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Prevalence and molecular characterisation of extended spectrum β-Lactamase resistant determinants in *Salmonella* spp. isolated from Swine, Tirupati district, Andhra Pradesh

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Abstract
Pigs and Pork serve as an important reservoir of *Salmonella* spp., which is still the second-greatest cause of food-associated hospitalizations globally. Hence, surveillance studies on antimicrobial resistance in *Salmonella* are essential. The aim of the present study is to determine the prevalence, antibiotic susceptibility profile and molecular characterization to detect resistant determinants in *Salmonella* spp. isolated from swine in Tirupati, Andhra Pradesh. *Salmonella* was identified using cultural and biochemical characteristics and positive isolates were assessed for antibiotic susceptibility and subjected to PCR assay. Collectively, 100 rectal swabs were obtained from pigs maintained on both organized and unorganized farms. We found 164 isolates as Enterobacteriaceae members, of which 28 isolates (17.07%) were *Salmonella*. Resistance to amoxicillin and colistin was found to be high (100%) followed by ceftriaxone (71.42%), gentamicin (50%), amoxicillin / clavulanic acid (32.14%) and enrofloxacin (10.71%). No isolate was found to be resistant to tetracycline. *blaTEM* (82.14%) is the most prevalent β – lactamase gene found in the gut microbiota of swine, followed by *blaCTX-M* group 2 (10.71%), *blaCTX-M* group 1 (7.14%) and *blaSHV* (3.57%) genes.
As the colistin is supposed to be the last resort antibiotic in human therapeutic usage. Hence, the validity of colistin resistance is further investigated by E-Test by selecting two isolates that showed colistin resistance by Disc diffusion test. However, the isolates showed sensitivity to colistin with MIC of 1µg/ml of colistin. The isolates showed disparity in colistin resistance when tested by disc diffusion method and E-test. Despite of phenotypic resistance to colistin detected by the Disc Diffusion method, the plasmid-mediated mcr^{-1} gene could not be found in any isolate.

Keywords: *Salmonella*, antimicrobial resistance, E-Test, antimicrobial resistance determinants

1. Introduction
Livestock is an essential component of the Indian agricultural production system and advances the nation's economy. There are 535.8 million Livestock in India (Singh and Kumari, 2017; Panda *et al.* 2022) [48, 43]. 9.06 million Pig population is present in India, of which 1.90 million are exotic / crossbreds and 7.16 million are indigenous/non-descripts. According to the livestock Census 2019 (Singh A, 2020) [47]. Swine constitute around 1.7% of total livestock. In India, rural areas account for around 90.27% of the pig population and urban areas account for 9.73%. Of the country's sources of animal protein, 7% comes from the pork production. Only north-eastern states, such as Assam and Nagaland, consume the majority of pork (Malik *et al.* 2020; Singh A, 2020) [38, 47].
In the family Enterobacteriaceae, *Salmonella* is a Gram-negative facultative rod-shaped bacterium. Members of the genus *Salmonella* spp. colonize gastro-intestinal tract of vertebrate hosts. Some species are widespread, while others evolved to survive only in certain type of hosts, with outcomes ranging from subclinical to systemic infection with high mortality (Giannella *et al.* 1997) [25]. A variety of biotic or abiotic reservoirs of *Salmonella* have been reported in the environment of pig production (Barber *et al.* 2002) [5].
Pigs and pork constitute an important reservoir of *Salmonella* spp. (Ferrari *et al.* 2019) [23]. Salmonellosis in swine has been almost caused by either *S.Choleraesuis* or by *S.Typhimurium*, which produces septicemia and enterocolitis respectively (Griffith *et al.* 2019) [26]. In pig farms, *Salmonella* infections may continue to persist in the surroundings of the herd for several months or even years. (Baloda *et al.* 2001) [4].

Salmonella transmission to humans through contaminated carcasses during slaughter makes *salmonella* carriage in pigs a serious food safety concern. *Salmonella* carriage in pigs is a significant food safety issue as *Salmonella* can be transmitted to humans through carcass contamination at slaughter (Barilli *et al.* 2018) [6]. *Salmonella* continues to be the second most common cause of food-borne hospitalizations globally, according to Ehuwa *et al.* (2021) [21].

An essential tool in the effective production of pork is antibiotics. (Cromwell, 2002) [16]. Antimicrobials are widely utilized for both therapeutic and non-therapeutic purposes, which includes growth promotion (Aarestrup, 2005) [1]. The highest levels of antimicrobial usage (AMU) were seen in chickens, followed by pigs and dairy cattle. However, pigs had the greatest AMU per kilogram of meat produced. Antimicrobial consumption by animals is currently twice than that used by humans (Cuong *et al.* 2018) [18].

Concerns have been expressed about the possibility of the establishment of bacterial populations resistant to antibiotics as a result of the widespread use of antibiotics treat diseases or as additives for animal feed (McDonald *et al.*, 1997; Witte, 1998) [39, 53] and aid in the clonal proliferation of bacteria that are multidrug resistant (MDR). Wide utilization of antibiotics led to inadequate assimilation of antibiotics in the animal body and an increased concentration of antibiotic residues in livestock manure. This would have detrimental effects on the environment's quality and the well-being of humans (Chen *et al.* 2018; Staley *et al.* 2020) [15, 50].

The public health in many countries is continuously at risk due to the rising prevalence of antibiotic resistant *Salmonella* infections, which have been observed on global basis (CDC, 2013) [13]. Consequently, the World Health Organization (WHO) has listed antibiotic resistant (AMR) *Salmonella* as one of the most critical bacteria (Tacconelli *et al.* 2018) [52].

Information on antibiotic resistance in *Salmonella* isolates of swine is scarce. The primary objective of the current investigation is to evaluate and determine the magnitude of antimicrobial resistance as well as resistant determinants.

