Journal of Pharmaceutical Research International

33(44B): 298-313, 2021; Article no.JPRI.74662
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

Abundance of Multiple-Antibiotic-Resistant *Salmonella* Strains in Fecal Samples of *Rhinoceros unicornis* of the Kaziranga National Park, India

Tilak Saha[1,2], Dibyendu Paul[1] and Ranadhir Chakraborty[1*]

[1]Omics Laboratory, Department of Biotechnology, University of North Bengal, Darjeeling-734013, India.
[2]Laboratory of Immunology and Microbiology, Department of Zoology, University of North Bengal, Darjeeling-734013, India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i44B32680
Editor(s):
(1) Paola Angelini, University of Perugia, Italy.
Reviewers:
(1) Deepak Macchindra Vikhe, Pravara Institute of Medical Sciences, India.
(2) Ch. Paramageetham, Sri Venkatsewara University, India.
Complete Peer review History: https://www.sdiarticle4.com/review-history/74662

Original Research Article

Received 11 July 2021
Accepted 21 September 2021
Published 23 September 2021

ABSTRACT

Aims: This study was undertaken to assess the abundance of multiple-antibiotic-resistant (MAR) *Salmonella* strains in fecal samples of *Rhinoceros unicornis* of the Kaziranga National Park (KNP), India.
Study Design: Antibiotic-resistance profile of the *Salmonella* isolates from fecal samples of rhinoceros was carried out by replica plating on Muller Hinton Agar (MHA) plates containing antibiotics. The presence of class 1 integrons in metallo-β-lactamase (MBL) producing *Salmonella* isolates was determined by multiplex PCR.
Place and Duration of Study: The study was carried out on rhinos of KNP situated in the Golaghat district of Assam, India in April 2015.
Methodology: Fresh rhino fecal samples (designated as 1R, 2R, 3R, 4R and 5R) were dilution plated onto MacConkey agar. Purified bacterial colonies were then streaked separately on bismuth sulphite (BS) agar plates. All black colonies which are characteric growth of *Salmonella* were selected and used to make master plates on Luria Agar. To determine the antibiotic-resistance

**Corresponding author: E-mail: rcnbusiliguri@gmail.com;*

profile of the isolates, master-plates of purified single colonies of *Salmonella* spp. were replicate-printed on plates containing antibiotics from the β-lactam, aminoglycoside, or quinolone groups. To detect the presence of an integron, a conserved segment polymerase chain reaction was used.

Results: 97.6% of the *Salmonella* isolates tested were resistant to >1 antibiotics (multidrug resistant or MAR). A total of 100 isolates from two fecal samples, 4R and 5R, were found to be imipenem resistant; 52 of them tested positive for the presence of MBLs. Five of the twenty MBL producing strains contained class 1 integrons.

Conclusion: Because *Salmonella* is usually spread by drinking contaminated water, it is likely that water bodies in KNP are contaminated with MAR *Salmonella* strains. In case of Salmonellosis outbreak among Indian one-horned rhinos, our antibiogram will assist the veterinarians to choose the appropriate regimen of antibiotics for the rhinos in the KNP.

Keywords: Rhinoceros; kaziranga national park; Salmonella; multiple-antibiotic-resistant; metallo-β-lactamase; class I integron.

1. INTRODUCTION

The greater Indian one-horned rhinoceros (*Rhinoceros unicornis*) is now mostly confined to the Kaziranga National Park (KNP) in Assam's Golaghat district. According to a 2012 estimate by the International Rhino Foundation (www.rhinos.org), KNP is home to approximately two-thirds of the world's population of one-horned rhinos. Indian rhinos can be found in a variety of environments, including marshes, alluvial plains, grasslands, and arid forests [1,2]. All rhino species require regular access to water [3]. They need to drink every day or every other day because they are hindgut fermenters with relatively fast gut transit times, which reduces the time for water resorption. Herbivorous animals rely on their gut microbiota for nutrition [4]. However, little is known about the microbial diversity in the rhinoceros' gastrointestinal tract (GIT). Rhinoceros, as a non-ruminant herbivore, can use fibrous plant matter through microbial fermentation in the hindgut. KNP is intertwined with four rivers. River water is a major source of bacteria, including antibiotic-resistant bacteria. Using molecular techniques, Bian and his colleagues [5] investigated the gut microbiota of the white rhinoceros. They used barcoded pyrosequencing to characterize 105,651 16S rRNA gene sequences obtained from fecal samples from five white rhinoceroses. They came to the conclusion that *Firmicutes* and *Bacteroidetes* were the most common phyla in the samples, which were mostly made up of unclassified bacteria. Enteritis and diarrhoea are common diseases caused by bacterial infection in the gastrointestinal tract. *Mycobacterium bovis*-related diseases have recently been reported in black rhinos in South Africa [6]. Infectious diseases have the potential to occur and spread in the rhino population of KNP. Because of the small number of founder populations, the current population (366 in numbers) has less genetic variability. Salmonellosis caused by *Salmonella* spp. infection in both black and Indian one-horned rhinoceroses (*Diceros bicornis*, *Rhinoceros unicornis*) is often fatal [7]. *Salmonella* is most likely the most dangerous pathogen in rhinos, causing enteritis and fatal septicemia. Many pathogenic *Salmonella* isolates have integrons containing antibiotic-resistant gene cassettes. Integrons are the prokaryotic mobile genetic elements that are responsible for acquisition and dissemination (both vertical and horizontal) of resistance genes [8,9]. Class 1 integrons, the best- characterized integrons with the *intI1* and *attI* loci at 5' end and a short antiseptic resistance gene (*qacE_1*), a sulfonamide resistance gene (*sulI*) and an open reading frame (ORF5) of unknown function in their 3' end, have frequently been reported in clinical and environmental isolates [10,11] and its prevalence is alarming for infections caused by the pathogens. In 2003, Lindstedt and his colleagues [12] found integrons with sizes 650, 1000, 1200, 1500, 1600, 1700, 2000 and 2100 bp in many of the isolates of *S. typhimurium* (97 %) and *S. enteritidis* (22%). Such isolates, therefore, pose a threat as a zoonotic pathogen with increased resistance to several antibiotics. In a retrospective survey of captive black, white and Indian rhinos in the United States, 11% demonstrated positive cultures with clinical signs due to *Salmonella* infection [13]. β -lactam resistant pathogens are being reported from different habitats. Yong and colleagues [14] identified clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. that produce metallo-β-lactamase (MBL).

Habitat destruction, overexploitation, invasive species, pollution, and infectious disease are the five most widely acknowledged causes of species loss [15]. Successful wildlife conservation necessitates a thorough understanding of each of the critical factors that contribute to species extinction and endangerment. There is an increasing demand for rigorous scientific tests to determine the role of an infectious disease and its impact at the individual, population, or species levels [16]. Many evidences suggest that infectious disease can drastically reduce population densities, causing them to become extinct due to other factors [17,18]. In this study, we found that the incidence and abundance of MAR (including imipenem resistance) *Salmonella* spp. were high in fecal samples from Indian rhinos, and that a significant proportion of MAR isolates carried class 1 integrons.

2. MATERIALS AND METHODS

2.1 Collection of Fecal Samples

Fresh fecal samples (approximately 200 g each) were collected in sterile plastic containers in April 2015 from five different locations in the Kohora range of KNP, between Daphlang and Kathphora (Fig.1) situated in the in the Golaghat district of Assam, India. The containers were delivered to the laboratory on dry ice and processed as soon as they arrived.

