isolates were analyzed by SDS-PAGE using 8% acrylamide resolving gel, stained with 0.1% Coomassie Brilliant Blue R-250 for 1 hour at 120 voltages and a 12% separating gel.<sup>16</sup>

#### **Bacteriocin gene detection**

The PCR reactions were done in a 25 µl volume comprising of 1.5 µl of MgCl<sub>2</sub> (15 mM), 0.5 µl of 1 mM dNTP, 2.5 µl of Taq buffer (5×), 0.5 µl of each primer (2.5 pM each) Col K- F; CAGAGGTCGCTGAACATGAA, Col K- R; TCCGCTAAATCCTGAGCAAT and Col 28b (L) – F; TGCATATTGAAAGCGTCAGC Col 28b (L) R; CAGGTTATCCCCTCTACCA described previously<sup>17</sup>, 0.2 µl of Taq DNA polymerase and 5 µl of the DNA sample. 14.8 µl PCR water was used to bring the final reaction volume to 25 µl. The thermocycler cycling conditions were 1 cycle of denaturation at 94 °C for 5 min, then 35 cycles of denaturation at 94 °C for 1 min, annealing at the primer specific T<sub>m</sub> (Col K and L at 58° C) for 1 min, elongation at 72 °C for 1 min, and final elongation at 72° C for 7 minutes. Amplified DNA fragments were run alongside a 100bp ladder on 2% agarose gels containing ethidium bromide and visualized under UV light.

## RESULT

A total of 257 Gram negative rod bacterial isolates were identified from 65 human rectal swabs and 62 cow dung samples. *Enterobacter* species was the most prevalent isolate identified while *Morganella* specie was the least. Figure 1.0 showed distribution of gram negative isolates from the samples collected. Out of the 257 Gram negative rods that were isolated, 21 were positive for bacteriocin activity against the *E. coli* O157:H7 (17 from human samples, 4 from cattle samples), seven isolates (7), presumptively identified as *Serratia* spp. (4), *Aeromonas* spp. (1) and *Proteus* spp. Two (2) isolates were confirmed to be producers of bacteriocins by induction at 254 nm. *Serratia marcescens* has the highest protein concentration of 2.032mg/ml, Col 28b gene was detected on the two *Serratia marcescens* with 460 as PCR product size (bp) while Col K gene was not detected from the isolates. Table 1 showed the occurrence of Gram Negative Rods with

Antimicrobial Activity against *E. coli* 157:H7. From the human rectal swab isolates, a total of 17 (13.6%) of 125 was active with *Serratia spp* having the highest number of activity. In the same vein, From the cow dungs swab isolates, a total of 4(3.03%) of 132 was active with *Serratia spp* having the highest number. The 21(8.2%) of 257 isolates showed bioactivity against the *E. coli* O157:H7 either before or after exposure to UV light. Table 2.0 shows bioactivity of UV induced isolates against ***E. coli* O157:H7**. Table 3.0 showed the protein concentration (mg/ml) in the bioactive Gram Negative Rods using Bradford method. *Serratia marcescens* has the highest protein concentration while *Proteus mirabilis* has lowest concentration. Table 4.0 showed biochemical reactions of Gram Negative Rods isolated using MICROBACT 12E. Figure 2 shows sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of extract obtained from bioactive Gram Negative Rods with the following abbreviation: M – Protein Marker; 1 – *Serratia marcescens* (RS 001); 2 - *Serratia marcescens* (RS 004); 3 – *Proteus mirabilis* (RS 052).

Comment [H6]: Why bold???

70.1 KDa – Bovine Serum Albumin; 49.2 KDa – Oval Albumin; 28.8 KDa – Pepsinogen, 19.6 KDa – Trypsinogen, 16.9 KDa – Lysozyme. Fig 3 showed Agarose Gel Electrophoresis for Colicin Gene Detection, M: 100 bp marker, 1: Negative control, lane 2-4: Col K with isolates RS 01, RS 04, RS 52 respectively, lane 5-7: Col 28b(L) with isolates RS 01, RS 04, RS 52 respectively and lane 8 positive control.

## DISCUSSION

Evidently researchers including <sup>1,3</sup> had recorded potential of many enteric not only *E. coli* but also many species of Gram-negative bacteria for bacteriocin productions, such as Klebicins of *Klebsiella pneumonia*, marcescins of *Serratia marcescens*, alveicins of *Hafnia alvei*, cloacins of *Enterobacter cloacae* and pyocins of *Pseudomonads* against pathogens. Production of bacteriocin-like compound from these 257 isolated enterics from human and cattle samples against *E.coli* 0157:H7 ATCC 35150 which is an enteric pathogens indicator organism revealed 21(8.1%) enterobacteriaceae out of 257 screened with, as a positive producer.