2. Materials and Methods

2.1 Sample collection, culturing of *Salmonella*

Using sterile swabs (Himedia, India), rectal swab samples were collected randomly from apparently healthy weaners (piglets of 4 to 6 weeks age), diarrhoeic weaners, rearing pigs (pigs from 10 weeks to 6 months) and slaughtered pigs. 100 rectal swab samples were collected, of which 66 and 34 samples were obtained from different pigs of organized and unorganized swine farms located in and around Tirupati, Andhra Pradesh.

Faecal samples were inoculated in Selenite-F broth and incubated at 37 ºC for 18-24 hours in order to isolate *Salmonella* spp. Broth cultures were inoculated onto MacConkey agar and incubated at 37ºC for 18-24 hours. Pale colour colonies were selected from each plate and cultured on Brilliant green agar and xylose lysine deoxycholate agar. Further, colonies were subjected to standard biochemical tests. Using conventional culture and biochemical techniques as described by Cruickshank *et al.* (1975) [17] and Bergey's manual of systematic bacteriology (Sneath and Holt, 2001) [49], all the isolates were identified to the genus level.

2.2 Antimicrobial sensitivity test

According to the recommendations of Clinical Laboratory Standard Institute (2014) standards, all the isolates were tested for antibiotic sensitivity using the disc diffusion method on Muller Hinton (MH) Agar (Bauer *et al.* 1966) [7], against the following antimicrobial discs: amoxicillin (30 µg), amoxicillin/clavulanic acid (30 µg), ceftriaxone (30 µg), colistin (10 µg), enrofloxacin (10 µg), gentamicin (10 µg) and tetracycline (30 µg).

According to CLSI guidelines, the double-disc synergy test (DDST) was used to confirm the presence of ESBL-producing organisms. Amoxicillin (30 mg) and cefotaxime (30 mg) were used alone, as well as amoxicillin/clavulanate (30/10 mg) and cefotaxime/clavulanate (30/10 mg) in combination. Subsequently, plates were incubated overnight, an increase in zone size of at least 5 mm was considered to be indicative of the formation of ESBLs. (Lalruatdiki *et al.* 2018) [31].

2.3 Colistin resistance by E-Test

The minimum inhibitory concentration (MIC) of the colistin-resistant isolates from the initial disc diffusion screening was determined using the colistin E-test strip (Biomerieux, India). Pure culture isolates were cultivated overnight at 37 °C in Luria Bertani (LB) broth and swabbed on MHA plates. According to the guidelines (CLSI, 2014), a MIC strip has been placed in the center of the plate and incubated for 24 hours at 37 ºC. MIC value of ≥ 4µg/ml is considered as resistant.

2.4 DNA extraction and determination of purity

According to Shakuntala *et al.* (2017) [46], the boiling and snap-chilling procedure was used to extract DNA with minor modifications. About 1.5 ml of overnight broth culture was centrifuged for 1 minute at 10,000 rpm. After the supernatant was discarded, the pellet was dissolved in 1ml of PBS and centrifuged at 10,000 rpm for 1 minute. supernatant was discarded and the pellet was re-suspended in 500 µl of sterile distilled water. The suspension was subsequently boiled for 10 minutes at 100°C and immediately subjected for snap chilling on crushed ice for 6 minutes. To remove cell debris, bacterial lysate was centrifuged for 1 minute at 10,000 rpm and the supernatant was stored at -20 °C. one µl (microliter) of the extracted DNA was used to test the optical density in Nanodrop (Thermo Scientific, USA). Pure DNA with an absorbance (A) ratio of 1.8 to 2.0 was further used in PCR reactions.

2.5 Detection of antibiotic resistance genes by PCR

PCR was standardized for the detection of genes associated with amoxicillin, ceftriaxone and colistin resistance. According to the guidelines of the Dallenne *et al.* (2010) [19] study, the DNA was analyzed for the presence of ESBL genes using a PCR assay with specified primers in a Bio-Rad thermal cycler. Two duplex PCRs were standardized targeting four important ESBL genes (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$ group 1 and 2 genes). Duplex PCR-I was used for detecting *bla*TEM (encodes broad and extended spectrum β- lactamases) and *bla*SHV (encodes broad and extended spectrum β-lactamases, whose sulphydrl group was inhibited by p-mercurichloro-benzoate) genes and duplex PCR-II was used to detect *bla*CTX- M group 1 and group 2 genes (encodes β-lactamases with predominant cefotaxime hydrolyzing abilities) in phenotypically amoxicillin and ceftriaxone-resistant isolates.

Oligonucleotide primers used and their respective amplicon sizes were given in Table 1. PCR was optimized to 25 µl reaction mixture under standardized cycle conditions, after initial experiments with variable component quantities, which include initial denaturation at 94°C for 10 minutes, 30 cycles

of denaturation at 94 °C for 40 s, annealing at 60 °C for 40 s, elongation at 72 °C for 1 m, final elongation at 72 °C for 7 m, and holding at 4 °C.

The genotypic resistance to colistin was studied by targeting *mcr-1* gene in colistin resistant isolates. Uniplex PCR for the detection of 309 bp product of *mcr-1* gene was carried out as per the procedure described by Liu *et al.* (2016) [35]. Table 1 lists the oligonucleotide primers used as well as the amplicon size.

Table 1: Primers used in present study

Target gene	Primer sequence	Amplicon size (bp)
bla_{TEM} gene	F: CATTTCCGTGTCGCCCTTATTC R: CGTTCATCCATAGTTGCCTGAC	800
bla_{SHV} gene	F: AGCCGCTTGAGCAAATTAAAC R: ATCCCGCAGATAAATCACCAC	713
bla_{CTX-M} group 1 gene	F: TTAGGAAATGTGCCGCTGTA R: CGATATCGTTGGTGGTACCAT	688
bla_{CTX-M} group 2 gene	F: CGTTAACGGCACGATGAC R: CGATATCGTTGGTGGTACCAT	404

PCR assay was carried in Bio-Rad thermal cycler under standard conditions, which include initial denaturation at 94 ℃ for 15 minutes, followed by 25 cycles of denaturation at 94 ℃ for 30 s, annealing at 58 ℃ for 90 s, elongation at 72 ℃ for 60 s and a final cycle of elongation at 72 °C for 10 minutes.

