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Phenotypic and Genotypic Detection of UV-induced Bacteriocin Production in Enteric Gram-Negative Rods and their Activity against *Escherichia coli* O157: H7

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Authors' contributions

This work was carried out in collaboration among all authors. Authors OOO, FMA, SBA and OAO did the study design. Author OOO, FMA and OJA did the laboratory work. Authors OOO and SBA wrote the first draft of the manuscript. Statistical analysis and literature searches were done by authors OOO, RAO, OJA and OCA, while author SBA, EFA and OAO did the proof reading of the manuscript. The final manuscript was read and approved by all authors.

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ABSTRACT

Background: Virulence and antibiotic resistance of bacterial pathogens are 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* 0157: 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* 0157: H7 due to their constitutive bacteriocin, however, on the 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 ≤ 2 classes of antibiotics and exhibited Gamma/alpha haemolysis.
Conclusion: This study revealed *Serratia marcescens* in this work to possess 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).

1. INTRODUCTION

Bacteriocins are small sized 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). Bacteriocins are generally active against the strains that lack the respective encoding plasmid and closely related to the producer [1]. These are different from classical antibiotics: they are ribosomal synthesized and have a narrow killing spectrum in term of its specificity to bacteria that lack their particular plasmid.2 It includes a variety of proteins in terms of different molecular sizes, mode of action, microbial targets, release to targeted microbes, and their immunity mechanisms [2].

Bacteriocin 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 [2,3]. Bacteriocins work better against their target bacteria than antibiotics at lower concentrations. Each bacteriocin has specific immunity gene to confer immunity to its producer, it has an unequal attribute for its mechanism of target cell resistance or sensitivity when comparing to antibiotics [4]. 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 [5]. Each bacteriocin possesses immunity protein which provides resistance to the 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 of the bacteriocin type [5].

Shiga toxin producing*E coli* O157: H7 is the most dangerous bacteria strain causing severe enteric diseases and death in humans and animals [6]. Haemorrhagic colitis in humans are caused 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 experienced by the infected person, which become worse 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.[7,8,9] The severe infection caused by *E. coli* O157: H7 can be terminated by the administration of bacteriocin produced by commensally non-pathogenic bacteria [1].

2. MATERIALS AND METHODS

2.1 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 the rectal sample, inserted into the 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 cattle from the cattle rearing settlement was aseptically collected using sterile cotton swabs and aseptically put into 5 ml sterile universal sampling bottles containing sterile nutrient broth and analysed within six hours of collection.

2.2 Isolation of Gram-NegativeBacteria from the Samples

Gram-negative rod bacteria were isolated from the human rectal swab (human origin) and cow dung (animal origin) samples using the streaking method.[10] 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 [11].

2.3 Bacteriocin Production Potential of the Gram-Negative 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 wassub-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 centrifuged to separate supernatant from the 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 BaCl2 added to 99.5 ml of 0.18 M H2SO4 as standard for comparison). One hundred microlitres of the solution were inoculated using spread plate method on to Mueller Hinton agar plates, sterile cork-borer was used to make 8 mm holes at the 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 the zone of inhibition form around the well, measured and recorded in mm [11].

2.4 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 [10,11,12,13] 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 into 8 mm wells bored at the 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 zones indicate the activity of bacteriocin positive isolates, they were subsequently selected for further studies and stored in glycerol broth at -20°C.

2.5 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. [11] Antibiotic susceptibility of the bacteriocin-producing isolates to various antibiotics including Ampicillin (10 µg), Amoxycillin/Clavulanic acid (30µg), Chloramphenicol (30µg), Tetracycline (30µg), Nalidixic acid (30µg), Gentamicin (10µg), Sulfamethoxazole/Trimethoprim (25µg) and Nitrofurantoin (300µg) were determined on Muller Hinton media using disc diffusion method for the standard procedure of the Clinical and Laboratory Standards Institute [14].

2.6 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) for the identification.

2.7 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. The method described previously was adopted.[15] 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 10µl aliquot was applied to a gel as well as the standard marker been loaded in the first gel lane. 100µl of the supernatant centrifuge isolates was suspended in electrophoresis loading buffer and heated for 5min at 100°C. The isolates were analysed 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.[16]

2.8 Bacteriocin Gene Detection

The PCR reactions was done in a 25µl volume comprise of 1.5µl of MgCl2 (15mM), 0.5µl of 1 mM dNTP, 2.5µL of Taq buffer (5×), 0.5 µL of each primer (2.5pM each) Col K- F; CAGAGGTCGCTGAACATGAA, Col K- R; TCCGCTAAATCCTGAGCAAT and Col 28b (L) – F; TGCATATTGAAAGCGTCAGC Col 28b (L) R; CAGGTTATCCCCTCTACCA described previously [17], 0.2µl of Taq DNA polymerase and 5µl of the DNA sample. 14.8µl PCR water was used to bring the final reaction volume to 25µL. 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 Tm (Col K and L at 58° C) for 1min, 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.

