

Original Research Article

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Pathogenic Bacteria Prevalence in a Selected Environmental Sample and their Sensitivity to Routine Antibiotics

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ABSTRACT

The prevalence of human pathogenic bacteria especially *Salmonella* species in cow dung as a selected environmental sample was investigated. Five different abattoirs in Anambra State, Nigeria were used. Potentially pathogenic organisms were isolated and identified and they include; *Salmonella enteritidis*, *Shigella sp.*, *Clostridium perfringens*, *Pseudomonas sp.*, *E. coli*, *Staphylococcus aureus*, *Vibrio cholerae* and *Vibrio parahemolyticus*. The susceptibility of the isolates to different antibiotics was tested and it was observed that *Pseudomonas sp.* was very sensitive to the antibiotics, Ceporex (10µg) and Tarivd (10µg) with 16mm zone of inhibition. *Clostridium sp.* was more susceptible to Levofloxacin (20µg) with 20mm zone of inhibition, *Staph. aureus* was more susceptible to Streptomycin (30µg) and Levofloxacin (20µg) with 18mm zone of inhibition, *E. coli* was more susceptible to Gentamycin (10µg) with 20mm zone of inhibition, *Salmonella enteritidis* was more sensitive to Augmentin (30µg) with 20mm zone of inhibition and *Shigella sp.* was more sensitive to Ciproflox (10µg) with 20mm zone of inhibition. The pathogenicity of these isolates was studied by infecting each on mice. There was death of two mice infected with *Clostridium perfringens*. Mice infected with *Shigella sp.*, suffered swollen of the scrotum and scrotal sac which was observed after dissection. Mice infected with *Pseudomonas sp.*, *Staphylococcus aureus*, and *Salmonella enteritidis* and *E. coli*, 25×10^8 , 8×10^8 , 20×10^8 , 10×10^8 cfu/ml of the infected organisms were recovered from their intestine respectively. This shows that the organisms colonized their intestine at high level and they shed them in their faeces, though the infections were asymptomatic at the stage.

Keywords

Prevalence, cow dung, pathogenic bacteria, Antibiotics, sensitivity.

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Introduction

Salmonella is a genus of rod-shaped, gram-negative, non-lactose fermenter, non-spore-

forming, predominantly motile enterobacteria with diameters around 0.7 to 1.5µm lengths, and flagella that grade in all

directions (ie peritrichous). They are chemoorganotrophs obtaining their energy from oxidation and reduction reactions using organic sources, and are facultative anaerobes. Most species produce hydrogen sulfide. Salmonella is closely related to the genus Escherichia and can be found in cold and warm-blooded animals (including humans), worldwide and in the environment (Abakpa *et al.*, 2015). They cause illnesses such as typhoid fever, paratyphoid fever, and food borne illness. Salmonella infections can be zoonotic and can be transferred between humans and animals. Many infections are due to ingestion of contaminated food. Salmonella species are facultative intracellular pathogens that enter cells via macropinosomes. Salmonella bacteria can survive for weeks outside a living body and they can be destroyed by heat at about 55°C for 10 m (Parker *et al.*, 2010).

Typhoid fever is a major cause of death worldwide. Typhoid fever is caused by the genus salmonella and is acquired by ingestion of food or water contaminated by faeces of infected man, animals. Typhoid fever continues to be a health problem in the world, affecting mainly poorer regions where sanitation and clean water are lacking (Watson *et al.*, 2015; Mutua *et al.*, 2015). Salmonella and other intestinal pathogens can be spread by the infected humans and animals by shedding them in their faeces. The symptoms of salmonella and other pathogenic bacterial infection which can be found in cattle vary widely, and in many cases infected animals show no symptoms at all. These symptoms depend on serotype, infection does, and immune status of the animals that are infected (Svenska, 2011). The symptoms that can be seen are diarrhea, abortions, fever, poor general condition, decreased appetite, pneumonia, blood poisoning, and death (Svenska, 2011; Waddington *et al.*, 2014). These bacteria

that can be found in cow dung can be isolated by using enrichment, selective and differential media. Application of non-composted (fresh) cow dung as manure in agricultural practice could potentially cause contamination of food stuffs with pathogenic bacteria like *salmonella* sp., *E. coli*, *Vibro* sp, *Listeria* sp, *Pseudomonas* sp., *Shigella* sp., *Staphylococcus aureus*, *Clostridium* sp. and fecal streptococcus. The primary objectives of this study is to determine the prevalence of salmonella and other intestinal pathogens in cow dung and the susceptibility of these bacteria isolates to different antibiotics as well as ascertain the microbiology index and safety of using fresh cow dung (non-composted) as organic manure.

