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Histopathological Effects of Enterotoxigenic *Klebsiella variicola* and *Enterobacter* Species Isolated from Iko River- Nigeria

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Abstract: ***The histopathological effect of enterotoxigenic Klebsiellavariicola and Enterobacter species isolated from Iko River- Nigeria was investigated using the rabbit ligated ileal loop assay. The isolates were coded 10, 11A, 12A, 13 and EC6. In a phylogenetic study using the 16S rRNA sequencing, isolates 12A, 13 and EC6 were identified as KlebsiellavariicolaF2R9 [T] (AJ783916), isolate 11A as Enterobacter ludwigiiEN-119 [T] (AJ853891), while 10 was identified to be either Enterobacter asburiaeJCM 6051 [T] (AB004744)/cancerogenus LMG 2693 [T](Z96078). All the isolates were found to be enterotoxigenic by causing fluid accumulation in segments of the rabbit ileum. Fluid aspirated from the ileal loops injected with crude enterotoxin were bloody and histopathological examination of the cut segments showed degeneration of the villi with cellular infiltration and exudates, necrosis and ulceration of the villi with hemorrhage.***

Keywords: Enterobacteria, rabbit ileal loop, enterotoxin, histopathology

1. Introduction

Klebsiella and *Enterobacter* are rod-shaped Gram-negative bacteria (enterobacteria) belonging to the family Enterobacteriaceae. Enterobacteria are ubiquitous in water, soil, decaying vegetation and occasionally food and, in many cases, form parts of the intestinal flora of humans and animals. They constitute the largest group of human pathogens, with the genera *Escherichia, Klebsiella, Enterobacter, Serratia, Citrobacter*and *Proteus* being members of the normal intestinal flora of humans and animals and may be isolated from a variety of environmental sources[8]. They have simple nutritional requirements, with MacConkey agar being used to isolate and differentiate members of this family into two groups: Pink coloured lactose fermenters and pale coloured non lactose fermenters[16]. The family Enterobacteriaceae contains a large number of genera (including *Klebsiella, Enterobacter, Escherichia coli, Salmonella, Shigella, Citrobacter*) which are genetically and biochemically related [16].

Many of these microorganisms which were sometimes dismissed as harmless commensals are today known to be responsible for major health problems worldwide. A limited number of species, including *E. coli, K. pneumoniae, Enterobacter aerogenes, Enterobacter cloacae, S marcescens,* and *P. mirabilis*, are responsible for most infections produced by this group of organisms [8].

2. Literature Review

Phylogenetic analysis of clinical, carriage and environmental isolates classically identified as *K. pneumoniae* demonstrated the existence of three main phylogenetic lineages (phylogroups) called KpI, KpII and KpIII as initially demonstrated based on the sequence analysisof *gyrA* and *parC* genes [3][2] and later by their association with specific families of chromosomal β-lactamase genes [9][6]. Subsequent taxonomic work has proposed the names *Klebsiellavariicola*, for phylogroupKpIII 15] and *K. quasipneumoniae* for phylogroupKpII. As a consequence, the name *K. pneumoniae* should be used only for strains that belong to phylogroupKpI- *K. pneumoniae sensustricto*[12]. It is difficult todistinguish *K.pneumoniae* from *K.quasipneumoniae* and *K variicola* by biochemical tests [15][7].Until now, *K. variicola* and *K. quasipneumoniae* strains have therefore been generally misidentified as *K. pneumoniae* in clinical microbiology laboratories. Currently, these three species can be more reliably differentiated by genotyping methods [3][2][1].

Klebsiella and *Enterobacter* belong to the lactose fermenting enterobacteria known as coliforms which include overt and opportunistic pathogens responsible for a wide range of infections. The increasing incidence of the coliforms and other Gram-negative organisms in diseases reflects in part a better understanding of their pathogenic potential but more importantly the changing ecology of bacterial diseases. The widespread and often indiscriminate use of antibiotics has created drug-resistant Gram-negative bacilli that readily acquire multiple resistance through transmission of drug resistance plasmids (R factors). Also, development of new surgical procedures, health support technology, and therapeutic regimens has provided new portals of entry and compromised many host defenses [8].

The genus *Enterobacter*is more specifically a nosocomial opportunistic pathogen and is sought out to be one of the many key causes for extraintestinal infections next to *E. coli*. A selection of enteric bacteria like *Enterobacter* infect infants, the elderly, and those who are in the terminal stages of other disease or are immune suppressed [11].