2.2 Processing of Fecal Samples for Bacteriology

The samples were pretreated according to standard methodology [19] with appropriate modifications for collection of rhino fecal samples. 8 g of faeces were suspended in a sterile plastic centrifuge tube containing 50 mL of sterile phosphate-buffered saline (PBS) (0.05 mol/l, pH 7.4). To remove the bacteria from the plant residue, the sample was vortexed for about 10 min. The suspension was then centrifuged for 5 minutes at 200 g, and the supernatant was collected in a separate 50 mL centrifuge tube. This procedure was carried out three times. Thus, 100-120 mL of supernatant was collected from each sample. The cells in the supernatant were collected and washed three times with 30 mL fresh PBS by centrifuging at 3000 g for 3 minutes. Finally, the washed cell pellets were resuspended in 10 mL of sterile PBS in one tube, divided into 1-mL aliquots.

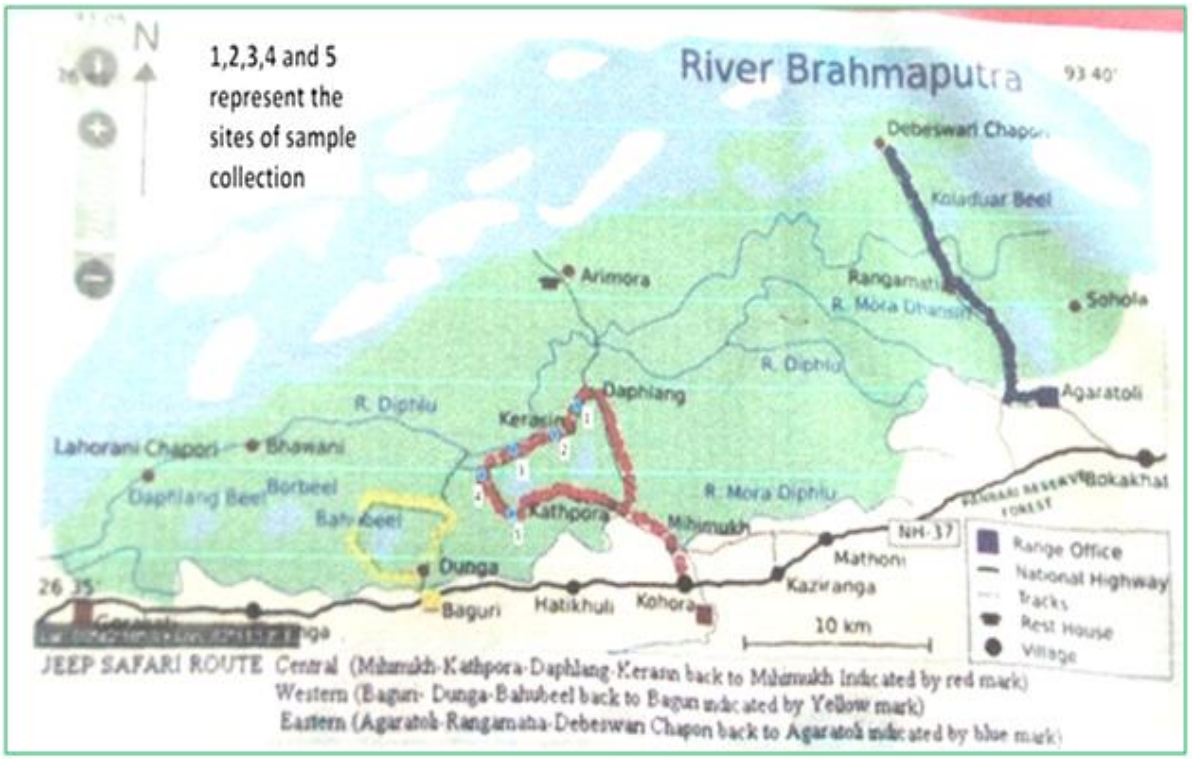

Fig. 1. Sites of sample collection at Kohora range during jeep safari through the trekkers' route, as depicted in the map (red)

2.3 Isolation of *Salmonella* spp. from Processed Fecal Samples

The bacteria-containing supernatant was serially diluted from 10^{-1} to 10^{-7}, and 0.1 mL suspensions from each dilution were spread onto MacConkey agar medium with a sterile glass spreader. The plates were then incubated overnight at 37ºC. The colourless bacterial colonies which grew on MacConkey agar (Fig. 2A) medium were then picked up with sterile tooth-picks and streaked for isolation of single colony on fresh Luria agar medium. With sterile toothpicks, the colourless single colonies were transferred onto bismuth sulphite (BS) agar plates. All of the black colonies (Fig. 2B) that grew on BS agar plates (possible *Salmonella* colonies) were used to make master plates on Luria Agar (LA) in groups of 50 with definite code numbers for each isolate. In this manner, 300 isolates were master plated in total. *Salmonella* isolates from five different fecal samples were labelled as 1R (1-50), 2R (1-100), 3R (1-50), 4R (1-50), and 5R (1-50).

2.4 Determination of Antibiotic Resistance Profile (ARP) of the Isolates

ARP of the isolates was determined using replica plating technique described previously [11]. Purified single colonies were singled out using sterile toothpicks for constructing master-plates for printing on Mueller-Hinton agar (MHA) plates containing ampicillin (25 µg/mL) or streptomycin (15 µg/mL) or oxy-tetracycline (25 µg/mL) or chloramphenicol (25 µg/mL) or kanamycin (25 µg/mL) or imipenem (10 µg/mL) or ciprofloxacin (10 µg/mL) or gentamycin (10 µg/mL) or azithromycin (25 µg/mL) or amoxicillin (20 µg/mL) or nalidixic acid (10 µg/mL). If the isolates grew on at least two different antibiotic-containing plates, they were considered MAR. Complete inhibition of growth in antibiotic plate(s) was considered as sensitive.

2.5 Testing of the Imipenem-Resistant Isolates for Active Presence of MBL(s)

MAR *Salmonella* isolates from two different samples (4R and 5R) were tested for the presence of active MBL(s) by replica plating isolates that formed colonies in MHA plates containing imipenem onto MHA plates containing both imipenem and EDTA. The method is a modification of the imipenem-EDTA Disk method for differentiation of MBL-producing *Pseudomonas* spp. and *Acinetobacter* spp., described by Yong et al., [15]. 200 µl of 50 mM EDTA was spread on each MHA plate containing imipenem (10g/mL) and allowed to dry in the incubator for 1 hour at 37 °C. The master plates, constructed with selected MAR isolates, were replicated onto three sets of MHA plates (set I: MHA plate with no antibiotic; set II: MHA plates containing imipenem; and set III: MHA plates containing both imipenem and EDTA). Plates were incubated at 37 °C overnight. The isolates