2.6 Agarose gel electrophoresis of PCR product

In a submerged gel electrophoresis apparatus (Genei), the PCR product that had been amplified was subjected to electrophoresis as described by Sambrook and Russel's (2002) [45]. Agarose gel (1.5%) was prepared in 1X TBE buffer in a microwave oven. After cooling to about 50 ℃, Ethidium bromide @ 0.5µg per ml was added to the agarose solution. The molten agarose was poured on the gel-casting platform and submerged with sufficient quantity of electrophoresis buffer (1X TBE). The amplified PCR products were slowly loaded into the wells of submerged gel. 3 µl of 100bp-1500bp DNA ladder (Genei™) was loaded as molecular weight marker in one well and subjected to electrophoresis at 80 mA and progress of mobility was monitored by the migration of dyes. An UV trans-illuminator was used to visualize the PCR result and gel doccumentation system (Alpha Innotech, Alpha imager Hp) was used for photographing.

3. Results

3.1 Prevalence of *Salmonella* spp.

Out of 100 rectal samples collected, 164 isolates of Enterobacteriaceae were recovered from swine of organized and unorganized farms located within and near Tirupati, Andhra Pradesh. Out of 164 isolates, 28 isolates (17.07%) were identified as *Salmonella* spp. The *Salmonella* isolates were pre enriched by inoculating rectal swabs into Selenite F broth (Fig. 1). The *Salmonella* spp. produced non- lactose fermenting pale colored colonies on MacConkey agar (Fig.2). On Brilliant Green agar, *Salmonella* isolates produce red colored colonies were obtained (Fig.3) and on XLD agar black centered colonies were observed (Fig. 4). The isolates were indole negative, Methyl Red positive, Voges Proskauer test negative, citrate positive, urease negative and on TSI slant, acidic butt (yellow) and alkaline slant (red) with H_2S production was seen (Fig. 5).

3.2 Antimicrobial sensitivity test

Out of 164 Enterobacteriaceae isolates, 28 (17.07) were characterized as *Salmonella* spp. All the isolates showed resistance to amoxicillin (100%) and colistin (100%). 20 (71.42%), 14 (50%) and 09 (32.14%) isolates were resistant to ceftriaxone, gentamicin and amoxicillin / clavulanic acid respectively. For enrofloxacin, 3 (10.71%) isolates showed resistance. None of the isolate showed resistance to tetracycline (Fig. 6a). 27 isolates (96.42%) were detected to produce ESBL (Fig. 6b).

The isolates exhibiting colistin resistance were further subjected to E-test, by using colistin E-test strip, in order to determine minimum inhibitory concentration (MIC). According to CLSI 2014 standards, MIC value of ≥ 4µg/ml is considered as resistant. Of 28 colistin resistant *Salmonella* isolates, two isolates were randomly selected to perform E-test. Tested *Salmonella* isolates were sensitive with MIC of 1µg/ml for colistin (Fig. 6c).

3.3 Multi drug resistance

Of 28 isolates tested for antimicrobial resistance, 23 isolates (82.14%) were MDR. When the isolates were concurrently resistant to at least three antimicrobials from distinct classes of antimicrobial agents, they were categorized as multidrug resistant (MDR) pathogens. A total of eleven different antimicrobial resistance patterns were recorded in swine enteric microbial isolates. Most predominant resistant patterns recorded were Amoxicillin-Colistin-Enrofloxacin – Ceftriaxone-tetracycline, Amoxicillin-amoxicillin/clavulanic acid-Gentamicin-Colistin-Ceftriaxone-Tetracycline and Amoxicillin-Gentamicin-Colistin-Enrofloxacin-Ceftriaxone - Tetracycline (Table 2).

3.4 Detection of antibiotic resistance genes by PCR

All 28 isolates showed phenotypic resistance to amoxicillin and the isolates were tested for the presence of ESBL genes (bla_{TEM}, bla_{SHV}, bla_{CTX-M} group 1 and group 2) (Fig.7 & 8). Among the 28 tested isolates, 23 (82.14%) were positive for bla_{TEM}, three (10.71%) were found to possess bla_{CTX-M} group-2, two (7.14%) isolates carried bla_{CTX-M} group-1 gene and one (3.57%) is positive for bla_{SHV} genes.

Uniplex PCR was used to detect the presence of the mcr-1 gene in the DNA recovered from Salmonella isolates that were phenotypically colistin resistant, as detailed under the materials and methods. Positive control for *mcr-1* gene was included in the test using the known DNA sample provided by Prof. Alberto Quesada Molina of the Department of Biochemistry, Biology Molecular and Genetics, and Faculty of Veterinary Medicine at the University of Extremadura in Aceres, Spain. Positive control yielded 309 bp PCR product for *mcr-1* gene (Fig. 9). None of the tested isolates revealed

presence of *mcr-1* gene and the genotypic resistance against colistin mediated by *mcr-1* gene could not be detected in the present study.

4. Discussion

Salmonella is one among major zoonotic pathogen in animals and humans. Zare *et al.* (2014) [56] have previously demonstrated that *Salmonella* may be found in the faeces of cattle and goats. *Salmonella* was found in pork-born food products, according to Jiang *et al.* (2021) [29], confirming that food producing animals might carry and shed the *Salmonella* through faeces. *Salmonella* could be transmitted from animals to the surrounding environment, according to reports by Abouzeed *et al.* (2000) [2] and Akoachere *et al.* (2009) [3].

The results of the current investigation demonstrated antimicrobial resistance and prevalence of extended spectrum β lactam (ESBL) resistant determinants in *Salmonella* spp. recovered from swine of both unorganized and organized farms that are present in and around Tirupati, Andhra Pradesh.

In the current study, 100 rectal swabs in total were processed to isolate *Salmonella* spp. Of 100 rectal swabs processed, 164 Enterobacteriaceae members were found, including 28 *Salmonella* species. The total prevalence of *Salmonella* spp. in the current study was 17.07%.

