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Identification of Changes During Infection with Gelatinase-Producing and Gelatinase-Defective Strains of *Enterococcus faecalis* Using Live-Animal Model

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Abstract: Haematological, enzymatic and histopathologic changes during gelatinase positive (gel^+) *Enterococcus feacalis* infection was assessed in an animal (albino rat) model using standard methods. The role of gelatinase in post-enterococcaemia was established. White Blood Cell (WBC) count, Packed Cell Volume (PCV) and platelets were significantly reduced (at $p \geq 0.05$) in gelatinase positive (gel^+) than in gelatinase-negagive (gel^-) compared to the controls. The enzymes Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Acid Phosphatase (ACP) and Alkaline Phosphatase (ALP) showed the following values 105, 43, 39.39 and 102.63 IU L^{-1}, respectively for the gel^+ infected animas, 108, 57, 164.6 and 428.94 IU L^{-1}, respectively for gel^- and 108, 67, 77.77 and 202.63 IU L^{-1}, respectively for the control. The results obtained for the bilirubin test were 18.5 mg dL^{-1}, total bilirubin and 7.83 mg dL^{-1} conjugated bilirubin for gel^+ infected animas, total and conjugated bilirubin recorded 7.4 and 2.46 mg dL^{-1}, respectively in gel^- infected animas and 5.55 and 4.92 mg dL^{-1}, respectively in the control. Histopathological changes within the individual groups varied and overall changes were less extensive than observed in animals infected with gel^+ *E. faecalis*. Thin section showed an overall loss of structural integrity. The results show areas of pronounced haemorrhage, necrosis with bacterial clusters and distortion in morphology. There was a striking difference in the severity of lesions between gel^- and gel^+ infected animal. However, in an intraperitoneal rat infection model, gel^+ strain was relatively less pathogenic. These findings highlight the importance of gelatinase as a pathogenic factor and are likely key determinants important to pathogenesis of pathogens.

Key words: Gelatinase, *Enterococcus faecalis*, infection, aminotransferase, bilirubin, phosphatase

INTRODUCTION

Infections caused by *Enterococcus faecalis* include bacteraemia, endocarditis, urinary tract and intra-abdominal infections (Dalal *et al*., 2008). These infections are therapeutically challenging (Gomes *et al*., 2008; Drobni *et al*., 2009). Enterococcaemia could be as a result of translocation across intact intestinal epithelial, intravenous lines (Gentry-Weeks *et al*., 2003), abscesses and urinary tract infections (Moy *et al*., 2006).

The risk factors for mortality associated with enterococcal bacteraemia include severity of illness, patient age and use of broad spectrum antibiotics (Drobni *et al*., 2009). Some of the enterococcal strains (45-68%) produce gelatinase which is an extra-cellular zinc-containing metalloproteinase (Roberts *et al*., 2004). Gelatinase can hydrolyze gelatin, collagen, fibrinogen, casein, haemoglobin, insulin, sex pheromone-related peptides and some other bioactive peptides (Nakayama *et al*., 2007). It is also responsible for inflamed pulps and periapical lesions in oral infections (Roberts *et al* ., 2004). Gelatinase has played an important role in the pathogenicity of most pathogenic bacteria.

The enzyme has been associated with disease progression due to its cytotoxic and tissue-destructive potential and inhibitory effects on phagocytes (Singh *et al*., 2005). Gelatinase production and activity are higher in clinical than faecal isolates from healthy volunteers (Engelbert *et al*., 2004; Zeng *et al*., 2005). This study investigated the enzymatic, haematological and histopathologic changes during gelatinase-positive *E. faecalis* infection in the animal model.

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MATERIALS AND METHODS

Source of organisms: The bacteria used in this study were collected from the Department of Microbiology, University of Ado-Ekiti, Nigeria. They included *Enterococcus faecalis* DMOF 0237 (Gelatinase-producing, gel^+) and *Enterococcus faecalis* DMOF 0093 (Gelatinase-defective, gel^-) strains. Identification was based on the protocol established by Bauer *et al.* (1998).

Source of experimental animals: Forty five 4 weeks old female albino Wistar rats were obtained form the Pre-clinical Animal House, University of Ibadan, Nigeria. The animals were acclimatized for 5 days and fed with grower's mash (TopFeed®) and adequately supplied with distilled water *ad libitum*. The animals were randomly assigned (in cages) into three groups: gel^+, gel^- and a control. Each group was made up of 15 rats.

Inoculation of experimental animals and establishment of enterococcaemia: An overnight culture of the isolates was grown in peptone water. The culture was concentrated by centrifugation at 14, 636 g for 15 min at 26°C. The bacterial pellet was re-suspended in 2 mL of normal saline to a final concentration of 10^9 cfu mL^{-1}. The bacterial suspension was diluted to 5.0×10^5 mL and 100 μL.

The rats were challenged intraperitoneally as described by Singh *et al.* (1998) and control group was injected with the same volume of sterile normal saline.

Enterococcaemia in the experimental animals was examined in the blood samples of the representative rats of each group. Blood was collected from each group, plated onto bile aesculin agar and inoculated at 37°C and observed for growth.