*Klebsiella,Enterobacter,*and *Serratia* species are frequent causes of bacteremia at some medical centers and also are frequently involved in infections associated with respiratory

tract manipulations, such as tracheostomy and procedures using contaminated inhalation therapy equipment. However,*Klebsiella* and *Serratia* species commonly cause infections following intravenous and urinary catheterization and infections complicating burns [8].

Enteropathogenic or enterotoxigenic bacteria have developed multiple strategies to counteract the natural or acquired host defenses, and to cause disease, either from accidental introduction of environmental bacteria into the host or from a long-term adaptation of an environmental bacterium to the new survival conditions of the digestive tract [14]. A variety of vehicles have been implicated in the spread of nosocomial pathogens. For example, *Klebsiella, Enterobacter*, and *Serratia* species have all been recovered in large numbers from hospital food, particularly salads, with the hospital kitchen being a primary source [8].

3. Materials and Methods

Culture and Isolation of microorganisms

The enterobacteria were isolated during a study to enumerate the coliform counts in water samples using the membrane filtration and most probable number (MPN) methods. The isolates were picked and maintained on nutrient agar slants prior to their identification using the 16S rRNA sequencing.

Preparation of isolates for enterotoxin production

The isolates were cultured for enterotoxin production as described by Ezurike*et al*., (2007) [5], using tryptic soy broth (Hardy diagnostic, USA). 10^5 cells per ml of the isolates were inoculated into 10 ml sterile tryptic soy broth and incubated at 37^0C for 48 h. This was thereafter centrifuged at 5000 rpm for 15 min. Cell free supernatants were then filter sterilized using a 0.22 µm membrane filters and the cell free filtrates used as crude toxin preparation.

Screening for enterotoxigenicity

The different enterobacteria were subjected to enterotoxin production test using the ligated ileal loop assay [10][5]. This was done using 6 to 8 weeks old rabbits. The rabbits were starved for 24 h before challenge while water was given *ad libitum*. They were thereafter anaesthetized with ketamine injection and chloroform but secured in dorsal recumbancy. Following a midline insicion, the ileum of the rabbit was tied into loops of 5 cm in length. With sterile syringes, 0.5 ml of each sterile crude enterotoxin was injected into the loops while one loop was inoculated with sterile saline to serve as control. The loops were put back and the incision was sutured. The rabbits were sacrificed after observation for 7 h and the ileum examined for fluid accumulation. Following the fluid aspiration, the dilatation index (DI) was calculated as the ratio of the volume of fluid to the length of the loop. A DI of ≥ 0.2 was adjudged positive.

Histopathological studies

a) **Tissue processing**: The sections were cut and each placed in 10% neutral buffered formaline inside bijou bottles for fixation.Fixation was carried out for 24-48 h prior to processing of the fixed tissues by dehydration in a series of ascending ethanol concentrations (70%, 80%, 90%, and absolute ethanol) for 4 min. They were thereafter dealcoholized and cleared in xylene for 30 min before being embedded in paraffin for another 30 min. The tissueswere cut with micro-tome machine into tiny sections of 4 µ in thickness for staining.

b) **Haematoxylin and eosin staining:** The cut sections were dewaxed in xylene and hydrated through descending ethanol concentration (absolute, 90%, 80%, 70%) to clear water. The tissues were then stained with haematoxylin (cole's) for 10 min, rinsed in water and differentiated in 1% acid alcohol. They were rinsed in water and blued in tap water for 5 min before staining with eosin for 30 sec [13].

4. Results and Discussion

The five enterobacteria were found to be enterotoxigenic with the fluid accumulation that occured in the ileal loops (figure 1). The volume of the fluid ranged from 0.2 to 0.3 and the ileal segments fluid were found to be bloody due to hemorrhage. Histopathological effects of the enterotoxin on loops inoculated with the crude enterotoxin ranged from degeneration of the villi with cellular infiltration and exudates on the mucosae to necrosis and ulceration of the villi with hemorrhage (figures 4 and 5). However, the control segment inoculated with sterile saline showed intact mucosa with prominent villi separated by crypts (C) and lined by simple columnar epithelium with intact basement membranes (figure 3).Although this work seems to be among the first to carry out enterotoxin production by some other enterobacteria [*K. variicola* F2R9 ^{T} (AJ783916), *E. ludwigii*EN-119 ^{T} (AJ853891), *E. asburiae*JCM 6051 ^{T} (AB004744)/*cancerogenus* LMG 2693 ^{T} (Z96078)] other than *Escherichia coli,* the histopathological changes observed in this study were similar to the histologic changes induced by staphylococcal enterotoxins (SEs) as reported in Ezurike*et al*., (2007)[5].