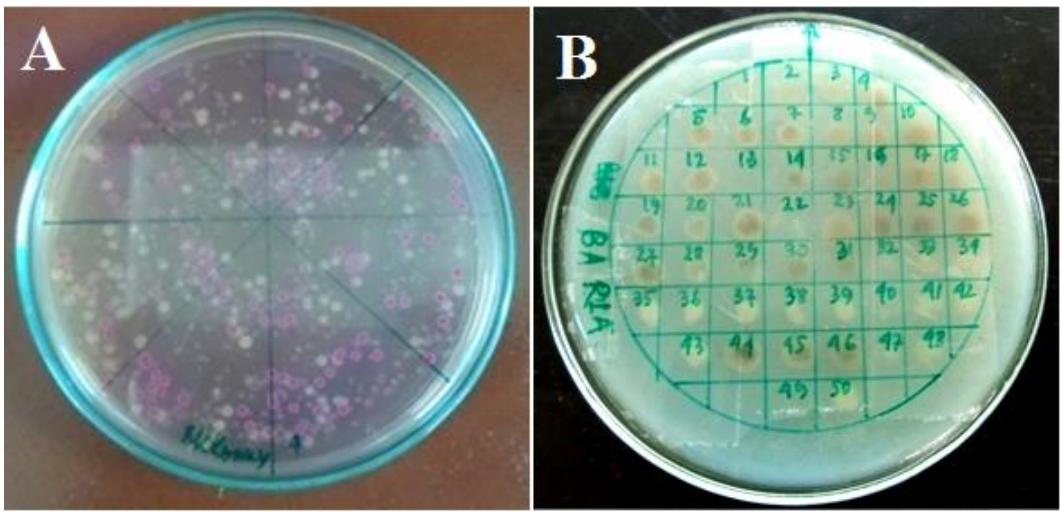

Fig. 2. Representative culture plates showing *Enterobacteriaceae* members isolated from the fecal sample of *Rhinoceros unicornis.* A- Colonies on MacConkey agar after dilution plating of fecal sample. B- Colourless colonies picked up from MacConkey agar plate and inoculated in Bismuth-Sulfite agar plate to confirm *Salmonella* isolates with characteristic black colonies

that did not grow in set III plates but did grow in plates containing imipenam were the only ones that produced MBLs (MBLs are inactivated when metal ions are chelated by EDTA present in the medium). The validity of this method was confirmed by using the EDTA-disk method on a few isolates that tested positive in our study.

2.6 Detection of Class 1 Integron by CS-PCR Method

To detect the presence of an integron, a conserved segment polymerase chain reaction (CS-PCR) was used, as described previously by Levesque et al [20]. Because the primers used in this PCR anneal, namely 5′CS (5′-GGCATCCAAGCAGCAAG-3′) and 3′CS (5′AAGCAGACTTGACCTGA- 3′), specifically in the 5′- and 3′-CS of class 1 integrons, the amplicons yielded contain gene cassettes [21] The template DNA was extracted from imipenam-resistant *Salmonella* isolates using the previously described method [11]. PCR reaction was carried out on Peltier Thermal cycler (BIO-RAD DNA engine). In all reactions, PCR set up containing the whole cell DNA of *Morgnella* Sp. TR90 (class 1 integron-bearing bacterium) was used as positive control. The genomic DNA of *Escherichia coli* JM109 (devoid of class 1 integron) and sterile distilled water were used as negative controls. The amplified products were visualized after electrophoresis through a 1% agarose gel containing ethidium bromide using TAE running buffer, and a 500-bp ladder (Bangalore Genei, India) was used as the molecular size marker.

3. RESULT

3.1 Isolation of *Salmonella* spp

Bacterial densities of five fecal samples, enumerated on MacConkey plates (used for the isolation of gram-negative bacilli including coliform organisms and enteric pathogens, on the basis of lactose fermentation) following dilution plating method, were as follows: 1R had 13.6 x 10^5 CFU/ mg fecal matter; 2R had 16.8 x 10^5 CFU/ mg fecal matter; 3R had 10.7 x 10^5 CFU/ mg fecal matter; 4R had 16.6 x 10^5 CFU/ mg fecal matter and 5R had 64 x 10^4 CFU/ mg fecal matter. *Salmonella* strains were isolated by randomly selecting distinct, colourless colonies manifesting on MacConkey plates at higher dilutions from each fecal sample and repeatedly dilution-streaked to obtain pure cultures after being confirmed on a highly selective medium, bismuth-sulfite-agar.

3.2 Antibiotic Resistance Profile of the *Salmonella* Strains

Tables 2 – 6 show the ARPs of *Salmonella* isolates per fecal sample, ranging from 1R to 5R. Surprisingly, 10% of R5 isolates tested positive for ARP and were resistant to all antibiotics tested. There were no R5 isolates that were resistant to fewer than four antibiotics. The results for R4 isolates were similar, except that no R4 isolate was found to be resistant to all 9 antibiotics tested. Aside from MAR isolates that were resistant to two to seven (out of nine) antibiotics, only a few isolates from R1, R2, and R3 samples that were resistant to only one (single) antibiotic. Only R2 had 2% of its isolates sensitive to all antibiotics out of all the samples. The acquisition of the MAR phenotype among *Salmonella* strains was high, with only 3% of the total strains being singly resistant. Except for one, no strains were sensitive to ampicillin, but an overwhelming number of strains (approximately 80%) were sensitive to two older generation antibiotics, gentamycin and streptomycin. Susceptibility to three antibiotics, azithromycin, ciprofloxacin, and gentamycin, varied noticeably. Most isolates from R2, R3, and R4 were azithromycin sensitive. Similarly, 100% of the isolates from R1, R2, and R3 were susceptible to ciprofloxacin (Table 1). It was observed that >90% of *Salmonella* strains, irrespective of the fecal samples, were carbapenem (imipenem) resistant. Carbapenem resistance is 'over-detected' in organisms from the family *Enterobacteriaceae* (where *Salmonella* should be of no exception). The carbapenem resistance in *Enterobacteriaceae* members can occur (i) regardless of the mechanism or (ii) due to production of carbapenemase [β-lactamases capable of hydrolyzing carbapenems, such as IMP (active on imipenem) or VIM (Verona integron-encoded MBLs) or NDM (New Delhi MBL)] or (iii) by a mechanism other than carbapenemase enzymes such as AmpC or ESBL with altered permeability due to porin mutations or efflux pumps [22]. MBLs are distinguished by the requirement for zinc ions in their active site, which can be used as a diagnostic marker because chelators such as EDTA inhibit MBL activity by binding zinc. MBLs have broad lactamase activity, including carbapenemase activity, but are inactive against monobactams [23].

Table 1. Abundance (%) of susceptible (0- antb) and antibiotic- resistant (1-9 antb) *Salmonella* strains in the fecal samples of *Rhinoceros unicornis* from Kaziranga [Magnitude of resistance is shown with numerical values (1-9) to show the number of antibiotics against which the respective strains were resistant]

Antibiotic / Sample	0 antb	1 antb	2 antb	3 antb	4 antb	5 antb	6 antb	7 antb	8 antb	9 antb
R1	-	4%	2%	2%	6%	4%	12%	6%	64%	-
R2	2%	2%	5%	4%	13%	9%	8%	37%	20%	-
R3	-	2%	14%	12%	40%	16%	10%	6%	-	-
R4	-	-	-	-	20%	14%	18%	38%	10%	-
R5	-	-	-	-	20%	30%	8%	18%	14%	10%