3. RESULTS

A total of 257 Gram-negative 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. Fig. 1 showed the distribution of Gram-negative isolates from the samples collected. Out of the 257Gram-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 level 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 the 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 the Bradford method. *Serratia marcescen* has the highest protein concentration while *Proteus mirabilis* has the lowest concentration.Table 4.0 showed biochemical reactions of Gram-negative rods isolated using MICROBACT 12E. Fig. 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). 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.

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
Aeromonas sp.	8	3	18	0
Chryseomonas sp.	1	0	4	0
Citrobacter sp.	21	1	6	0
Edwardsiella sp.	1	0	1	0
Enterobacter sp.	23	1	20	0
Erwinia sp.	3	1	1	0
Escherichia coli	15	1	15	0
Flavobacterium sp.	1	0	5	0
Klebsiella sp.	7	1	2	1
Morganella sp.	0	0	1	0
Proteus sp.	4	2	4	1
Providencia sp.	2	1	5	0
Pseudomonas sp.	13	0	12	0
Salmonella sp.	6	0	5	0
Serratia sp.	15	6	17	2
Stenotrophomonas sp.	2	0	9	0
Vibrio sp.	1	0	7	0
Yersinia sp.	2	0	0	0
Total	**125**	**17**	**132**	**4**

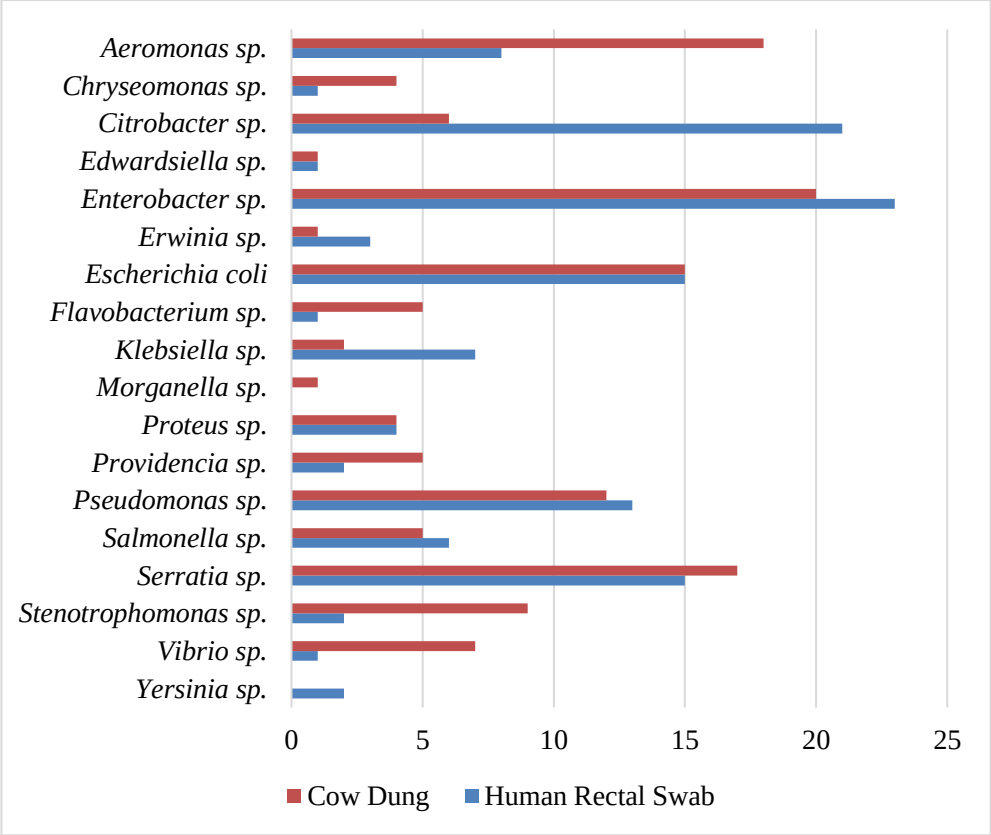

Fig. 1. Distribution of Gram-negative rods in human rectal swabs and cow dung

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 at254 nm	