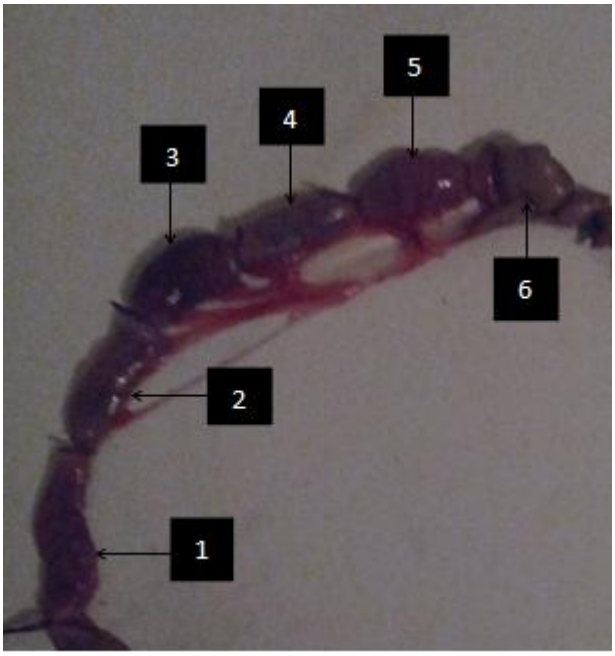

Figure 1: Ligated segments of rabbit ileum after injection with crude enterotoxin preparations from different enterobacteria. 1 = section inoculated with EC6, 2 = section inoculated with isolate 10, 3 = section inoculated with isolate 11A, 4 = section inoculated with isolate 12A, 5 =

section inoculated with isolate 13, 6 = section inoculated with sterile saline. Segments with DI of ≥ 0.2 were adjudged positive [10][5]..

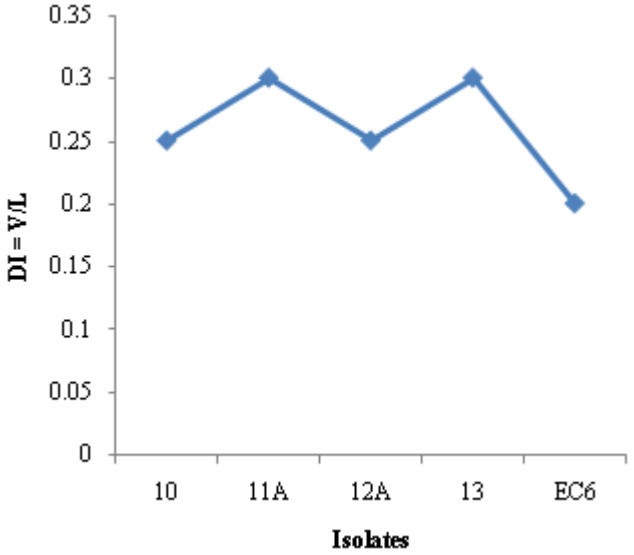

Figure 2: Enterotoxin production by the isolated enterobacteria

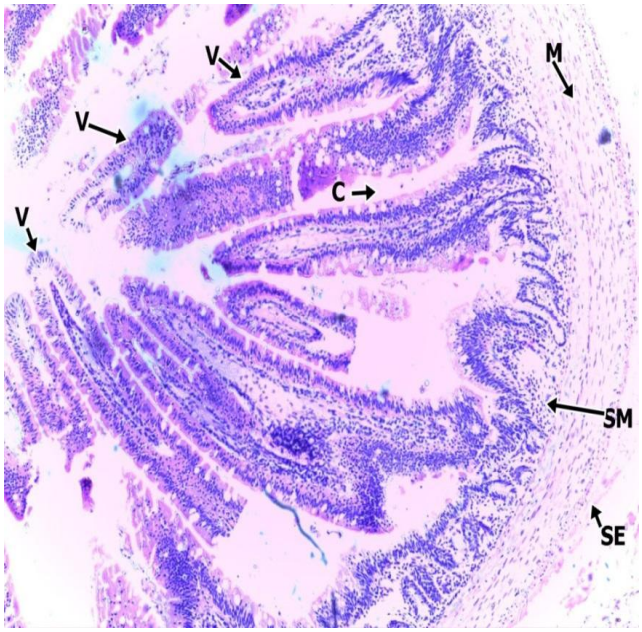

Figure 3: Section of control rabbit ileum showing prominent layers consisting of the serosa (SE), muscular coat (M), submucosa (SM), and the mucosa. The mucosa consists of prominent villi separated by crypts (C) and lined by simple columnar epithelium with intact basement membranes, x 100.