Materials and Methods

Sample collection

A total of 20 samples of fresh cow dung was collected and processed. The samples were collected from five different sites (Abattoirs) in Anambra State. The five slaughter houses from where the samples were collected are Nkpor market abattoir, Umunya abattoir, Kwata abattoir and two different abattoirs in Amansi. Five samples were collected from the cow intestine after slaughter and the other fifteen from the fresh cow dung deposits. The samples were collected with sterilized stainless spoon into wide mouth sterile sample bottles (Nwankwegu *et al.*, 2016a). The samples were transported in ice pack to the Microbiology laboratory UNIZIK immediately. Names were assigned to each of the sample from the study sites, based on the common name given to their locations.

Microbial enumeration

Different selective and differential media were used for the isolation of the bacterial pathogens found in the cow dung. The

media used were Salmonella – Shigella agar, Mannitol salt agar, Blood agar, Eosine Methylene blue agar, MacConkey agar, Thiosulfate – Citrate- Bile salts – Sucrose agar (TCBS), Brain Heart infusion agar, Nutrient agar, Nutrient broth, Selenite F broth and Peptone water. All these media were prepared according to the manufacturers' direction. 25g of each sample of cow dung was dissolved in 225ml of peptone water in conical flask. The conical flask were shaken to mix very well for 30 m and allowed to stand for 18 h at room temperature. A loopful of the broth culture of cow dung was streaked on a sterile agar plates. The plates were inverted and incubated at room temperature for 18 – 24 h. The colonies characteristics were recorded. Colonies were subcultured to obtain pure culture and then stored in agar slant.

Isolation of *Salmonella* and *Shigella* spp

25g of the cow dung was dissolved in 225ml of peptone water and allowed to stand for about 18 h at room temperature. 5ml of the peptone broth culture were then transferred to 20ml of selenite F broth in 3 different Erlenmeyer's flasks. They were incubated at 37⁰C for 24 h. The broth culture was than plated out on SS agar using streak plate method.

Blackish colonies (*Salmonella*) and colourless colonies (*Shigella*) that developed after 18 – 24 h of incubation at room temperature were isolated and purity checked by repeated streaking on fresh SS agar plates following incubation at room temperature (Raghavendra *et al.*, 2009; Resende *et al.*, 2014).

Isolation of *Escherichia coli*

25g of the cow dung was dissolved in 225ml of peptone water and allowed to stand for

about 18 h at room temperature. The broth culture was then plated out on EMB agar using a streak plate method. Green metallic sheen colonies were isolated and subcultured into MacConkey agar to get pure culture. Discrete pinkish colonies that developed after incubation at room temperature were isolated selected.

Isolation of *Staphylococcus aureus*

25g of the cow dung was dissolve in 225ml of sterile peptone water, and allowed to stand for about 18h at room temperature. The broth culture was plated out on mannitol salt agar using streak plate method. The plates were incubated at room temperature for 18-24 h. White to deep yellow colonies that developed on the plates were isolated and sub-cultured to obtain pure colonies (Ghaderpour *et al.*, 2014).

Isolation of *Clostridium* Sp.

25g of the cow dung was dissolved in 225ml of sterile peptone water, and allowed to stand for about 18h at room temperature. The broth culture was then plated out on Brain Heart Infusion agar using streak plate method. The plates were incubated at 37⁰C for 24 h in anaerobic condition. Yellow colonies that developed on the plates were sub-cultured on fresh Brain Heart Infusion agar in anaerobic condition using candle jar incubation method. Pure cultures of the isolates were obtained (Alfa *et al.*, 2014).

Isolation of *Vibrio* Sp

25g of the cow dung was dissolved in 225ml of sterile peptone water and allowed to stand for about 18h at room temperature. The broth culture was thereafter plated out on T.C.B.S agar using streak plate method. The plates were incubated aerobically at room temperature for 18-24 h. Yellow and green colonies that developed were isolated and subcultured.

