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Evaluation and Antimicrobial Susceptibility Pattern of Pathogenic Bacteria in Poultry Wastes

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Abstract: In the present study, the prevalence of pathogenic bacteria from poultry environment was investigated. Potentially pathogenic organisms were isolated and identified notably; *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 20 mm zone of inhibition, *Staph. aureus* was more susceptible to Streptomycin (30µg) and Levoflaxacin (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*, $25x10^8$, $8x10^8$, $20x10^8$, $10x10^8$ cfu/ml of the infected organisms were recovered from their intestine respectively. It seems 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: Poultry, Prevalence, Pathogenic Bacteria, Sensitivity

1. Introduction

Poultry is one of the fastest growing agricultural sectors in the contemporary Nigeria [1]. This leads to significant raise in the production of broilers at the rate of 8 to 10% per annum [2] with an annual turnover of 30,000 crores [3]. The advantages of low investment and the requirement of small area have increased the number of poultry shops and creating employment opportunities [4]. This also leads to the generation of huge quantities of poultry wastes usually composed of broiler and layers, feathers, bones, blood, hatchery debris and dead birds. These wastes pose serious environmental pollution problems through microbial infection, offensive odours, promotion of flies and rodent breeding [5]. Again some of the diseases often suffered by the poultry birds could possess certain zoonotic quality with both acute and chronic manifestations. There are no proper disposing units for these wastages in developing countries like Nigeria but attempts are in process to reuse these materials as beneficiary ones such as fertilizers and animal feed supplements [6]. As these wastes are composed of tissues and blood, we have hypothesized that these deposited wastes may serve as a reservoir for the multiplication of several pathogenic microorganisms that can cause severe disease outbreaks to both man and animals. At present, there is paucity of scientific information on the potential microbial pathogens associated with poultry environmental samples. Hence, the present study was carried out to identify the potential pathogens that can survive in these poultry environmental samples which are capable of predisposing man to infections.

2. Materials and Methods

2.1. Sample Collection

A total of ten samples of fresh poultry wastes was collected and processed. The samples were collected from five different sites in Arroma farm Anambra State and composite sample obtained. The samples were collected with sterilized stainless spoon into wide mouth sterile sample bottles [7]. The samples were transported in ice pack to the Microbiology laboratory, Nnamdi Azikiwe University Awka, Anambra State, Nigeria immediately. Samples were appropriately labelled for identification conveniences.

2.2. 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. 25 g of sample of poultry wastes was dissolved in 225 ml 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 poultry waste 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.

2.2.1. Isolation of Salmonella and Shigella Spp

25 g of sample was dissolved in 225 ml of peptone water and allowed to stand for about 18 h at room temperature. 5 ml of the peptone broth culture were then transferred to 20 ml of selenite F broth in 3 different Erlenmeyer's flasks. They were incubated at 37°C 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 [8, 9].

2.2.2. Isolation of Escherichia Coli

25 g of the poultry waste was dissolved in 225 ml 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 [9, 10].

2.2.3. Isolation of Staphylococcus Aureus

25 g of sample was dissolve in 225 ml of sterile peptone water, and allowed to stand for about 18 h 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 [10, 11].

2.2.4. Isolation of Clostridium Sp

25 g of sample was dissolved in 225 ml of sterile peptone water, and allowed to stand for about 18 h at room temperature. The broth culture was then plated out on Brain Heart Infusion agar using streak plate method. The plates were incubated at 37°C 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 [11].

2.2.5. Isolation of Vibrio Sp

25 g of sample was dissolved in 225 ml of sterile peptone water and allowed to stand for about 18 h 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 [11].

2.2.6. Isolation of Pseudomonas Sp

25 g of sample was dissolved in 225 ml of sterile peptone water and allowed to stand for about 18 h 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 coloures that developed were isolated. All isolates were subjected to conventional biochemical characterizations in our laboratory [10].

2.3. 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 Mc Farland 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 [9, 12]. 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 m of applying the discs, the plates were inverted and incubated for 24 h at room temperature [12, 13, 14, 15]. The clear zones that developed around each disc were measured as the zones of inhibition on the basis of CLSI guideline.