Frequency of bacteriocin production in this enteric strains has been significantly low in comparing with report of <sup>11</sup> that recorded 26.8% of the total gram-negative isolates for antibacterial activity against *E.coli* 0157:H7 likewise <sup>18</sup> reported that more than 30% of lactose-fermenting gram-negative bacteria isolated from the River Seine produced one or more colicins, which were active against *E. coli* K12, and found it amazing that, 98% of these strains were susceptible to classic antibiotic which similar to bacteriocinogenic strains in this study that were found highly sensitive to antibiotics used in this study. <sup>17</sup> out of 21 bacteriocinogenic producers in this study are from human rectal, this suggest that bacteriocinogeny is more frequent among commensal human isolates than animal isolates which related to the findings of <sup>19,20</sup> found more colicinogenic isolates among humans isolates (50%) than animal isolates (16%).

Ultra violet ray induction of the 21 active species against *E.coli* O157: H7 yield seven isolates comprising of five *Serratia marcescens*, one *Proteus mirabilis* and one *Aeromonas iwoffni*, that produced bacteriocin-like content after exposure to Ultra-violet ray,<sup>1</sup> listed *Serratia marcescens* as one of bacteria capable of producing bacteriocin. *Serratia marcescens* has been also confirmed to produce bacteriocins (marcensin) upon induction with ultraviolet ray.

The *Serratia marcescens* in this study possess Col 28b gene when screened for gene detection which correspond to <sup>21</sup> who reported one case of a chromosomally encoded colicin, colicin-like bacteriocin 28b produced by *Serratia marcescens*, a colicin very homologous to pore-forming colicins, likewise others <sup>21,22</sup> reported that bacteriocin 28b from *S. marcescens* binds to *Escherichia coli* outer membrane proteins, also reported,<sup>12, 23</sup> isolated and characterized bacteriocin L from *S. marcescens* and <sup>24</sup> stated that *Serratia marcescens* produces an endonuclease with extraordinarily high specific activity that is released into the surrounding medium for studies on gene regulation, protein secretion, endonuclease action, and protein structure as a result of which it has found many applications in biotechnology. The molecular weight of the isolate fall within 15 kDa to 80 kDa that support <sup>25</sup> report that purified *S. marcescens* N28b synthesized and secreted a bacteriocin, with a molecular mass of 45 kDa, which was capable of inhibiting the growth of *Escherichia coli*.

The *Proteus mirabilis* strain in this work was negative to Colicin K gene used as showing in figure 2 above. Colicin K has been isolated by <sup>26</sup> when working on colicinogenic strain of *E. coli* K 12, Colicin K is a relatively homogeneous substance having a molecular weight of approximately 45,000Da. The report of <sup>27</sup>, was about the nature of the colicin K derived from *Proteus mirabilis* Col K +. It was shown that the bacteriocin produced by the colicinogenic *Proteus* in the presence of mitomycin C is a protein of low molecular weight resembled to colicin K derived from the *E. coli* K 235 bacillus having the same immunological and bactericidal specificities. The two bands of

molecular weight of the *Proteus mirabilis* in this study was between the standard marker of 28.9KDa - 49.2KDa and 70.1KDa though not been purified.

## CONCLUSION

This study revealed *Serratia marcescens* in this work to possessed an antimicrobial molecule of bacteriocinogenic type with possession of Col 28b gene, non-hemolytic, and sensitive to antibiotics which suggests it's possible use as an inexpensive and inoffensive therapeutic agent (probiotics) in treating *E. coli* O157:H7 infections such as Haemorrhagic colitis.

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Table 1: Occurrence of Gram Negative Rods with Antimicrobial Activity against *E. coli* 157:H7

Presumptive Isolates Identity	Human Rectal Swabs		Cow Dungs	
	Total	Total Active	Total	Total Active
<i>Aeromonas</i> sp.	8	3	18	0
<i>Chryseomonas</i> sp.	1	0	4	0
<i>Citrobacter</i> sp.	21	1	6	0
<i>Edwardsiella</i> sp.	1	0	1	0
<i>Enterobacter</i> sp.	23	1	20	0
<i>Erwinia</i> sp.	3	1	1	0
<i>Escherichia coli</i>	15	1	15	0
<i>Flavobacterium</i> sp.	1	0	5	0
<i>Klebsiella</i> sp.	7	1	2	1
<i>Morganella</i> sp.	0	0	1	0
<i>Proteus</i> sp.	4	2	4	1
<i>Providencia</i> sp.	2	1	5	0
<i>Pseudomonas</i> sp.	13	0	12	0
<i>Salmonella</i> sp.	6	0	5	0
<i>Serratia</i> sp.	15	6	17	2
<i>Stenotrophomonas</i> sp.	2	0	9	0
<i>Vibrio</i> sp.	1	0	7	0
<i>Yersinia</i> sp.	2	0	0	0
<b>Total</b>	<b>125</b>	<b>17</b>	<b>132</b>	<b>4</b>