Isolation of *Pseudomonas* sp

25g of the cow dung was dissolved in 225ml of sterile peptone water and allowed to stand for about 18h at room temperature. The broth culture was then plated on EMB agar using streak plate method. The plates were incubated aerobically at room temperature for 18-24 h. Pinkish colonies that developed on EMB agar were isolated and subcultured on cetrimide agar. The creamy to yellow coloured colonies that developed were isolated. All isolates were subjected to conventional biochemical characterizations in our laboratory.

Antibiotic sensitivity test

The clinical and Laboratory Standard Institute (CLSI), disc diffusion method was used for the antibiotic sensitivity test. The turbidity of the inoculums of various isolates was made to be equivalent to 0.5 of McFarland standard and each of the isolates was inoculated onto the surface of sterile Muller-Hinton agar plates using a sterile swab in order to ensure even distribution of inoculums. The plates were allowed to dry and commercially procured Gram positive and negative antimicrobial discs with different concentrations were placed on the surface of the agar plates. After 30 min of applying the discs, the plates were inverted and incubated for 24 h at room temperature (Agwu *et al.*, 2015; Loiki *et al.*, 2016; Assouhoun-Dieni *et al.*, 2001). The clear zones that developed around each disc were measured as the zones of inhibition on the basis of CLSI guideline.

Pathogenicity test

Laboratory animal

Three months old immunocompetent albino mice (male) weighing between 30 and 33g, bred in Chris poultry farm Awka were used.

They were housed in 9 different metal cages and fed prior to infection. Two of the mice in the cage served as control.

Inoculum Preparation

The bacteria isolated from the cow dung; *E. coli*, *Salmonella enteritidis*, *Pseudomonas* sp., *Staphylococcus aureus*, *Shigella* sp., *Clostridium pefringes.*, *Vibrio cholerae* and *Vibrio parahaemolyticus* were used. They were cultured differently in conical flask containing 80ml of sterile nutrient broth, overnight at 37°C inside shaker. At mid logarithmic growth phase, 5ml of each suspension was transferred to 25ml of another sterile nutrient broth. The bacterial suspensions were grown at 37°C inside the shaker until an optical density of 1.0 at a wavelength of 620nm was achieved. Subsequently, 2ml of each suspension was washed (Centrifuged at 3000 rpm for 30 min) twice in 2ml sterile isotonic saline, which corresponded to 3×10^8 cfu/ml. The inocula were used for animal inoculation.

Animal Inoculation

The mice were infected using oral and intraperitoneal routes. 0.1ml saline suspension of the inocula of different isolates was inoculated orally on 8 set of mice. The other 8 mice were inoculated intraperitoneally. The two control mice were inoculated with 0.1 saline, one oral and the other intraperitoneally. The mice were fed and observed for pathological signs for 14 d. At the end of 14 d, the survived mice were dissected and their intestine, lungs, and livers harvested. The organs of the mice that died after inoculation were also harvested for analysis. 2g of each intestine was weighed and ground in 2ml of saline with mortar and pestle. The number of the infecting organisms in the intestine was determined by plating after 10^{-5} serial

dilution. The organs were also homogenized in 1ml of sterile distilled water. The homogenated organs were serially diluted using ten-fold dilution with sterile water and the number of organisms was determined by plating. The antibiotic sensitivity results of the isolates were used as the marker to noting if the organisms injected were the ones isolated from the organs.

Statistical analysis

Statistical analysis was carried out on the values obtained from the experimental study using statistical package for social science (SPSS; version 21.0). One way analysis of variance (ANOVA) was used. P-values test of significance carried out at 95% level of confidence (Nwankwegu *et al.*, 2016b).

Results and Discussion

Microbial spp

Potentially pathogenic bacteria were isolated from the cow dung. The isolates were characterized and identified as members of the genera *Salmonella*, *Shigella*, *Vibrio*, *Staphylococcus*, *Escherichia* and *Pseudomonas* and *Clostridium* (Table 1).