Table 2. Antibiotic resistance profile of 50 isolates from 1R fecal sample

Strain	Amp	Strep	Tet	Cam	Kan	Imp	Cip	Gen	Azm	Amx	Nal	Resistant against number of antibiotics
1	+	-	+	+	+	+	-	-	+	+	+	8
2	+	-	+	+	+	+	-	-	+	+	+	8
3	+	-	+	+	+	+	-	-	+	+	+	8
4	+	-	+	+	+	+	-	-	+	+	+	8
5	+	-	+	+	+	+	-	-	+	+	+	8
6	+	-	+	-	+	+	-	-	+	+	+	7
7	+	-	+	+	+	+	-	-	+	+	+	8
8	+	-	+	+	+	+	-	-	+	+	+	8
9	+	-	+	+	+	+	-	-	+	+	+	8
10	+	-	+	+	+	+	-	-	+	+	+	8
11	+	-	+	+	+	+	-	-	+	+	+	8
12	+	-	+	+	+	+	-	-	+	+	+	8
13	-	-	+	-	-	-	-	-	-	-	-	1
14	+	+	+	+	-	+	-	-	-	+	-	6
15	-	+	+	+	-	+	-	-	-	+	+	6
16	+	-	+	-	-	-	-	-	-	-	-	2
17	+	-	+	-	-	+	-	-	-	+	+	5
18	+	+	+	-	-	+	-	-	-	+	+	6
19	+	+	+	+	-	+	-	-	-	+	-	6
20	+	+	+	+	-	+	-	-	-	+	-	6
21	+	-	+	+	+	+	-	-	+	+	+	8
22	-	-	-	-	-	+	-	-	-	-	-	1
23	+	+	+	+	-	+	-	-	+	+	+	8
24	+	+	+	+	-	+	-	-	-	+	+	7
25	+	+	+	+	-	+	-	-	-	+	+	7
26	+	-	+	+	+	+	-	-	+	+	+	8
27	+	-	+	+	+	+	-	-	+	+	+	8
28	+	+	+	-	+	-	-	-	-	+	+	6
29	+	-	+	-	-	-	-	-	-	+	+	4
30	+	-	+	-	-	-	-	-	-	+	+	4
31	+	+	+	-	-	-	-	-	-	+	+	5
32	+	+	-	-	-	-	-	-	-	-	+	3
33	+	+	-	-	-	-	-	-	-	+	+	4
34	+	-	+	+	+	+	-	-	+	+	+	8
35	+	-	+	+	+	+	-	-	+	+	+	8
36	+	-	+	+	+	+	-	-	+	+	+	8
37	+	-	+	+	+	+	-	-	+	+	+	8

Strain	Amp	Strep	Tet	Cam	Kan	Imp	Cip	Gen	Azm	Amx	Nal	Resistant against number of antibiotics
38	+	-	+	+	+	+	-	-	+	+	+	8
39	+	-	+	+	+	+	-	-	+	+	+	8
40	+	-	+	+	+	+	-	-	+	+	+	8
41	+	-	+	+	+	+	-	-	+	+	+	8
42	+	-	+	+	+	+	-	-	+	+	+	8
43	+	-	+	+	+	+	-	-	+	+	+	8
44	+	-	+	+	+	+	-	-	+	+	+	8
45	+	-	+	+	+	+	-	-	+	+	+	8
46	+	-	+	+	+	+	-	-	+	+	+	8
47	+	-	+	+	+	+	-	-	+	+	+	8
48	+	-	+	+	+	+	-	-	+	+	+	8
49	+	-	+	+	+	+	-	-	+	+	+	8
50	+	-	+	+	+	+	-	-	+	+	+	8

Amp- Ampicillin, Tet-tetracyclin, Strep-Streptomycin, Kan-Kanamycin, Cam-Chloramfenicol, Imp-Imipenem, Cip-Ciprofloxacin, Gen-Gentamycin, Azm-Azithromycin, Amx-Amoxicillin, Nal-Nalidixic acid; +, resistant; -, sensitive; X, inconclusive result

Table 3. Antibiotic resistance profile of 100 isolates from 2R fecal sample

Strain	Amp	Strep	Tet	Cam	Kan	Imp	Cip	Gen	Azm	Amx	Nal	Resistant against number of antibiotics
1	+	-	+	+	+	+	-	-	-	+	+	7
2	+	-	+	+	+	+	-	-	-	+	+	7
3	+	-	+	+	+	+	-	-	-	+	+	7
4	+	-	+	+	+	+	-	-	-	+	+	7
5	+	-	-	+	-	+	-	-	-	+	+	5
6	+	-	+	+	+	+	-	-	-	+	+	7
7	+	-	+	+	+	+	-	-	-	+	+	7
8	+	-	+	+	+	+	-	-	-	+	+	8
9	+	-	+	+	+	+	-	-	-	+	+	7
10	+	+	+	+	-	+	-	X	-	+	+	7
11	+	-	+	+	-	+	-	-	-	+	+	6
12	+	-	+	+	+	+	-	-	-	+	+	7
13	+	-	+	+	+	+	-	-	-	+	+	7
14	+	-	+	+	+	+	-	-	-	+	+	7
15	+	-	+	+	+	+	-	-	-	+	+	7
16	+	-	+	+	+	+	-	-	-	+	+	7
17	+	-	+	+	+	+	-	-	-	+	+	7
18	+	-	+	+	+	+	-	-	-	+	+	7
19	-	-	-	+	-	-	-	-	-	-	+	2
20	+	+	+	-	-	-	-	-	-	-	-	3
21	+	+	+	+	+	+	-	-	-	+	+	8
22	+	-	+	+	+	+	-	-	-	+	+	7
23	+	+	+	+	+	+	-	-	-	+	+	8
24	+	-	+	+	+	+	-	-	-	+	+	7
25	+	+	+	+	+	+	-	-	-	+	+	8
26	+	+	+	+	+	+	-	-	-	+	+	8
27	+	-	+	+	-	+	-	-	-	+	+	6
28	+	+	+	+	+	+	-	-	-	+	+	8
29	+	-	+	+	+	+	-	-	-	+	+	7
30	+	-	+	+	+	+	-	-	-	+	+	7

Strain	Amp	Strep	Tet	Cam	Kan	Imp	Cip	Gen	Azm	Amx	Nal	Resistant against number of antibiotics
31	+	+	-	+	+	+	-	-	-	+	+	7
32	+	+	+	+	+	+	-	-	-	+	+	8
33	+	-	-	+	+	+	-	-	-	+	+	7
34	+	-	-	+	+	+	-	-	-	+	+	7
35	+	-	-	+	+	+	-	-	-	+	+	7
36	+	-	-	+	+	+	-	-	-	+	+	7
37	+	+	+	-	+	+	-	-	-	+	-	6
38	+	-	-	+	+	+	-	-	-	+	+	6
39	+	-	-	-	+	+	-	-	-	+	-	5
40	+	-	-	+	-	+	-	-	-	+	-	4
41	+	-	-	+	-	+	-	-	-	+	-	4
42	+	-	-	+	-	+	-	-	-	+	-	4
43	-	-	-	-	-	-	-	-	-	-	+	1
44	+	-	-	+	-	-	-	-	-	-	-	2
45	+	-	-	+	-	-	-	-	-	-	-	2
46	+	-	-	-	-	-	-	-	-	-	-	1
47	-	-	-	-	-	-	-	-	-	-	-	0
48	-	-	-	-	-	-	-	-	-	-	-	0
49	+	-	-	+	-	-	-	-	-	-	-	2
50	+	-	-	+	-	-	-	-	-	-	-	2
51	+	-	+	+	+	+	-	-	-	+	+	7
52	+	-	+	-	-	+	-	-	-	+	+	5
53	+	-	+	+	-	+	-	-	-	+	+	6
54	+	-	+	-	-	+	-	-	-	+	+	5
55	+	-	-	+	-	+	-	-	-	+	-	4
56	+	-	+	-	-	+	-	-	-	+	+	5
57	+	-	-	+	-	+	-	-	-	+	+	4
58	+	-	-	-	-	+	-	-	-	+	+	4
59	+	-	+	-	-	+	-	+	-	+	+	6
60	+	-	-	+	-	+	-	-	-	+	-	4
61	+	+	-	-	-	X	-	-	-	+	+	4
62	+	-	+	-	+	+	-	-	-	+	-	5
63	+	+	+	+	-	+	-	-	-	+	-	6
64	+	-	+	-	-	+	-	-	-	+	+	5
65	-	-	+	+	-	+	-	-	-	+	+	5
66	+	-	-	+	-	+	-	-	-	+	-	4
67	+	-	-	+	-	+	-	-	-	+	-	4
68	+	-	+	+	-	+	-	-	-	-	-	4
69	+	-	-	-	-	+	-	-	-	+	+	4
70	+	+	+	-	-	+	-	+	-	-	-	5
71	+	-	-	-	-	+	-	-	-	+	-	3
72	+	+	+	+	-	+	-	+	-	+	+	8
73	+	+	+	+	-	+	-	+	-	+	+	8
74	+	+	+	+	-	+	-	+	-	+	+	8
75	+	+	+	+	-	+		+	-	+	-	7
76	+	+	+	+	-	+	-	+	-	+	-	7
77	+	+	+	+	-	+	-	+	-	+	+	8
78	+	-	+	+	+	+	-	-	-	+	+	7
79	+	-	+	+	+	+	-	-	-	+	+	7
80	+	+	+	+	+	+	-	-	-	+	+	8
81	+	-	+	+	+	+	-	-	-	+	+	6
82	+	-	+	+	+	+		-	-	+	+	7
83	+	-	+	+	+	+	-	-	-	+	+	7