Table 2: Bioactivity of UV Induced Isolates against *E. coli* O157:H7

*Isolate Code	Isolate Identity	Before UV Exposure		UV Exposure at 302 nm		#UV Exposure at 254 nm	
		Raw Broth	Centrifuged Broth	Raw Broth	Centrifuged Broth	Raw Broth	Centrifuged Broth
¥RS 065	<i>Aeromonas sp.</i>	25	0	0	0	<b>10</b>	<b>12</b>
¥RS 067	<i>Aeromonas sp.</i>	13	0	0	0	0	0
¥RS 121	<i>Aeromonas sp.</i>	14	0	0	0	0	0
¥RS 034	<i>Citrobacter sp.</i>	0	12	0	0	0	0
¥RS 089	<i>Enterobacter sp.</i>	12	0	0	0	0	0
¥RS 048	<i>Erwinia sp.</i>	12	16	0	0	0	0
¥RS 010	<i>Escherichia coli</i>	0	14	0	0	0	0
¥RS 071	<i>Klebsiella sp.</i>	18	0	0	0	0	0
£CF 094	<i>Klebsiella sp.</i>	14	0	0	0	0	0
¥RS 052	<i>Proteus sp.</i>	22	13	0	0	<b>11</b>	<b>12</b>
¥RS 091	<i>Proteus sp.</i>	18	0	0	0	<b>10</b>	<b>11</b>
£CF 128	<i>Proteus sp.</i>	12	0	0	0	0	0
¥RS 038	<i>Providencia sp.</i>	15	16	0	0	0	0
¥RS 001	<i>Serratia sp.</i>	15	19	0	0	<b>25</b>	<b>27</b>
¥RS 004	<i>Serratia sp.</i>	12	17	0	0	<b>20</b>	<b>20</b>
¥RS 005	<i>Serratia sp.</i>	0	19	0	0	<b>13</b>	<b>20</b>
¥RS 066	<i>Serratia sp.</i>	15	0	0	0	<b>10</b>	<b>14</b>
¥RS 086	<i>Serratia sp.</i>	16	14	0	0	0	0
¥RS 088A	<i>Serratia sp.</i>	12	0	0	0	0	0
£CF 092	<i>Serratia sp.</i>	10	0	0	0	0	0
£CF 124	<i>Serratia sp.</i>	10	0	0	0	0	0

\* Table 3: Protein Concentration (mg/ml) in the Bioactive Gram Negative Rods using Bradford

Method

Isolates code	Isolates Identity	Protein concentration (mg/ml)
RS 001	<i>Serratia marcescens</i>	2.032
RS 004	<i>Serratia marcescens</i>	1.784
RS052	<i>Proteus mirabilis</i>	0.475

**Table 4.0: Biochemical Identification Result of Gram Negative Rod Isolated Using MICROBACT 12E**

Isolate Code	Lysine	Ornithine	H <sub>2</sub> S	Glucose	Mannitol	Xylose	ONPG	Indole	Urease	V.P	Citrate	TDA	Result summation	Percentage (%)	Identity
RS 001		7			0			7			6		7076	99.20	<i>Serratia marcescens</i>
RS 004		7			2			7			6		7276	99.28	<i>Serratia marcescens</i>
RS 005		7			0			5			6		7056	93.85	<i>Serratia marcescens</i>
RS 052		3			0			7			6		3076	93.06	<i>Proteus mirabilis</i>
RS 065		5			0			3			6		5036	49.50	<i>Aeromonas iwoffni</i>
RS 066		6			0			4			6		6046	99.69	<i>Serratia marcescens</i>
RS 086		7			0			5			6		7056	93.85	<i>Serratia marcescens</i>

Legend:H<sub>2</sub>S: Hydrogen sulphide, V.P: Voges Proskauer, O.N.P.G: o-Nitrophenyl-p-galactopyranoside