2.4. Pathogenicity Test

2.4.1. Laboratory Animal

Three months old immunocompetent albino mice (male)

weighing between 30 and 33 g, bred in Nkwegu farm Abakaliki, Ebonyi Sate were used. They were housed in 9 different metal cages each containing five mice and fed prior to infection. Five of the mice in the cage served as control.

2.4.2. Inoculum Preparation

The bacteria isolated from the poultry wastes; E. coli, Salmonella enteritidis, Pseudomonas sp., Staphylococcus aureus, Shigella sp., Clostridum pefringes., Vibrio cholerae and Vibrio parahaemolyticus were used. They were cultured differently in conical flask containing 80 ml of sterile nutrient broth, overnight at 37°C inside shaker. At mid logarithmic growth phase, 5 ml of each suspension was transferred to 25 ml 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 620 nm was achieved. Subsequently, 2 ml of each suspension was washed (Centrifuged at 3000 rpm for 30 m) twice in 2 ml sterile isotonic saline, which corresponded to $3x10^8$ cfu/ml. The inocula were used for animal inoculation [16].

2.4.3. 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. 2 g of each intestine was weighed and ground in 2 ml 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 1 ml 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.

2.5. 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 (Error bars) [17].

3. Results

3.1. Microbial Spp

Potentially pathogenic bacteria were isolated from the poultry wastes. The isolates were characterized and identified as members of the genera *Salmonella, Shigella, Vibrio, Staphylococcus, Escherichia and Pseudomonas and Clostridium* as shown in Table 1 [5].

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: Glucose	Sorbitol	Mannitol	Sucrose	Lactose	Maltose	Raffinose	Organisms
Pinkish on MA & green metallic sheen on EMB	Short rod	-	+	-	+	-	+	-	ND	-	+	-	++	++	++	++	++	++	++	*E. coli*
Black on SS agaro	Short rod	-	-	-	+	+	+	-	ND	-	+	-	++	++	++	-	-	++	++	*S. enteritidis*
Colourless on SS agar	Short rod	-	+	+	-	-	-	-	ND	+	+	-	++	++	++	+	-	++	+	*Shigelle sp.*
Yellow on TCBS	Curved rod	-	+	ND	-	+	+	-	ND	+	+	-	+	+	+	_	_	_	_	*V. cholera*
Green on TCBS	Curved rod	-	+	ND	-	+	+	+	ND	+	-	-	+	+	+	_	_	_	_	*V. Parahaemolyticus*
Creamy on Brain Heart infusion	Long rod	+	-	+	-	+	-	+	ND	+	-	+	++	+	+	++	_	_	_	*C. perfringens*
Pinkish on EMB	Rod	-	-	-	-	+	+	+	ND	+	-	-	+	++	++	++	+	+	-	*Pseudomans sp.*
White to deep yellow on MSA	Cocci	+	-	-	-	+	-	+	+	-	+	ND	+	+	-	+	-	-	-	*S. auerus*

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

3.2. 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.

3.3. 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 were recovered from the intestine.

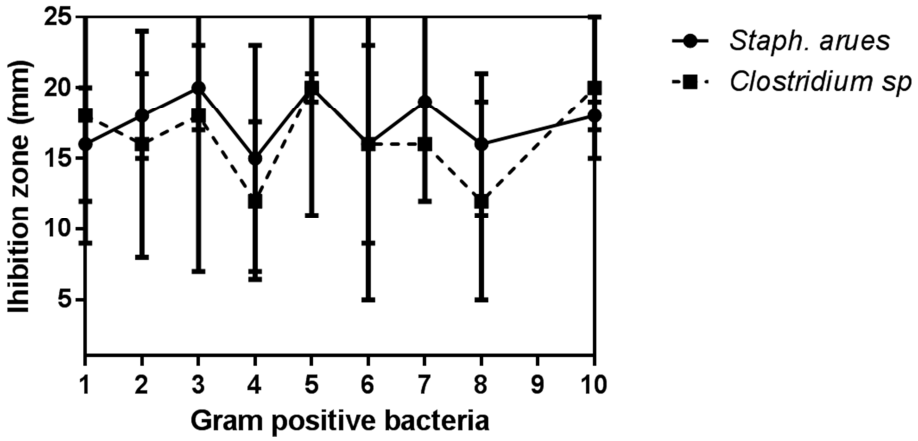

Fig. 1. *Antibacterial susceptibility pattern (error bars) of Gram positive bacteria (mm).*