The recovery of *Salmonella* from swine faeces is expected as pigs and pork constitute an important reservoir of *Salmonella* spp. (Ferrari *et al.* 2019) [23]. The present study is in accordance with studies of Li *et al.* (2019) [34] who reported the prevalence of *Salmonella* at the rate of 17.43%. Other studies reported the prevalence at about 11.81% and 11.93% (Li *et al.* 2013) [33]. Whereas studies of Cai *et al.* (2016) [11] reported the prevalence over 70%. *Salmonella* spp. prevalence might vary depending on sample size, sampling location, season of the year and other climatic and environmental variables (Keelara *et al.* 2013; Bondo *et al.* 2016) [30, 10].

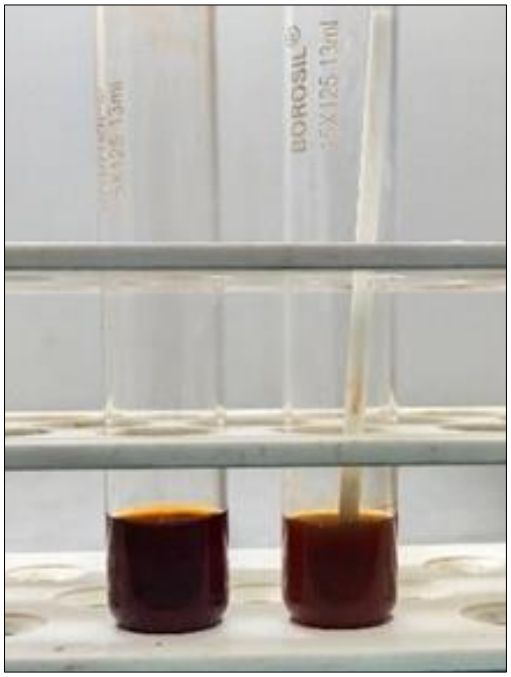


Fig 1: Pre-enrichment of *Salmonella* spp. in selenite- F broth

Fig 2: Non-lactose fermenting pale coloured colonies of *Salmonella* spp. on MacConkey agar

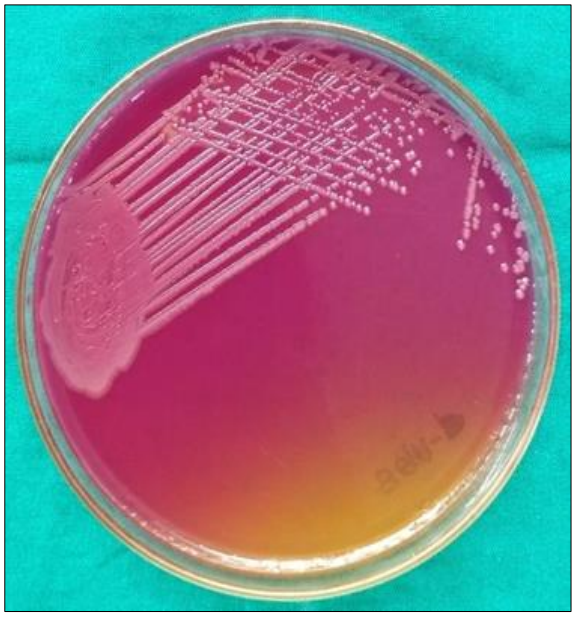


Fig 3: *Salmonella* spp. showing red coloured colonies on BGA plate

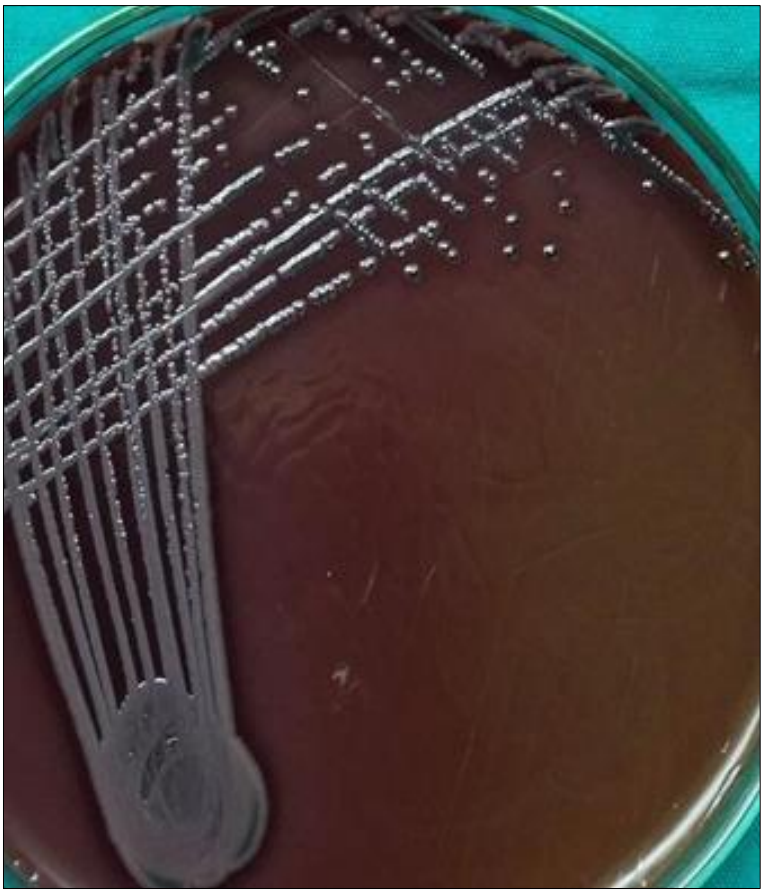


Fig 4: *Salmonella* spp. showing black centred colonies on XLD agar plate with H_2S production