		Raw Broth	Centrifuged Broth	Raw Broth	Centrifuge Broth	Raw Broth	Centrifuged Broth
¥RS 065	*Aeromonas* sp.	25	0	0	0	10	12
¥RS 067	*Aeromonas* sp.	13	0	0	0	0	0
¥RS 121	*Aeromonas* sp.	14	0	0	0	0	0
¥RS 034	*Citrobacter* sp.	0	12	0	0	0	0
¥RS 089	*Enterobacter* sp.	12	0	0	0	0	0
¥RS 048	*Erwinia* sp.	12	16	0	0	0	0
¥RS 010	*Escherichia coli*	0	14	0	0	0	0
¥RS 071	*Klebsiella* sp.	18	0	0	0	0	0
£CF 094	*Klebsiella* sp.	14	0	0	0	0	0
¥RS 052	*Proteus* sp.	22	13	0	0	11	12
¥RS 091	*Proteus* sp.	18	0	0	0	10	11
£CF 128	*Proteus* sp.	12	0	0	0	0	0
¥RS 038	*Providencia* sp.	15	16	0	0	0	0
¥RS 001	*Serratia* sp.	15	19	0	0	25	27
¥RS 004	*Serratia* sp.	12	17	0	0	20	20
¥RS 005	*Serratia* sp.	0	19	0	0	13	20
¥RS 066	*Serratia* sp.	15	0	0	0	10	14
¥RS 086	*Serratia* sp.	16	14	0	0	0	0
¥RS 088A	*Serratia* sp.	12	0	0	0	0	0
£CF 092	*Serratia* sp.	10	0	0	0	0	0
£CF 124	*Serratia* sp.	10	0	0	0	0	0

Table 3. Protein concentration (mg/ml) in the bioactive Gram-negative rods using BradfordMethod

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

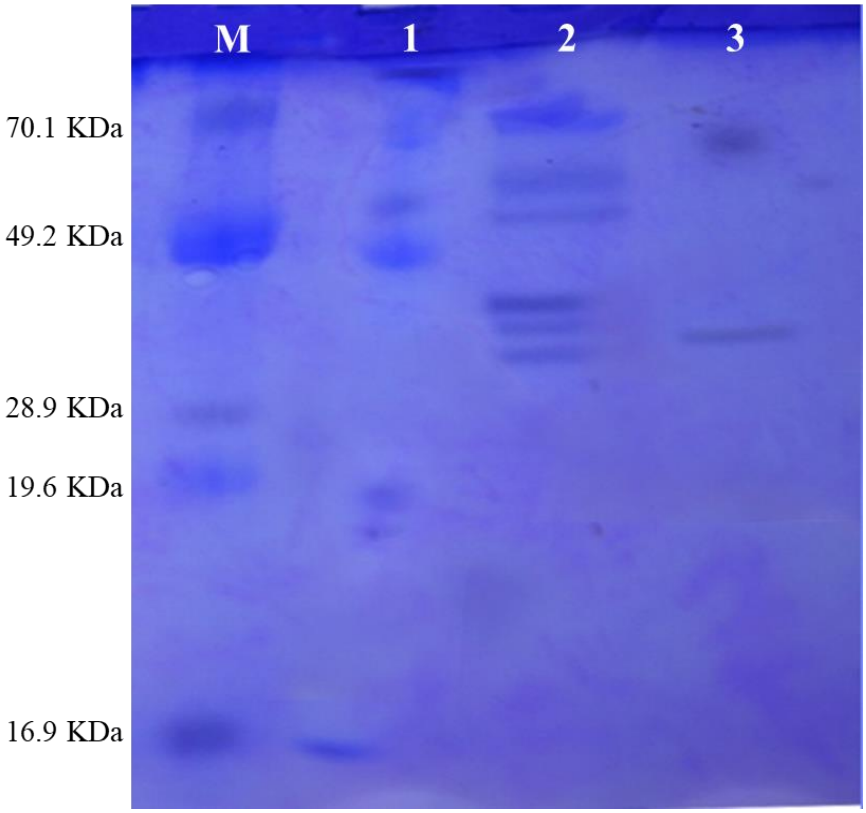

Fig. 2. SDS-PAGE Analysis of Extract Obtained from Bioactive Gram Negative Rods
M – ProteinMarker; 1 – Serratia marcescens (RS 001); 2 - Serratia marcescens (RS 004); 3 – Proteus mirabilis (RS 052). 70.1 KDa – Bovine Serum Albumin; 49.2 KDa – Oval Albumin; 28.8 KDa – Pepsinogen, 19.6 KDa – Trypsinogen,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