Resistance = or < 10mm zone of inhibition
Sensitive = or > 15mm zone of inhibition
Legend: 1= CPX, 2 = NB, 3 = CN, 4 = AML, 5= S, 6= RD, 7 = E, 8 = CH, 9 = APX, 10 = Lev

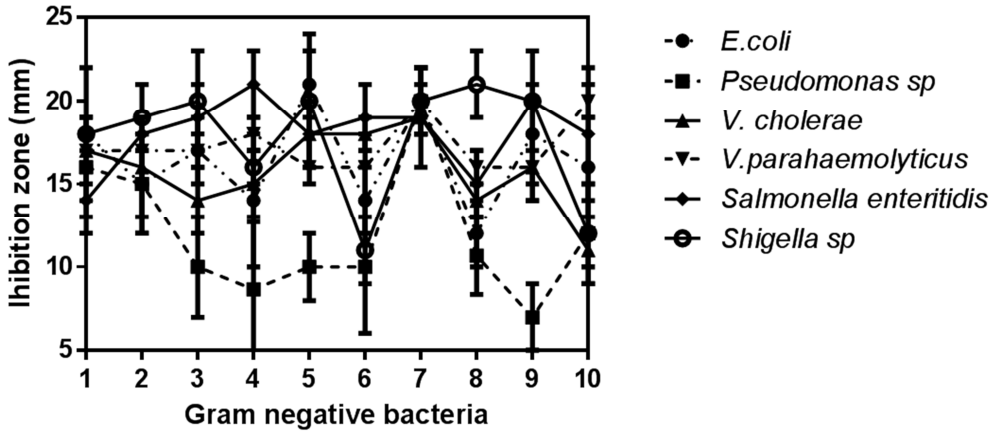

Fig. 2. *Antibacterial susceptibility pattern of Gram negative bacteria (mm).*

Resistance = or < 10mm zone of inhibition
Sensitive = or > 15mm zone of inhibition
Legend: 1= OFX, 2 = REF, 3 = CPX, 4 = AU, 5= CN, 6= S, 7 = CEP, 8 = NA, 9 = SXT, 10 = PN

Table 2. *Number of organisms recovered after dissection of orally infected mice (cfuml^{-1}).*

Organisms	**Liver (10^6)**	**Kidney (10^6)**	**Lung (10^6)**	**Intestine (10^8)**
E. coli	4	4	Nil	10
Clostridium sp.	1	Nil	2	2
V. cholerae	Nil	Nil	Nil	2
V. parahaemolyticus	Nil	Nil	Nil	1
Salmonella enteritidis	Nil	50	Nil	20
Shigella sp.	Nil	3	7	17
Staph. Aureus	2	1	3	8
Pseudomonas sp.	Nil	24	1	25

Table 3. *Number of organisms recovered after dissection of mice infected intraperitoneally (cfuml^{-1}).*

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

4. Discussion

The virulence of any microorganisms is a direct function of its infectious doze otherwise referred to as threshold of infections as well as its size of inhibition zones on in vitro susceptibility assay. The microbial loads and zones of inhibitions produced in the present study were enough to establish such fact. The isolates were characterized and identified as *Shigella sp., Salmonella enteritidis, Pseudomonas sp., Clostridium perfringes, 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 birds and the mice often act as 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 [19]. 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 [20]. 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 [21]. 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 [22].

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 mice 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. As for the mice 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.

5. Conclusion

The present study revealed that some poultry wastes are potential reservoirs of human pathogen and as such should be given proper disposal system. This also 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.

The poultry waste 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.

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