Strain	Amp	Strep	Tet	Cam	Kan	Imp	Cip	Gen	Azm	Amx	Nal	Resistant against number of antibiotics
84	+	-	+	+	+	+	-	-	-	+	+	7
85	+	-	-	-	-	+	-	-	-	+	-	3
86	+	-	+	+	+	+	-	-	-	+	+	7
87	+	-	+	+	+	+	-	X	-	+	+	7
88	+	-	+	+	+	+	-	-	-	+	+	7
89	+	-	+	+	+	+	-	-	-	+	+	7
90	+	-	+	+	+	+	-	-	-	+	+	8
91	+	+	+	+	+	+	-	X	-	+	+	8
92	+	+	+	+	+	+	-	X	-	+	+	8
93	+	-	-	-	-	+	-	-	-	+	-	3
94	+	+	+	+	+	+	-	-	-	+	+	8
95	+	-	+	+	+	+	-	-	-	+	+	7
96	+	-	X	-	-	+	-	-	-	+	+	4
97	+	+	+	+	+	+	-	X	-	+	+	8
98	+	+	+	+	+	+	-	-	-	+	+	8
99	+	+	+	+	+	+	-	X	-	+	+	8
100	+	+	+	+	+	+	-	X	-	+	+	8

Amp- Ampicillin, Tet-tetracyclin, Strep-Streptomycin, Kan-Kanamycin, Cam-Chloramfenicol, Imp-Imipenem, Cip-Ciprofloxacin, Gen-Gentamycin, Azm-Azithromycin, Amx-Amoxicillin, Nal-Nalidixic acid; +, resistant; -, sensitive; X, inconclusive result

Table 4. Antibiotic resistance profile of 50 isolates from 3R fecal sample

Strain	Amp	Strep	Tet	Cam	Kan	Imp	Cip	Gen	Azm	Amx	Nal	Resistant against number of antibiotics
1	+	-	-	+	-	+	-	-	-	+	-	4
2	+	-	-	+	-	+	-	-	-	+	-	4
3	+	-	-	-	-	+	-	-	-	+	-	3
4	+	-	-	+	-	+	-	-	-	+	+	5
5	+	+	+	+	-	+	-	-	X	+	+	7
6	+	-	-	+	-	+	-	-	-	+	+	5
7	+	-	-	-	-	+	-	-	-	+	-	3
8	+	-	-	-	-	+	-	-	-	+	-	4
9	+	-	-	-	-	+	-	-	-	+	-	3
10	+	+	+	+	-	+	-	-	X	+	-	6
11	+	+	+	+	X	+	-	-	-	+	-	6
12	+	-	-	+	-	+	-	-	-	+	+	5
13	+	+	+	+	-	+	-	-	X	+	-	6
14	+	+	+	-	+	+	-	+	-	+	-	7
15	+	-	-	-	-	-	-	-	-	+	-	2
16	+	-	-	-	-	-	-	-	-	+	-	2
17	+	-	-	-	-	-	-	-	-	+	-	2
18	+	-	-	-	-	+	-	-	-	+	-	3
19	+	-	-	-	-	-	-	-	-	X	-	1
20	+	-	-	+	-	+	-	-	-	+	+	5
21	+	-	-	+	+	+	-	-	-	+	+	6
22	+	-	-	-	-	-	-	-	-	+	-	2
23	+	-	-	-	-	-	-	-	-	+	-	2
24	+	-	-	-	-	-	-	-	-	+	-	2
25	+	-	-	-	-	-	-	-	-	+	-	2
26	+	-	-	-	-	+	-	-	-	+	-	3

Strain	Amp	Strep	Tet	Cam	Kan	Imp	Cip	Gen	Azm	Amx	Nal	Resistant against number of antibiotics
27	+	-	+	-	-	+	-	-	-	+	-	4
28	+	-	-	+	-	+	-	-	-	X	+	4
29	+	-	-	+	-	+	-	-	-	X	+	4
30	+	-	-	+	-	+	-	-	-	X	+	4
31	+	-	-	+	-	+	-	-	-	X	+	4
32	+	-	-	+	-	+	-	-	-	X	+	4
33	+	-	-	+	-	+	-	-	-	X	+	4
34	+	-	-	+	-	+	-	-	-	X	+	4
35	+	-	-	+	-	+	-	-	-	X	+	4
36	+	-	-	+	-	+	-	-	-	X	+	4
37	+	-	-	+	-	+	-	-	-	X	+	4
38	+	-	-	+	-	+	-	-	-	X	+	4
39	+	-	-	+	-	+	-	-	-	X	+	4
40	+	-	-	+	-	+	-	-	-	X	+	4
41	+	-	-	+	-	+	-	-	-	X	+	4
42	+	-	-	+	-	+	-	-	-	X	+	4
43	+	-	-	+	-	+	-	-	-	X	+	4
44	+	+	+	+	-	+	-	-	-	+	-	6
45	+	-	-	+	-	+	-	-	-	+	+	5
46	+	-	-	-	-	+	-	-	-	+	-	3
47	+	-	-	+	-	+	-	-	-	+	+	5
48	+	-	-	+	-	+	-	-	-	+	+	5
49	+	+	+	+	X	+	-	-	-	+	+	7
50	+	-	-	+	-	+	-	-	-	+	+	5

Amp- Ampicillin, Tet-tetracyclin, Strep-Streptomycin, Kan-Kanamycin, Cam-Chloramfenicol, Imp-Imipenem, Cip-Ciprofloxacin, Gen-Gentamycin, Azm-Azithromycin, Amx-Amoxicillin, Nal-Nalidixic acid; +, resistant; -, sensitive; X, inconclusive result