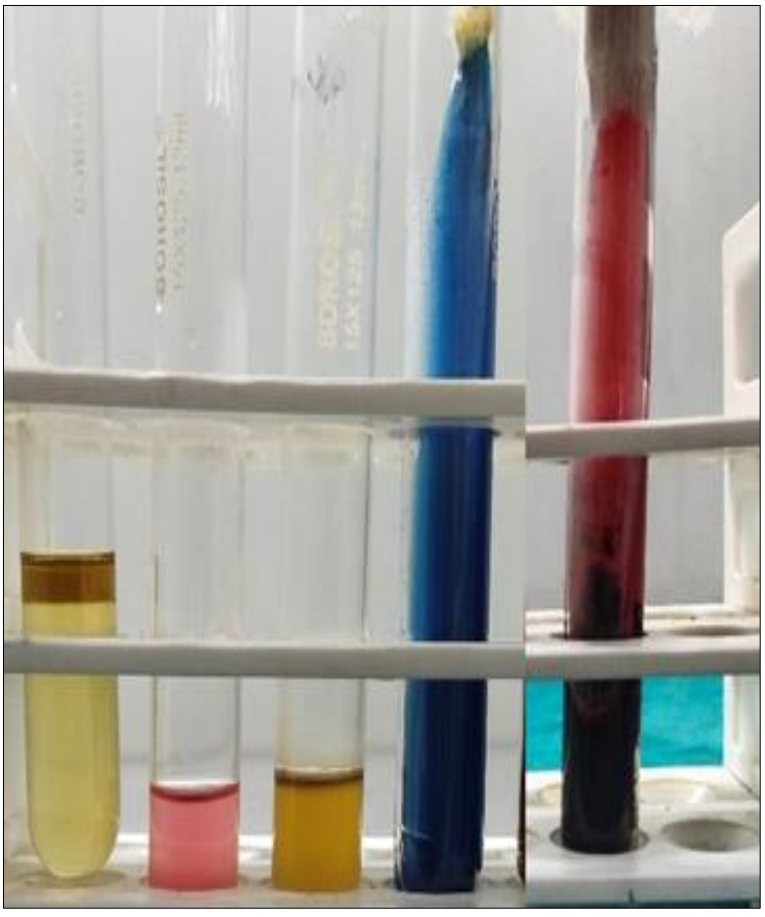
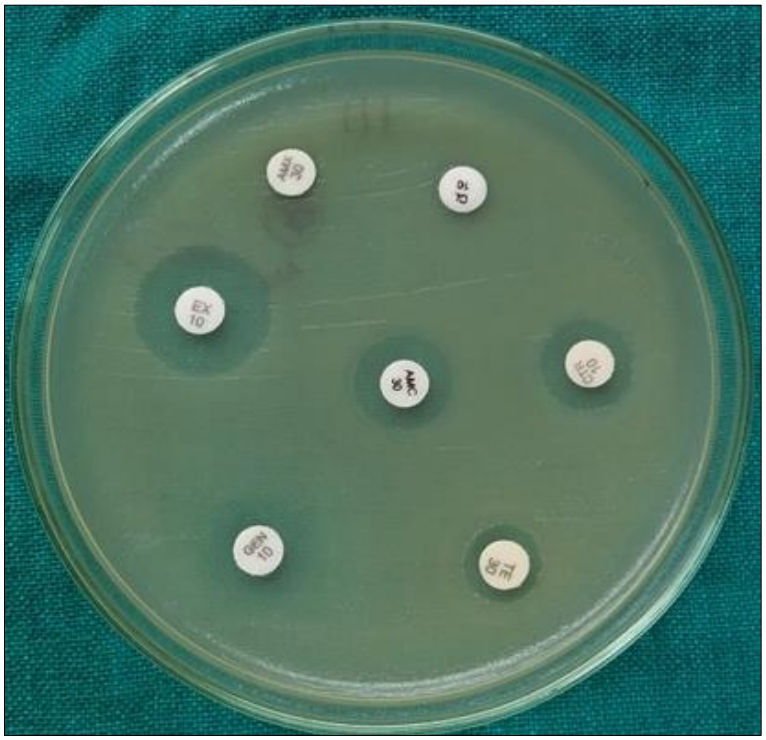


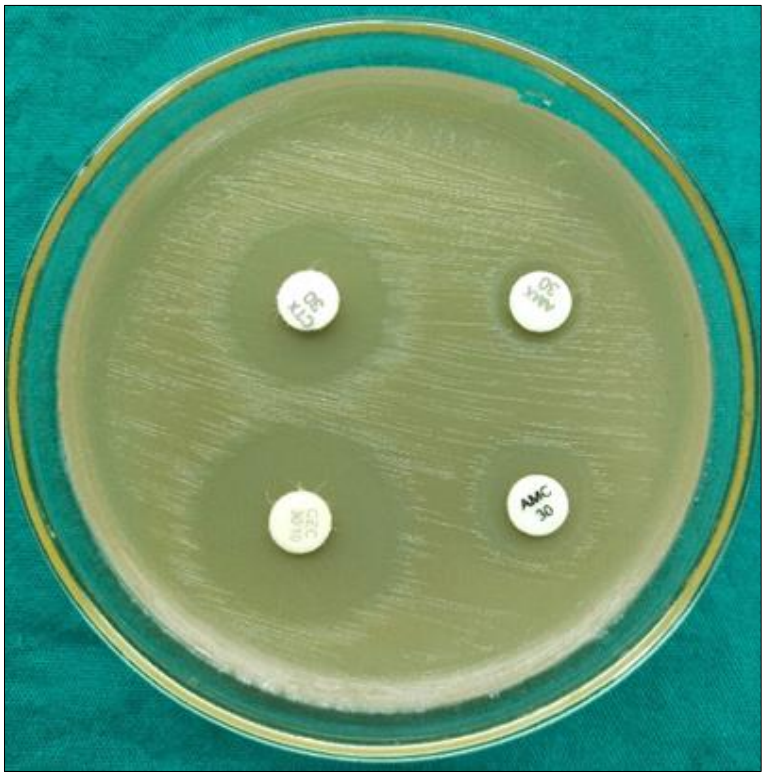
Fig 5: *Salmonella* spp. showing IMVIC tests (- + - +), TSI agar test (R/Y/H2S+ve) and urease test (-ve)