Sensitivity Test

The antibiotic sensitivity reactions on the isolates are presented in Fig 1 and 2. *Staphylococcus aureus* was highly sensitive to the antibiotic Streptomycin and Levofloxacin, *Salmonella enteritidis* was highly sensitive to Augmentin, *Clostridium perfringens* is highly sensitive to Levofloxacin, *Pseudomonas sp* was highly sensitive to Tarivid and Ceporex, *Vibrio cholerae* is highly sensitive to Gentamycin and Tarivid. *V. parahaemolyticus* was highly sensitive to Amplicin and *Shigella sp* is highly sensitive to Ciproflox.

Pathogenicity

After the oral and intraperitoneal infection of mice, there was death of two mice which were infected with *Clostridium sp*. Others were asymptomatic carriers because they showed no symptoms of disease, but they shed the organisms in their faeces. The mouse infected with clostridium sp. orally died on the first day while the other mouse infected with the same organism (intraperitoneally) died on the 3rd day and hairs on the skin were raised. Mouse infected with *staphylococcus aureus* intraperitoneally suffered from intraperitoneal lesion. The mice infected with *Shigella sp.*, showed enlargement and swollen of scrotum and scrotal sac after dissection. Bacterial numbers recovered from the intestine and organs of the infected mice are shown in Tables 2 and 3, more number of the organisms was recovered from the intestine.

Eight human pathogens were isolated in cow dung collected from five different abattoirs in Anambra State, namely Nkpor- market, Umunya, Kwata and two different abattoirs at Amansi, Awka. The isolates were characterized and identified as *Shigella sp.*, *Salmonella enteritidis*, *Pseudomonas sp.*, *Clostridium perfringens*, *E. coli*, *Staph. aureus*, *V. cholerae* and *V. parahaemolyticus* (Table 1).

It has been noted that in the livestock sector, different types of farm animals are capable of caring a wide range of zoonotic pathogen (18). The cow and the mice often act asymptomatic carriers of human pathogens such as *E. coli* and *Salmonella enteritidis* which are rarely detected during routine anti-mortem examination. Their wastes may contain high concentrations of the organisms (Christian, 2012).

Table.1 Biochemical identities of bacterial isolates

Colour of the colonies	Shape	Gram stain	Indole	Voges-proskaver test	Methylred test	Citrate	Motility	Urease	Coagulase test	Oxidase test	Catalase test	Spore stain	Sugar fermentation						Organisms	
													Glucose	Sorbitol	Mannitol	Sucrose	Lactose	Maltose		Raffinose
Pinkish on MA & green metallic sheen on EMB	Short rod	-	+	-	+	-	+	-	ND	-	+	-	++	++	++	++	++	++	++	<i>E. coli</i>
Black on SS agar	Short rod	-	-	-	+	+	+	-	ND	-	+	-	++	++	++	-	-	++	++	<i>S. enteritidis</i>
Colourless on SS Agar	Short rod	-	+	+	-	-	-	-	ND	+	+	-	++	++	++	+	-	++	+	<i>Shigelle sp.</i>
Yellow on TCBS	Curved rod	-	+	ND	-	+	+	-	ND	+	+	-	+	+	+	-	-	-	-	<i>V. cholera</i>
Green on TCBS	Curved rod	-	+	ND	-	+	+	+	ND	+	-	-	+	+	+	-	-	-	-	<i>V. Parahaemolyticus</i>
Creamy on Brain Heart infusion	Long rod	+	-	+	-	+	-	+	ND	+	-	+	++	+	+	++	-	-	-	<i>C. perfringens</i>
Pinkish on EMB	Rod	-	-	-	-	+	+	+	ND	+	-	-	+	++	++	++	+	+	-	<i>Pseudomans sp.</i>
White to deep yellow on MSA	Cocci	+	-	-	-	+	-	+	+	-	+	N D	+	+	-	+	-	-	-	<i>S. auerus</i>

Key
 + = positive
 - = Negative
 ND = Not determined
 ++ = Positive with gas production