Table 5. Antibiotic resistance profile of 50 isolates from 4R fecal sample

Strain	Amp	Strep	Tet	Cam	Kan	Imp	Cip	Gen	Azm	Amx	Nal	Resistant against number of antibiotics
1	+	-	-	+	-	+	+	+	-	+	-	6
2	+	-	-	+	-	+	+	+	-	+	-	6
3	+	-	-	-	-	+	+	-	-	+	-	4
4	+	-	-	+	-	+	+	+	-	+	-	6
5	+	-	-	+	-	+	+	-	-	+	-	5
6	+	-	-	-	-	+	+	-	-	+	-	4
7	+	+	-	+	-	+	+	+	-	+	-	7
8	+	-	-	-	-	+	+	-	-	+	-	4
9	+	-	-	-	-	+	+	-	-	+	-	4
10	+	-	-	-	-	+	+	-	-	+	-	4
11	+	-	-	+	-	+	+	-	-	+	-	5
12	+	-	-	-	-	+	+	-	-	+	-	4
13	+	-	-	+	-	+	+	-	-	+	-	5
14	+	-	-	-	-	+	+	-	-	+	-	4
15	+	-	+	-	-	+	+	-	-	+	-	5
16	+	-	+	+	-	+	+	+	-	+	-	7
17	+	-	-	X	-	+	+	-	-	+	-	4
18	+	-	-	+	-	+	+	-	-	+	X	5
19	+	-	+	+	-	+	+	+	-	+	X	7

Strain	Amp	Strep	Tet	Cam	Kan	Imp	Cip	Gen	Azm	Amx	Nal	Resistant against number of antibiotics
20	+	-	+	+	-	+	+	+	-	+	X	7
21	+	-	+	+	-	+	+	+	-	+	X	7
22	+	-	+	+	-	+	+	+	-	+	-	7
23	+	-	+	+	-	+	+	+	-	+	-	7
24	+	-	+	+	-	+	+	+	-	+	X	7
25	+	-	+	+	-	+	+	+	-	+	X	7
26	+	-	+	+	-	+	+	+	-	+	+	8
27	+	-	+	+	-	+	+	+	-	+	-	7
28	+	-	+	+	-	+	+	+	-	+	-	7
29	+	-	+	+	-	+	+	+	-	+	-	7
30	+	-	+	+	-	+	+	+	-	+	-	7
31	+	-	+	+	-	+	+	+	-	+	-	7
32	+	-	+	+	-	+	+	+	-	+	X	7
33	+	-	+	+	-	+	+	+	-	+	X	7
34	+	-	+	+	-	+	+	+	-	+	+	8
35	+	+	+	+	-	+	+	+	-	+	-	8
36	+	-	+	+	-	+	+	+	-	+	-	7
37	+	+	-	+	-	+	+	+	-	+	-	7
38	+	-	-	-	-	+	+	+	-	+	-	5
39	+	+	-	+	-	+	+	+	-	+	+	8
40	+	-	-	+	-	+	+	+	-	+	X	6
41	+	-	-	+	-	+	+	+	-	+	-	6
42	+	-	-	+	-	+	+	+	-	+	-	6
43	+	-	-	-	-	+	+	-	-	+	-	4
44	+	-	-	+	-	+	+	+	-	+	-	6
45	+	-	-	X	-	+	+	-	-	+	-	4
46	+	-	-	+	-	+	+	+	-	+	-	6
47	+	-	+	+	-	+	+	+	-	+	+	8
48	+	-	+	+	-	+	+	-	-	+	+	7
49	+	-	+	-	-	+	+	-	-	-	+	5
50	+	-	-	+	-	+	+	-	-	+	+	6

Amp- Ampicillin, Tet-tetracyclin, Strep-Streptomycin, Kan-Kanamycin, Cam-Chloramfenicol, Imp-Imipenem, Cip-Ciprofloxacin, Gen-Gentamycin, Azm-Azithromycin, Amx-Amoxicillin, Nal-Nalidixic acid; +, resistant; -, sensitive; X, inconclusive result

Table 6. Antibiotic resistance profile of 50 isolates from 5R fecal sample

Strain	Amp	Strep	Tet	Cam	Kan	Imp	Cip	Gen	Azm	Amx	Nal	Resistant against number of antibiotics
1	+	-	+	-	-	+	+	-	-	+	-	5
2	+	-	+	-	-	+	+	-	-	+	-	5
3	+	-	+	-	-	+	+	-	-	+	-	5
4	+	-	+	-	-	+	+	-	-	+	-	5
5	+		-	-	-	+	+	-	-	+	-	4
6	+	-	-	-	-	+	+	-	-	+	-	4
7	+	-	+	-	-	+	+	-	-	+	-	5
8	+	-	-	-	-	+	+	-	-	+	-	4
9	+	-	-	-	-	+	+	-	-	+	-	4
10	+	-	-	-	-	+	+	-	-	+	-	4
11	+	-	-	-	-	+	+	-	-	+	-	4
12	+	-	-	-	-	+	+	-	-	+	-	4

Strain	Amp	Strep	Tet	Cam	Kan	Imp	Cip	Gen	Azm	Amx	Nal	Resistant against number of antibiotics
13	+	-	+	-	-	+	+	-	-	+	-	5
14	+	-	-	-	-	+	+	-	-	+	-	4
15	+	+	X	+	-	+	+	-	-	+	+	7
16	+	+	+	-	+	+	+	-	-	+	-	7
17	+	-	X	-	+	+	+	-	-	+	-	5
18	+	-	-	-	-	+	+	-	-	+	-	4
19	+	-	+	-	-	+	+	-	-	+	-	5
20	+	-	+	-	+	+	+	-	-	+	+	7
21	+	-	+	+	+	+	+	-	-	+	X	7
22	+	-	+	+	+	+	+	-	-	+	+	8
23	+	-	+	+	+	+	+	-	-	+	+	8
24	+	-	+	+	X	+	+	X	-	+	+	7
25	+	-	+	+	+	+	+	+	-	+	+	9
26	+	+	+	+	-	+	+	-	-	+	+	8
27	+	-	+	-	-	+	+	-	-	+	X	5
28	+	-	+	-	-	+	+	+	-	+	-	6
29	+	-	+	-	-	+	+	+	-	+	-	6
30	+	-	+	+	-	+	+	-	-	+	-	6
31	+	+	+	+	+	+	+	X	-	+	+	9
32	+	-	+	+	-	+	+	X	-	+	+	7
33	+	+	+	+	-	+	+	X	-	+	+	8
34	+	-	+	+	X	+	+	+	-	+	+	8
35	+	-	+	+	-	+	+	X	+	+	+	8
36	+	-	+	+	+	+	+	X	+	+	+	9
37	+	-	+	-	-	+	+	-	-	+	-	5
38	+	-	+	-	-	+	+	-	-	+	-	5
39	+	-	+	-	-	+	+	X	-	+	-	5
40	+	-	+	-	-	+	+	-	-	+	-	5
41	+	-	+	-	-	+	+	+	+	+	-	7
42	+	-	+	-	-	+	+	X	-	+	-	5
43	+	-	-	X	-	+	+	-	-	+	-	4
44	+	-	+	+	-	+	+	-	-	+	-	6
45	+	-	-	+	-	+	+	-	-	+	X	5
46	+	-	+	+	-	+	+	-	-	+	+	7
47	+	-	+	+	+	+	+	-	-	+	+	8
48	+	+	-	+	+	+	+	+	-	+	+	9
49	+	+	-	+	-	+	+	+	+	+	+	9
50	+	-	-	+	-	+	+	-	+	+	+	7

Amp- Ampicillin, Tet-tetracyclin, Strep-Streptomycin, Kan-Kanamycin, Cam-Chloramfenicol, Imp-Imipenem, Cip-Ciprofloxacin, Gen-Gentamycin, Azm-Azithromycin, Amx-Amoxicillin, Nal-Nalidixic acid; +, resistant; -, sensitive; X, inconclusive result

3.3 Screening of Imipenem-Resistant *Salmonella* Strains with Active Presence of MBL(s)

The active presence of MBLs was determined in 100 isolates of 4R and 5R samples (Fig. 3A and B). The MBLs were found in 40% and 64% of the imipenem-resistant strains from the 5R and 4R samples, respectively.