4.1 Identification and characterization of *Salmonella* spp. isolated from rectal swabs of swine



Amoxicillin - 8 mm (R)
Amoxicillin/clavulanic acid -14 mm (I)
Ceftriaxone -14 mm (R)
Colistin - 0 mm (R)
Enrofloxacin - 18 mm (S)
Gentamicin - 13 mm (I)
Tetracycline - 11 mm (R)

Fig 6a: On Muller Hinton Agar plate, *Salmonella* spp. showing



Amoxicillin - 7 mm (R)
Amoxicillin/clavulanic acid - 14 mm (I)
Cefotaxime - 18 mm (R)
Cefotaxime/ clavulanic acid - 25 mm (S)

Fig 6b: DDST of ESBL producing *Salmonella* spp. showing (> 5mm increase in zone size is +ve for ESBL)

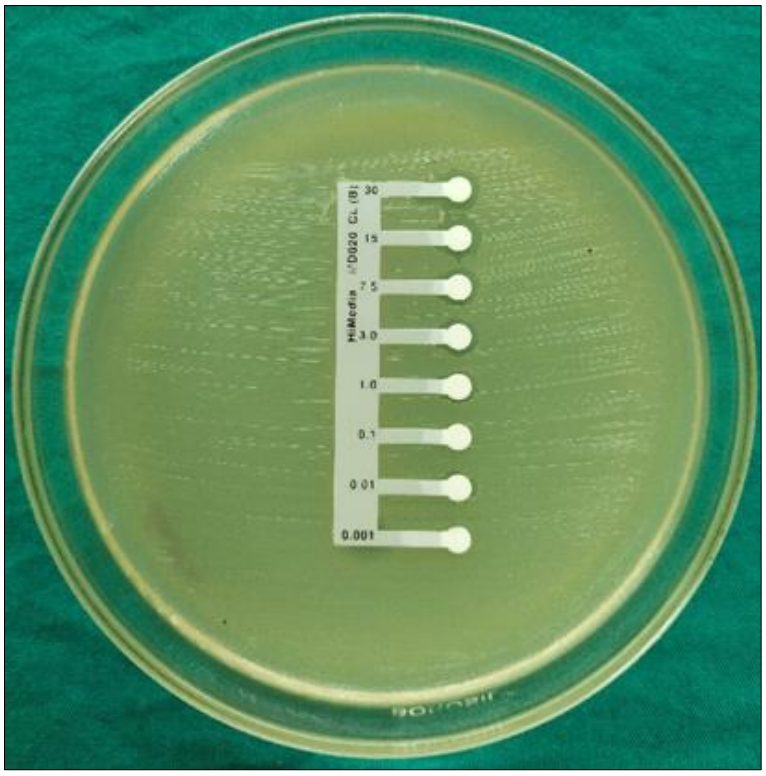


Fig 6c: Colistin MIC showing sensitivity in *Salmonella* spp. isolates (MIC: 1 µg/ml)

Fig 6: Antibiotic resistance patterns of *Salmonella* spp. on Muller Hinton Agar plate (S-Sensitive; I-Intermediate, R-Resistant)

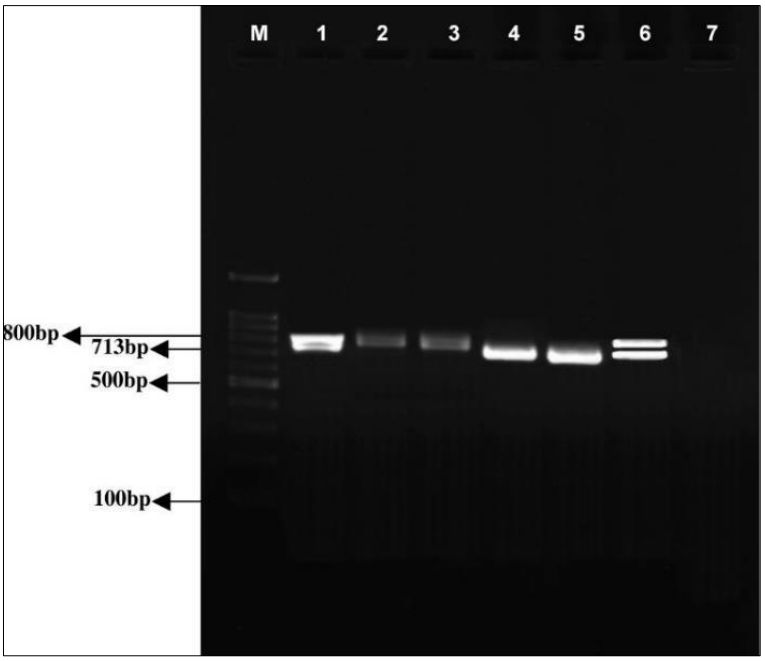
Table 2: Antimicrobial resistance patterns of MDR gut microbiota of swine

S. No.	Antimicrobial resistance patterns	No. of *Salmonella* isolates showing multidrug resistance.
R1	AMX-GEN-CL-CTR	1
R2	AMX-CL-EX-TE	1
R3	AMX-CL-CTR-TE	2
R4	AMX-AMC-GEN-CL-CTR	1
R5	AMX-AMC-CL-CTR-TE	1
R6	AMX-GEN-CL-CTR-TE	2
R7	AMX-CL-EX-CTR-TE	4
R8	AMX-AMC-GEN-CL-CTR-TE	4
R9	AMX-AMC-CL-EX-CTR-TE	1
R10	AMX-GEN-CL-EX-CTX-TE	4
R11	AMX-AMC-GEN-CL-EX-CTR-TE	2
	TOTAL	23

AMX-Amoxicillin, AMC-Amoxicillin/clavulanic acid, CL-Colistin, EX-Enrofloxacin, CTR-Ceftriaxone, GEN-Gentamicin, TE-Tetracycline.