4. DISCUSSION

Evidently, researchers including [1,3] 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 the bacteriocin-like compound from these 257 isolated enteric organismsfrom 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 11 that recorded26.8% of the total gram-negative isolates for antibacterial activity against *E.coli* 0157:H7 likewise [18] 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. 17 out of 21 bacteriocinogenic producers in this study are from human rectal, this suggests that

Table 4. Biochemical identification result of Gram-negative rod isolated using MICROBACT 12E

Isolate code	Lysine	Ornithine	H_2S	Glucose	Mannitol	Xylose	ONPG	Indole Table 3: confirmatory identity of the positive isolates after UV exposure using Microbact 12E	Urease	V.P	Citrate	TDA	Result summation	Percentage (%)	Identity
RS 001	7				0			7			6		7076	99.20	*Serratia marcescens*
RS 004	7				2			7			6		7276	99.28	*Serratia marcescens*
RS 005	7				0			5			6		7056	93.85	*Serratia marcescens*
RS 052	3				0			7			6		3076	93.06	*Proteus mirabilis*
RS 065	5				0			3			6		5036	49.50	*Aeromonas iwoffni*
RS 066	6				0			4			6		6046	99.69	*Serratia marcescens*
RS 086	7				0			5			6		7056	93.85	*Serratia marcescens*

Legend:H_2S: Hydrogen sulphide, V.P: Voges Proskauer, O.N.P.G: o-Nitrophenyl-p-galactopyranoside

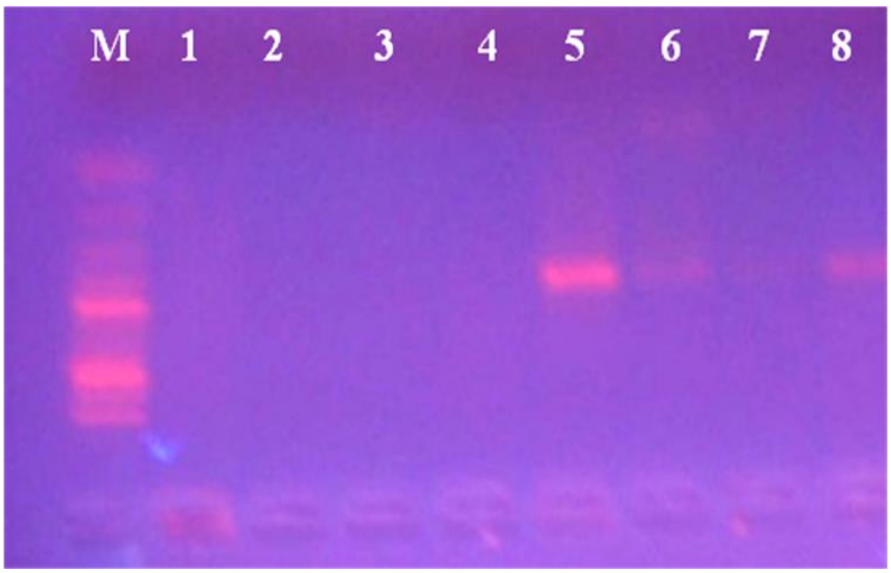

Fig. 3. Agarose gel electrophoresis for colicin gene detection

M: 100 bp marker, 1: Negative controllane 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

bacteriocinogeny is more frequent among commensal human isolates than animal isolates. Several researchers found more colicinogenic isolates among human isolates (50%) than animal isolates (16%) [19, 20].

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

The *Serratia marcescens* in this study possess Col 28b genewhen screened for gene detectionwhich corresponds toGuasch et al.[21] who reported one case of a chromosomally encoded colicin, colicin-like bacteriocin 28b produced by *Serratiamarcescens*, a colicin very homologous to pore-forming colicins, likewise others[21,22]reported that bacteriocin 28b from *S. marcescens* binds to *Escherichia coli* outer membrane proteins, also reported[12][23]isolated and characterized bacteriocin L from *S. marcescens* and [24] 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 falls within 15 KDa to 80 KDa that support [25] reports 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 Fig. 2. Colicin K has been isolated by [26] 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 [27], 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 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.

5. CONCLUSION

This study revealed *Serratia marcescens* in this work to possess an antimicrobial molecule of bacteriocinogenic type with possession of Col 28b gene, non-hemolytic, and sensitive to

antibiotics which suggests it's possible to use as an inexpensive and inoffensive therapeutic agent (probiotics) in treating *E. coli* O157:H7 infections such as Haemorrhagic colitis.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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