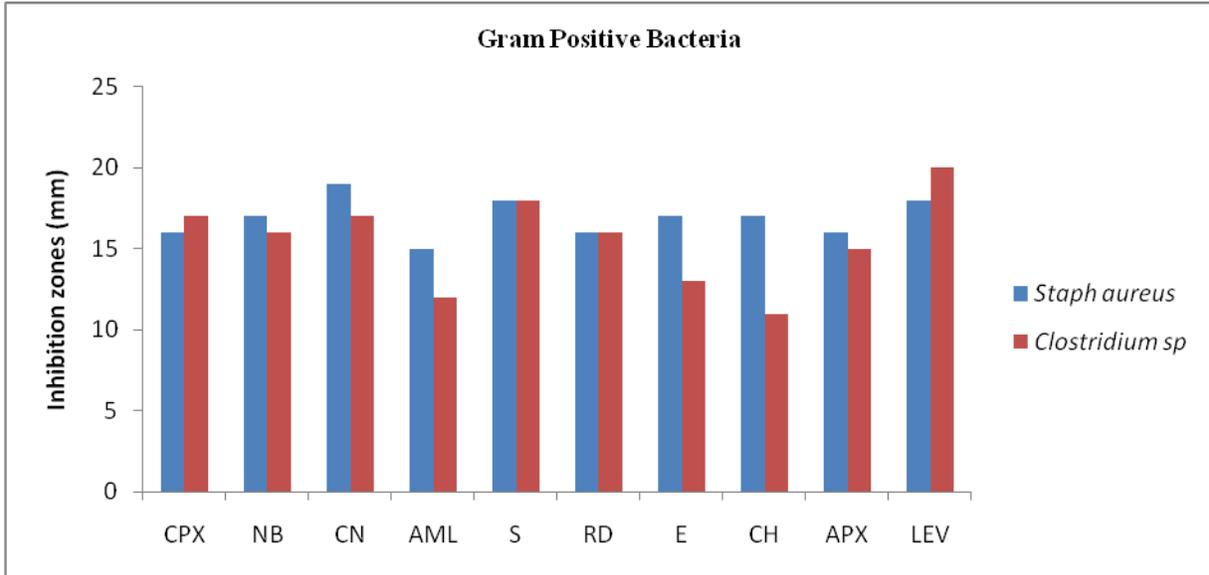
Table.2 No. of organisms recovered after dissection of orally infected mice (cfuml-1)

<i>Organisms</i>	Liver (10 ⁶)	Kidney (10 ⁶)	Lung (10 ⁶)	Intestine (10 ⁸)
<i>E. coli</i>	4	4	Nil	10
<i>Clostridium sp.</i>	1	Nil	2	2
<i>V. cholera</i>	Nil	Nil	Nil	2
<i>V. parahaemolyticus</i>	Nil	Nil	Nil	1
<i>Salmonella enteritidis</i>	Nil	50	Nil	20
<i>Shigella sp.</i>	Nil	3	7	17
<i>Staph. aureus</i>	2	1	3	8
<i>Pseudomonas sp.</i>	Nil	24	1	25

Table.3 No of organisms recovered after dissection of mice infected intraperitoneally (cfuml-1)

<i>Organisms</i>	Liver (10 ⁶)	Kidney (10 ⁶)	Lung (10 ⁶)	Intestine (10 ⁸)
<i>E. coli</i>	2	1	Nil	3
<i>Clostridium sp.</i>	2	1	1	2
<i>V. cholera</i>	Nil	Nil	Nil	Nil
<i>V. parahaemolyticus</i>	Nil	Nil	Nil	Nil
<i>Salmonella sp.</i>	Nil	20	Nil	50
<i>Shigella sp.</i>	Nil	1	3	7
<i>Staph. aureus</i>	2	1	2	3
<i>Pseudomonas sp.</i>	Nil	6	Nil	2