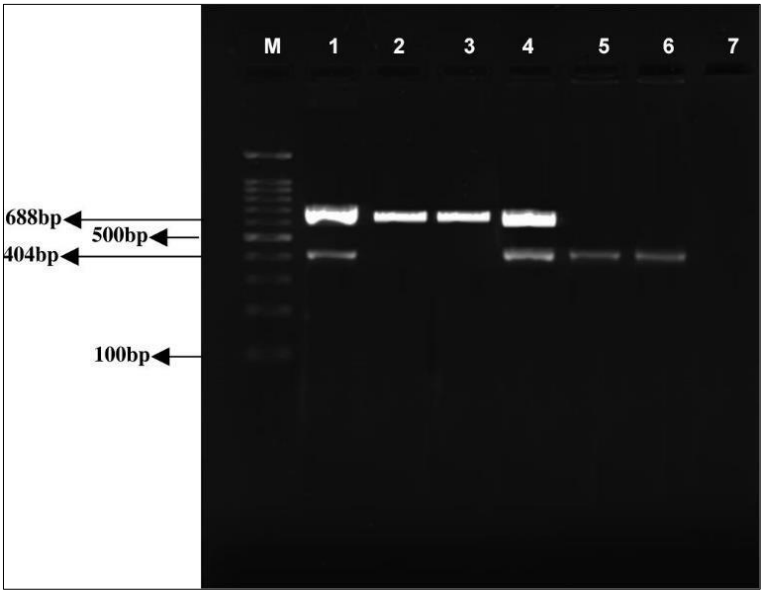
Antimicrobial resistance in *Salmonella* poses a threat to human health and reduces the choice of antimicrobials available which can even result in treatment failure (Threlfall, 2002) [54]. The present study reported high antimicrobial resistance to amoxycillin (100%) and colistin (100%) followed by ceftriaxone (71.42%), gentamicin (50%) and amoxicillin/clavulanic acid (32.14%). For enrofloxacin, 10.71% isolates showed resistance. None of the isolate showed resistance to tetracycline. 27 isolates (96.42%) were detected to produce ESBL genes by DDST method as described in materials and methods.

High antimicrobial resistance rates in *Salmonella* spp. were also reported by other workers. Cameron-Veas *et al.* (2018) [12] from Spain, demonstrated the multi drug resistance in *Salmonella* isolates from conventional pig farms. Percentage of resistance to tetracycline, ampicillin, ciprofloxacin and gentamicin was 98, 58, 50 and 20% respectively. None of the isolate exhibited resistance to colistin. Su *et al.* (2018) [51] tested 104 *Salmonella* isolates from diarrhoeic pigs. Most of the isolates were resistant to trimethoprim-sulfamethoxazole (100%), ampicillin (80.8%), tetracycline (76.9%) followed by ceftiofur (46.2%) and enrofloxacin (14.4%). 70.2% of the isolates exhibited varying scales of multi drug resistance.



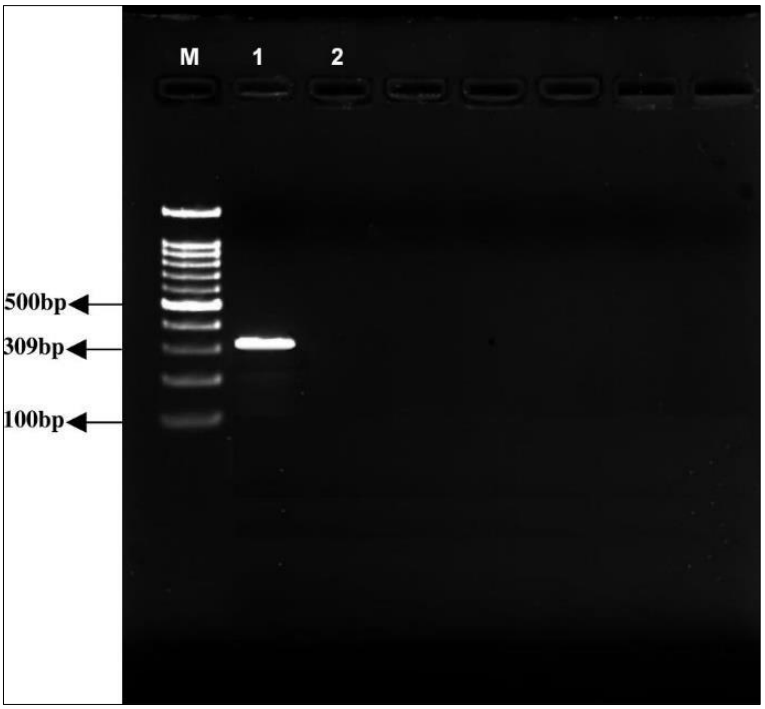
Lane M: Molecular weight marker (100-1500bp)
Lane 1: Positive control for *bla*$_{TEM}$ (800bp) and *bla*$_{SHV}$ (713bp)
Lane 2 to 6: Swine microbiota carrying *bla*$_{TEM}$ (800bp) and *bla*$_{SHV}$ (713bp) genes
Lane 7: Negative control

Fig 7: Detection of *bla*$_{TEM}$ and *bla*$_{SHV}$ genes



Lane M: Molecular weight marker (100-1500bp)
Lane 1: Positive control for *bla*$_{CTX-M}$ group-I (688bp) and group II (404bp) genes
Lane 2 to 6: Swine microbiota carrying *bla*$_{CTX-M}$ group-I (688bp) and group II (404bp) genes
Lane 7: Negative control

Fig 8: Detection of *bla* $_{CTX-M}$ group-I and group-II genes



Lane M: Molecular weight marker (100-1500bp)
Lane 1: Positive control for *mcr-1* gene (309bp)
Lane 2: Negative control

Fig 9: Standardization of uniplex PCR for the detection of *mcr-1* gene

Chen *et al.* (2019) [14] from China, demonstrated that isolation rates of *Salmonella* were more in swine (44%) than in chicken. Resistance to tetracycline (73.04%) was the highest, followed by 66.96% to ampicillin and 59.13% to doxycycline. A high rate of resistance to tetracycline (96.5%), ampicillin (84.4%), amoxicillin (85.2%) and ceftriaxone (8.6%) was reported by Jiang *et al.* (2021) [29] from China.