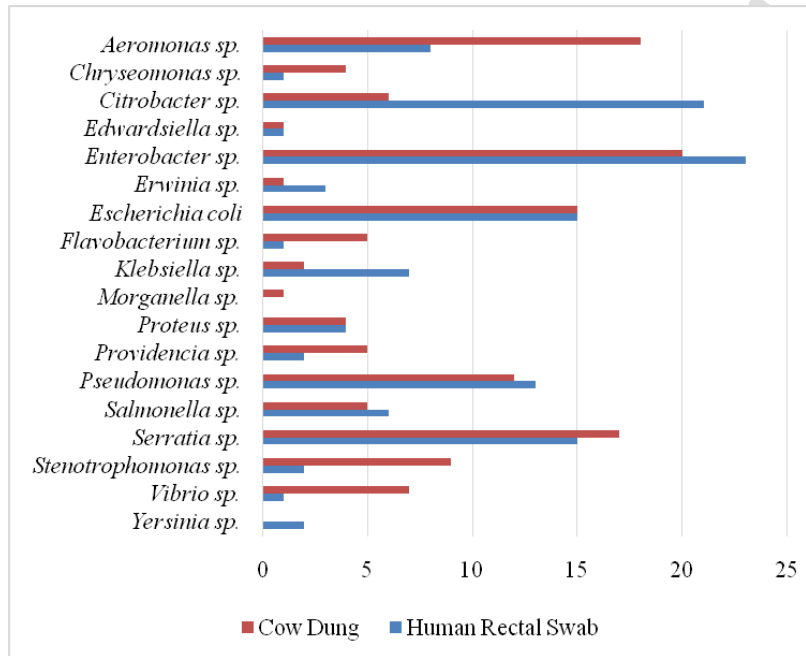


Figure 1: Distribution of Gram Negative Rods in Human Rectal Swabs and Cow Dung

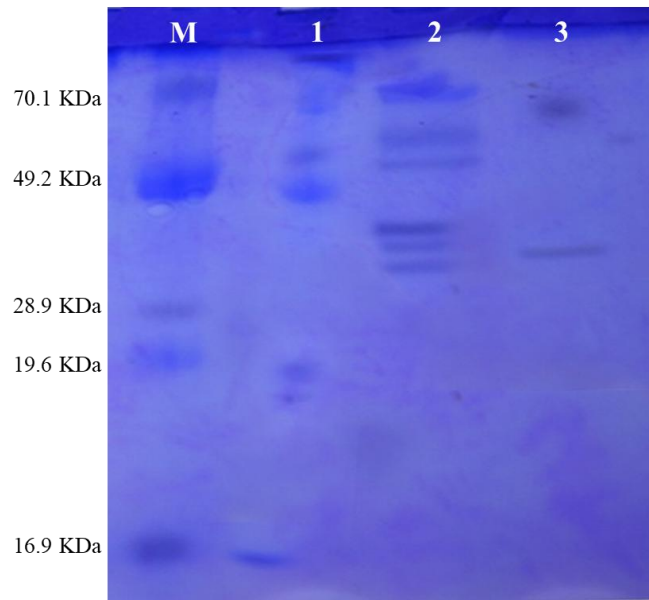


Figure 2: SDS-PAGE Analysis of Extract Obtained from Bioactive Gram Negative Rods. M – Protein Marker; 1 – *Serratia marcescens* (RS 001); 2 - *Serratia marcescens* (RS 004); 3 – *Proteus mirabilis* (RS 052). 70.1 KDa – Bovine Serum Albumin; 49.2 KDa – Oval Albumin; 28.8 KDa – Pepsinogen, 19.6 KDa – Trypsinogen, 16.9 KDa – Lysozyme.

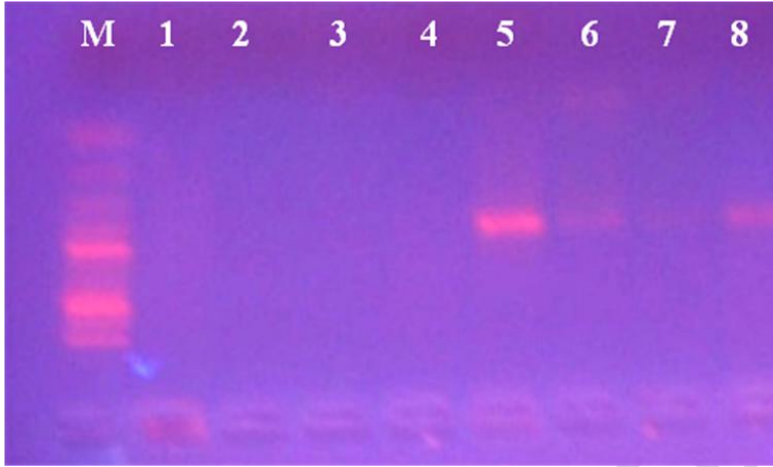


Fig 3 Agarose Gel Electrophoresis for Colicin Gene Detection, M: 100 bp marker, 1: Negative control, lane 2-4: Col K with isolates RS 01, RS 04, RS 52 respectively, lane 5-7: Col 28b(L) with isolates RS 01, RS 04, RS 52 respectively and lane 8 positive control