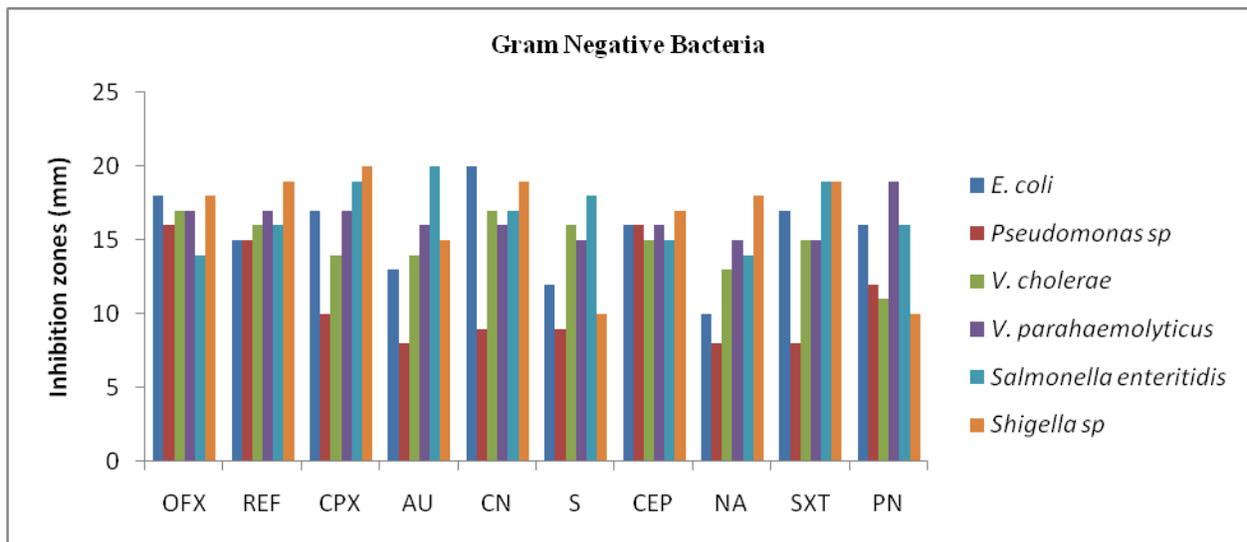
Fig.1 Antibacterial susceptibility pattern of Gram positive bacteria (mm)



Resistance = or < 10mm zone of inhibition

Sensitive = or > 15mm zone of inhibition

Fig.2 Antibacterial susceptibility pattern of Gram negative bacteria (mm)



Resistance = or < 10mm zone of inhibition

Sensitive = or > 15mm zone of inhibition

Previous study reported that mice are able to survived high doses of *Salmonella* enteric serovar Typhi administered by various routes whereas this bacterium causes a systemic infection and typhoid fever in humans (Bollen *et al.*, 2008). In a different study, it was reported 100% death of mice after 1 to 8 h oral infection of mice with *Clostridium perfringens* type D isolated from sheep and goat which causes enterotoxemia in them and death of mice after intraduodenal inoculation of this same organism (Fernandez- Miyakawa *et al.*, 2007). Multiplication of *Staphylococcus aureus* and formation of lesion which was observed when the mice were sacrificed after six days of intravenous inoculation has also been reported.

The antibiotic sensitivity reactions of the isolates shows that *Staphylococcus aureus* was highly sensitive to the antibiotic Streptomycin and Levofloxacin, *Salmonella enteritidis* is highly sensitive to Augmentin, *Clostridium perfringens* is highly sensitive to Levofloxacin, *Pseudomonas sp* is highly sensitive to Tarivid and Ceporex, *Vibrio cholerae* is highly sensitive to Gentamycin and Tarivid, *V. parahaemolyticus* is highly sensitive to Amplicin and *Shigella sp* is highly sensitive to Ciproflox. After the oral and intraperitoneal infection of mice, there was death of two mice which were infected with *Clostridium sp*. Others were asymptomatic carriers because they showed no symptoms of disease, but they shed the organisms in their faeces. The mouse infected with *Clostridium sp*. orally, died on the first day while the other mouse infected with the same organism (intraperitoneally) died on the 3rd day and hairs on the skin were raised. On the mouse infected with *Staphylococcus aureus* intraperitoneally, there was intraperitoneal lesion. The mice infected with *Shigella sp.*, showed enlargement and swollen of scrotum and

scrotal sac after dissection. The mice infected with *V. cholerae* and *V. parahaemolyticus*, low level of the organisms were recovered from their intestine.

In conclusion, this study shows that some animals are asymptomatic carriers of human pathogen and infection may depend on serotype and immune status of the animals that are infected.

Fresh cow dung was found to contain potentially pathogenic bacteria such as *Clostridium perfringens*, *Staphylococcus aureus*, *Salmonella enteritidis*, *Shigella sp.*, *Pseudomonas sp.*, *Vibrio cholera* and *Vibrio parahaemolyticus*. The susceptibility test result shows that the antibiotic levofloxacin is very effective to gram positive isolates and ceporex, reflacin and tarivid are very sensitive to gram negative isolates.

Salmonella which is a human pathogen was found to be asymptomatic to the experimental animal. The mice shed the organisms in their faces as carriers.

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