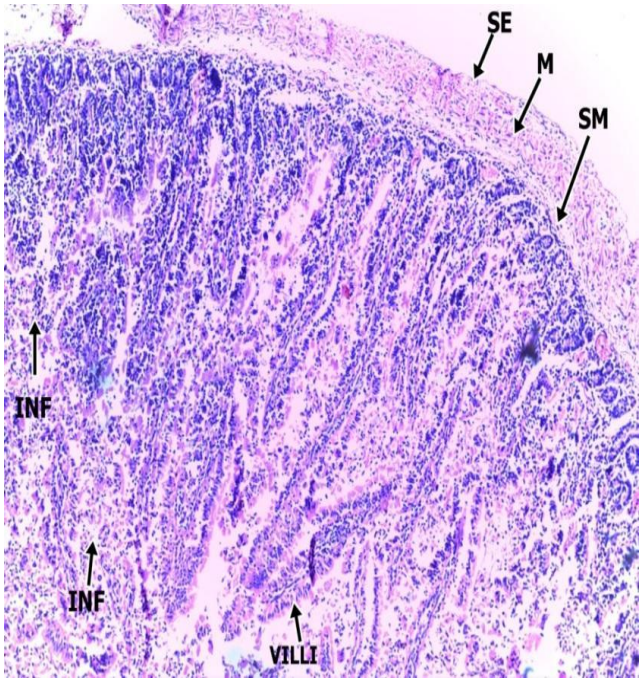

Figure 4: Section of the rabbit ileum injected with crude enterotoxin showing degeneration of the villi (villi arrow) with cellular infiltration and exudates (INF arrow) on the mucosae. x 100.

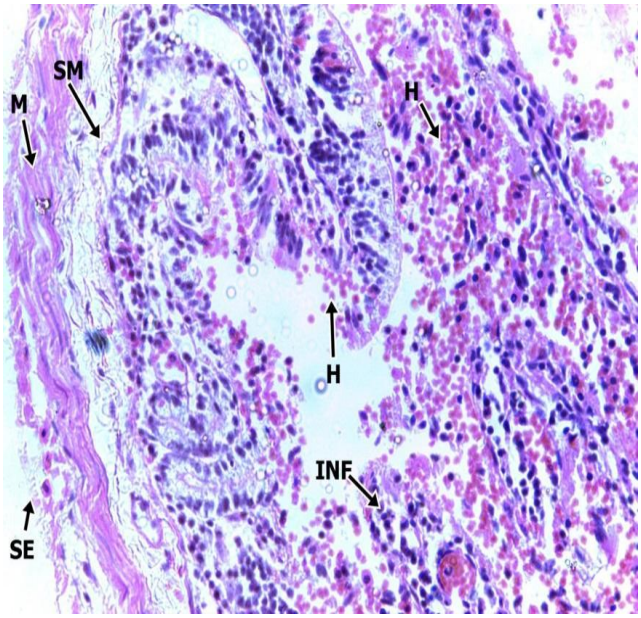

Figure 5: Section of the rabbit ileum injected with crude enterotoxin showing cellular infiltration(INF), necrosis and ulceration of the villi with hemorrhage (H with arrow).x 400.

5. Conclusion

It has been reported in Guentzel, (1996) thatenterotoxigenic strains of *Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter*have been isolated from infants and children with acute gastroenteritis and the enterotoxins of some of these organisms are of the heat-labile and heat-stable types and have other properties in common with the *E. coli* toxins. In this study, it has been confirmed as reported that other members of the enterobacteria other than the popular *E. coli*

can also be enterotoxigenic with some histopathological effects as seen with *Klebsiellavariicola*F2R9 ^{T} (AJ783916), *Enterobacter ludwigii*EN-119 ^{T} (AJ853891), *Enterobacter asburiae* JCM 6051 ^{T} (AB004744)/*cancerogenus*LMG 2693 ^{T} (Z96078).

6. Recommendation

According to Brisse and Verhoef, (2001)[3], and Rosenbluet*het al*., (2004)[15], *K. variicola* has been underreported in the literature due to the difficulty of distinguishing *K. variicola* from *K. pneumoniae* by classical methods used in clinical laboratories for species determination of the genus *Klebsiella*. Also previous investigations have shown that approximately 20% of human isolates thought to be *K. pneumoniae* are in fact *K. variicola*/KpIII or *K. quasipneumoniae*/KpII [2][15]. This therefore shows the need for a better way of identifying the genus *Klebsiella* to the species level other than the classical methods used in most laboratories.

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