In the present study, highest resistance was reported to colistin even though colistin is not used in pig farming. Colistin is considered as one among few last resort antibiotics to treat carbapenemase producing Enterobacteriaceae and multidrug resistant bacterial infections. As the colistin is supposed to be the last resort antibiotic in human therapeutic usage, the validity of colistin resistance in the present study was further investigated. E-Test was performed by selecting two isolates that showed colistin resistance by Disc diffusion test. However, the isolates showed sensitivity to colistin with MIC of 1µg/ml of colistin. The isolates showed disparity in the results when tested by disc diffusion method and E-test.

The disc diffusion approach was often challenging to interpret for medicines like colistin and polymyxin B, which diffuse weakly, according to observations from Biemer, (1973) [9]. In these circumstances, Broth dilution or agar dilution techniques were used to estimate colistin's MIC. Similar to the current study, several investigations have demonstrated the unreliability of disk diffusion to determine colistin susceptibility (Gales *et al.* 2001; Nicodemo *et al.* 2004; Tan and Ng, 2006) [24, 41, 53]. Lo Ten Foe *et al.* (2007) [36] from Netherlands, demonstrated considerable error rates and poor reproducibility for the same isolate over subsequent assessments.

The majority of the ESBL-producing organisms in the current investigation were also discovered to be co-resistant to tetracycline, fluoroquinolones and aminoglycosides. When the isolates were concurrently resistant to at least three antimicrobial drugs from distinct classes of antimicrobial agents, they were classified as multidrug resistant (MDR) organisms (De Koster *et al.* 2021) [20]. In the present study, 82.14% of the investigated isolates were reported for multi drug resistance. Most predominant resistant patterns recorded in swine *Salmonella* were Amoxicillin – Colistin – Enrofloxacin – Ceftriaxone -tetracycline, Amoxicillin – Amoxicillin /clavulanic acid – Gentamicin - Colistin - Ceftriaxone -Tetracycline and Amoxicillin – Gentamicin - Colistin – Enrofloxacin - Ceftriaxone -Tetracycline. The resistance of ESBL-producing enteric bacteria to other classes of antibiotics, such as aminoglycosides, quinolones, tetracycline, sulphonamides, trimethoprim and chloramphenicol, was also demonstrated by Perez *et al.* 2007 [44] which is in agreement with the results of present study.

Cameron-Veas *et al.* (2018) [12] reported 71.21% of MDR in *Salmonella* isolates recovered from conventional pig farms. Su *et al.* (2018) [51] demonstrated multi drug resistance in 70.20% of *Salmonella* isolates from diarrhoeic pigs. Chen *et al.* (2019) [14] from China, reported 50.43% multi drug resistance in *Salmonella* isolates (58/115).

Given that enrofloxacin is frequently used to treat enteric infections brought on by MDR strains, its prevalence (20.74%) raises serious concerns (Hopkins *et al.* 2005) [28]. Additionally, quinolones-resistant bacteria have been shown to impact human health when consumed through contaminated food (Fabrega *et al.* 2008) [22]. Historical widespread use of penicillin (amoxicillin) and tetracycline in animals may account for the high resistance to these drugs (Chen *et al.* 2019) [14]. Our study revealed a 78.65% resistance to ceftriaxone. While another study revealed a low ceftriaxone resistance rate of 8.6% (Mthembu *et al.* 2021) [40]. An extended-spectrum antibiotic, ceftriaxone has been approved for treating Gram-negative bacterial infections in humans. Hence, ceftriaxone resistance in farm animals may lead to the spread of zoonotic ceftriaxone-resistant bacteria through food (Jiang *et al.* 2021) [29].

Among the 28 tested isolates, the predominant ESBL gene detected was *bla$_{TEM}$* (82.14%), followed by *bla$_{CTX-M}$* group-2 (10.71%), *bla$_{CTX-M}$* group-1 (7.14%) and *bla$_{SHV}$* (3.57%) genes. The results of the present study were consistent with those of a study by Li *et al.* (2016) [32] in South China, where *blaTEM* (73.7%), *blaCTX-M-1* (15.8%), and *blaSHV* (5.3%) were the most prevalent β-lactamase types found in Enterobacteriaceae members. However, the present study also found *blaCTX-M* group 2 along with other three genes, in the gut microbiota of swine. Gundran *et al.* (2020) [27] from Philippines, reported *bla*$_{TEM}$ (91.67%) and *bla$_{CTX-M}$* (91.67%) genes as most prevalent ESBL genes, followed by *bla$_{SHV}$* gene (60.42%). Bernreiter-Hofer *et al.* (2021) [8] reported *bla$_{TEM}$* (56%) and *bla$_{CTX-M}$* group 1 (13.71%) prevalence in *E. coli* isolates retrieved from diseased pigs.

In the present study, the genotypic resistance to colistin was studied by targeting *mcr-1* gene in colistin resistant isolates. However, none of the isolate tested in the present study was detected positive for *mcr*-1gene. According to Luo *et al.* (2017) [37], chromosomal mutations in the genes (mgrB, phoPQ, and pmrAB) that would confer lipid A alterations may be the cause of the phenotypic colistin resistance in the absence of colistin-encoding mobile elements (*mcr* genes). Hence, colistin resistance recorded in the present study by disc diffusion method was may be either due to mutations in genes that causes lipid-A modifications which further confers colistin resistance or due to the disparity between antimicrobial susceptibility testing methods, as no colistin resistance was recorded when tested by E-test.

5. Conclusion

The present study reported the prevalence of ESBL producing genes (*bla*$_{TEM}$. *bla*$_{SHV}$, *bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-2}$), in healthy pig population in Tirupati, A.P. The presence of these genetic determinants in commensal flora of healthy pigs suggests their potential to transmit ESBL genes between species or within species of animals. Additionally, there is a significant risk that these resistant bacteria may spread from healthy pigs and their pens to people working within farms, the environment and other animals that have access to pigs and pig excreta. As it is more likely to enter the food chain and thus causes the transmission of resistance determinants to humans, this commensal multidrug-resistant bacteria have the potential to be a significant opportunistic pathogen and poses a significant risk to both pig productivity and public health.

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