3.4 Detection of Class 1 Integrons in *Salmonella* Strains Showing Active Presence of MBL(s)

Class 1 integrons were detected in only five of the 20 MBL producing strains chosen for the CS-PCR assay: 4R2, 4R7, 4R9, 4R10, and 4R15 (Fig. 4).

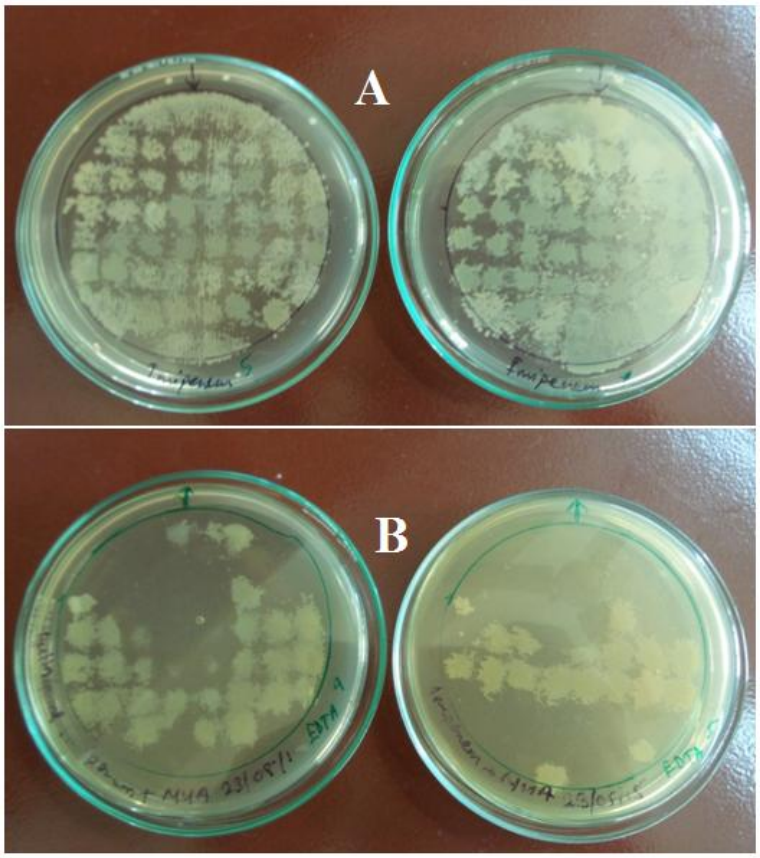

Fig. 3. Representative replica plates for screening of *Salmonella* isolates from '4R' and '5R' samples for metallo-β-lactamase activity. A- 4R and 5R isolates that grew on Imipenem plates; B- 4R and 5R isolates that grew on Imipenem+EDTA plates

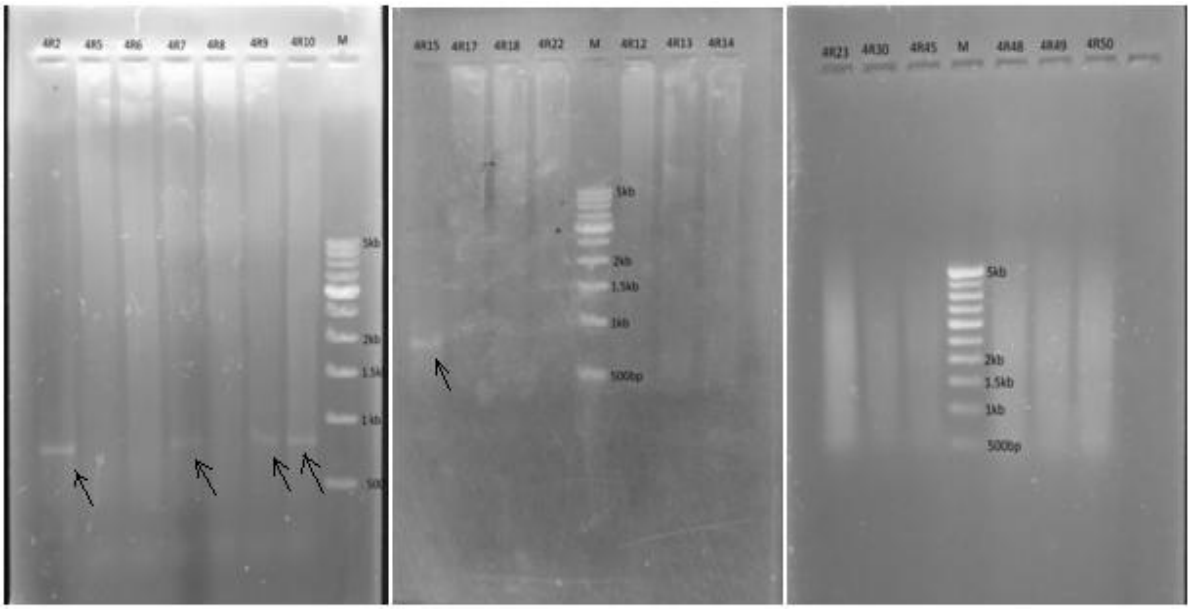

Fig. 4. CS-PCR assay showing five out of twenty metallo-β-lactamase producing MAR *Salmonella* strains found positive for the presence of Class 1 integron (amplification band indicated by arrow)

4. DISCUSSION

In most cases, all animals have a large population of bacteria in their gastrointestinal tract. Many of these commensal bacteria are not pathogenic. However, under certain conditions, these bacteria, some of which may be pathogenic and linked to disease outbreaks in various domesticated species. *Salmonella* spp., an *Enterobacteriaceae* member, is known to cause disease as a result of the stress caused by altered circumstances such as nutritional stress. The ability of these bacteria to cause disease is linked to the weakened body defences that occur during stressful times. Salmonellosis is a known disease that occurs in rhinoceros under similar conditions.

All Kaziranga Rhinos' feces contained a high abundance of multiple-antibiotic-resistant *Salmonella* strains. The fecal matter is discharged in a specific location by a specific Rhino. As a result, the dung sites are indicative of specific Rhinos that live in the Kaziranga forest area (Fig. 1). It is highly likely that KNP water bodies are contaminated with MAR *Salmonella* strains. Antibiotic resistance in a high number of strains indicates a high degree of gene transfer and acquisition of resistance genes in their genome. The data presented in this paper will provide valuable baseline information to veternerians of the National park. Furthermore, this information would be extremely valuable to conservationists. The vast majority of the *Salmonella* strains isolated from five different fecal samples were multidrug resistant (MAR) (Tables 2 - 6). EDTA was used to test the presence of MBL in antibiotic resistant isolates of 4R and 5R samples (Fig. 3). 40% of the antibiotic resistant strains from 4R and 64% of the antibiotic resistant strains from 5R were found to produce the MBL gene (Fig. 3A and B). A total 20 MBL producing samples were screened for the presence of class 1 integrons. Out of these 20 isolates, 5 (4R2, 4R7, 4R9, 4R10, and 4R15) were positive for class 1 integron (Fig. 4). The 650 bp amplicon found in the five isolates were reported to contain a fragment of the *sat*1 gene, which in its full version gives resistance to streptothricin (data not shown). PCR mapping of integrons may reveal several novel combinations of resistance genes [20]**.** It is highly predictive that the water bodies of KNP are contaminated with MAR *Salmonella* strains.

In their examination of 223 critically endangered species, Smith et al. [24] revealed that in the majority of cases, there was insufficient data regarding infectious disease or its effects, preventing any conclusion from being drawn regarding infectious disease as a contributing threat. At the most basic systematic level, it is estimated that only a small fraction of bacterial diversity in wild animals has been identified. A similar lack of understanding affects our understanding of wildlife-affecting viruses, parasites, and fungi [25]. Recent advances in molecular biology and microbiology have allowed for the detection and identification of hosts of novel microorganisms, many of which are pathogenic. Understanding the dynamics of disease-mediated species declines is critically important to the education of conservation professionals and is therefore critical to conservation missions concerned with a wide variety of species and habitats.

5. CONCLUSION

Our findings provide the first direct evidence that rhinos in the KNP are infected with MAR *Salmonella* strains. If rhinos are subjected to physiological stress, such as nutritional deficiency, the infected gut flora may wreak havoc on the animal population. The data presented in this report will provide valuable baseline information to the National Park's veterinarians. Furthermore, this information would be extremely valuable to conservationists. An evidence-based understanding of the load of plausible disease-causing agents in this vulnerable species' population will aid in the prioritisation of conservation efforts.

DISCLAIMER

One of the authors, D.P. was provided with the thesis grant provided by the Department of Biotechnology, Government of India. None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

ACKNOWLEDGEMENTS

The authors would like to give special thanks to the team of forest guide of KNP for providing fresh fecal samples of *Rhinoceros unicornis.*

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Sarma PK, Mipun BS, Talukdar BK, Kumar R, Basumatary AK. Evaluation of habitat suitability for Rhino (*Rhinoceros unicornis*) in Orang National Park using geo-spatial tools. International Scholarly Research Notices; 2011.
2. Mukherjee T, Sharma LK, Saha GK, Thakur M, Chandra K. Past, present and future: Combining habitat suitability and future landcover simulation for long-term conservation management of Indian rhino. Scientific Reports. 2020;10(1):1-12.
3. Hazarika BC, Saikia PK. Habitat Clafissification and Habitat Utilization of Great Indian One-Horned Rhino (*Rhinoceros unicornis Lin*.) in Rajib Gandhi Orang National Park. Proceeding, National Seminar on 'use of Remote sensing and GIS for Wild Life Studies. 2009;53-60.
4. Kartzinel TR, Hsing JC, Musili PM, Brown BR, Pringle RM. Covariation of diet and gut microbiome in African megafauna. Proceedings of the National Academy of Sciences. 2019;116(47): 23588-23593.
5. Bian G, Ma L, Su Y, Zhu W. The microbial community in the faeces of the white rhinoceros (*Ceratotheriumsimum*) as determined by barcoded pyrosequencing analysis. PLoS ONE. 2013;8(7):e70103. DOI: 10.1371/journal.pone.0070103
6. Espie IW, Hlokwe TM, Gey van Pittius NC. Pulmonary infection due to Mycobacterium bovisin a black rhinoceros (*Dicerosbicornis minor*) in South Africa. J Wildl Dis. 1999;45:1187.
7. Char KL, Ramanathan S, Rao MRK, Rao KC, Rao SV. Salmonellosis in adult Indian rhinoceroses (*Rhinoceroses unicornis*). The Journal of Zoo Animal Medicine. 1984;15(4):155-157.
8. Reechia GD, Hall R. Gene cassettes: A new class of mobile element, Microbiol. 1995;141:3015–3027.
9. Chakraborty R, Kumar A, Bhowal SS, Mandal AK, Tiwary BK, et al. Diverse Gene Cassettes in Class 1 Integrons of Facultative Oligotrophic Bacteria of River Mahananda, West Bengal, India. PLoS ONE. 2013;8(8):e71753. DOI: 10.1371/journal.pone.0071753
10. Fluit AC, Schmitz FJ. Class 1 integrons, gene cassettes, mobility, and epidemiology, Eur. J. Clin. Microbiol. Infect. Dis. 1999;18:761–770.
11. Mukherjee S, Chakraborty R. Incidence of class-1 integrons in multiple antibiotic-resistant Gram-negative copiotrophic bacteria from the River Torsa in India. Res Microbiol. 2006;157:220–226.
12. Lindstedt BA, Heir E, Nyga˚rd I, Kapperud G. Characterization of class I integrons in clinical strains of Salmonella enterica subsp. Entericaserovars Typhimurium and Enteritidis from Norwegian hospitals. J Med. Microbiol. 2003;52:141–149.
13. Miller M, Schille B, Pancake C. Salmonellasurveillance in a herd ofasymptomatic captive black rhinoceros (*Dicerosbicornis*) using fecal culture and PCR. J Zoo Wildl Med. 2008;39:56–60.
14. Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, et al. Imipenem EDTA disk method for differentiation of metallo-β-Lactamase producing clinical isolates of Pseudomonas spp. and Acinetobacter spp. J. of Cli. Microbiol. 2002;40(10):3798–3801. DOI: 10.1128/JCM.40.10.3798-3801.2002
15. Wilcove DS, Rothstein D, Dubow J, Phillips A, Losos E. Quantifying threats to imperiled species in the United States. Bio-Science. 1998;48:607–615.
16. Lafferty KD, Holt RD. How should environmental stress affect the population dynamics of disease? Ecology Letters. 2003;6:654–664.
17. De Castro F, Bolker B. Mechanisms of disease-induced extinction. Ecology Letters. 2005;8:117–126.
18. Gerber LR, McCallum H, Lafferty KD, Sabo JL, Dobson AP. Exposing extinction risk analysis to pathogens: is disease just another form of density dependence? Ecological Applications. 2005;15:1402–1414.
19. Wang J, Ji H, Zhang D, Liu H, Wang S, et al. Assessment of probiotic properties of Lactobacillus plantarum ZLP001 isolated from gastrointestinal tract of weaning pigs. Afr. J. Biotechnol. 2011;10:11303–11308. DOI: 10.5897/AJB11.255
20. Le´vesque C, Piche´ L, Larose C, Roy PH. PCR Mapping of integrons reveals several novel combinations of resistance genes. Antimicrob Agents Chemother. 2003;39P185–91.
21. Martinez P, Freizo AC, Fluit FJ, Schmitz VSC, Grek J, et al. Class I integrons in gram negative isolates from different European hospitals and association with

decreased susceptibility to multiple antibiotic compounds, J. Antimicrob. Chemother. 1998;42:689–696.
22. Ranjan VK, Mukherjee S, Thakur S, Gupta K, Chakraborty R. Pandrug-resistant Pseudomonas sp. expresses New Delhi metallo-β-lactamase-1 and consumes ampicillin as sole carbon source. Clinical Microbiology and Infection. 2021;27(3): 472-e1.
23. Bush K, Bradford PA. β-Lactams and β-lactamase inhibitors: an overview. Cold Spring Harbor Perspectives in Medicine. 2016;6(8):a025247.
24. Smith KF, Sax DF, Lafferty KD. Evidence for the role of infectious disease in species extinction and endangerment. Conservation Biology. 2006;20(5):1349–1357.
25. MacPhee RDE, Greenwood AD. Infectious Disease, Endangerment, and Extinction Inter J Evol Biol. 2013;Article ID 571939. Available:http://dx.doi.org/10.1155/2013/571939

Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle4.com/review-history/74662