Assessment of haematological parameters: Haematological changes were determined after 7, 15 and 21 days of infection. The Packed Cell Volume (PCV), Red Blood Cell (RBC), White Blood Cell (WBC), platelets, Haemoglobin (Hb), Mean Cell Haemoglobin Concentration (MCHC), Mean Cell Volume (MCV) and Mean Cell Haemoglobin (MCH) were determined using the methods described by Cheesbrough (2003).

Determination of enzyme activity: Alkaline Phosphatase (ALP) and Acid Phosphatase (ACP) were monitored according to Bergmeyer and Bernt (1974) while Aspartate Transaminase (AST) and Alanine Transaminase (ALT) were determined using Randox® enzyme kits.

Tissue processing for histopathological studies: The experimental animals were sacrificed 3 weeks after challenge; tissue specimens were collected and processed for histopathological studies using the method of Gentry-Weeks *et al.* (2003). Microscopic examination of the tissues was carried out as described by Baker and Silverton (1998). The slides were examined for pathological changes with Leitz microscope (Lux, Germany) with camera attachment.

RESULTS AND DISCUSSION

The role of gelatinase in post enterococcaemia was demonstrated in the results obtained from this study. The WBC count, PCV platelets and RBC count were significantly lower (at $p\geq0.05$) in the experimental animals than in the control. The White Blood Cell count (WBC) showed a drastic reduction within the 1st 3 weeks in rats challenged with gel^+ (8000-5200 mm^{-3}) compared to both the control (11500-12,000 mm^{-3}) and gel^- group (8000-8600 mm^{-3}) (Table 1).

The platelet counts also showed a drastic reduction in animals that received both gel^+ and gel^- strains. Increase in WBC is associated with bacterial infections which may also result from leukemia, tissue necrosis and proteolytic activity of cell (Zeng *et al.*, 2005).

The differences in WBC both in the control the controls and the experimental groups (gel^+ and gel^-) is shown in Table 2. The WBC which was drastically lower (47 and 43.3% in week 2nd and 3rd, respectively) in animals challenged with gel^+ *E. faecalis* may be due to cytotoxic effect indicative of the tissue-destructive

Table 1: Haematological changes in rats during enterococcal infection

	Treatment groups								
	Gel^+			Gel^-			Control		
Parameters	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3
WBC ($\times10^3$ mm^3)	8.00	6.40	5.20	8.00	8.50	8.60	11.50	12.00	12.00
Platelets ($\times10^4$)	7.00	5.50	5.00	6.20	5.80	6.00	6.00	7.40	7.50
Packed cell volume (%)	42.00	38.00	46.00	40.00	38.00	51.00	38.00	48.00	47.00
Heamoglobin (g dL^{-1})	11.32	11.89	13.20	12.03	12.97	14.20	10.20	11.20	11.60
RBC (10^6)	8.40	8.02	2.10	9.80	7.50	2.80	2.00	2.00	2.20
MCHC (g L^{-1})	0.27	0.31	0.29	0.30	0.34	0.28	0.27	0.23	0.25
MCV (10^{12} L)	5.00	4.74	21.90	4.08	5.07	18.21	19.00	24.00	21.36
MCH (10^{12} L)	1.35	1.48	6.29	1.23	1.73	5.04	5.10	5.60	5.27

WBC = White Blood Cell, RBC = Red Blood Cell, MCHC = Mean Cell Hemoglobin Concentration, MCH = Mean Cell Hemoglobin, MCV = Mean Cell Volume

Table 2: Difference in WBC count in gel$^+$ and gel$^-$ *Enteroccus faecalis*

Weeks	Gel$^+$ (%)	Gel$^-$ (%)
1	26.1	26.1
2	46.7	29.2
3	43.3	28.3

Table 3: Changes in marker enzymes during enterococcal infection in rats

	Treatment groups								
	Gel$^+$			Gel$^-$			Control		
Parameters (U L^{-1})	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3
AST	133.40	144.45	108.11	148.54	89.45	91.72	120.02	109.75	105.38
ALT	94.80	72.77	67.00	77.34	70.92	57.54	48.03	52.44	43.73
AST/ALT	1.41	1.99	1.61	1.92	1.26	1.59	2.50	2.09	2.41
ACP	67.05	69.21	40.99	56.37	37.53	64.63	45.75	56.02	39.39
ALP	92.59	70.02	101.83	64.25	81.34	208.45	109.05	89.57	102.63
Bilirubin									
Total	7.04	5.04	5.55	7.45	14.58	18.50	4.95	7.01	5.55
Conjugated	5.69	4.68	4.92	7.45	4.62	7.35	3.61	6.03	4.92

AST = Aspartate Transaminase, ALT = Alanine Transaminase, ALP = Alkaline Phosphatase, ACP = Acid Phosphatase

potential of gel$^+$, bone marrow depression or the inhibitory effects of gelatinase (Roberts *et al*., 2004). The reduction in WBC by 28% in week 2nd and 3rd observed animals challenged with gel$^-$ may contribute to the overwhelming effects of enterococcal bacterial infection (Cheesbrough, 2003). The gel$^+$ treated animals recorded the least amount of PCV, haemoglobin concentration and RBC count with the values of 46%, 13.2 g dL^{-1} and 2.1×10^6 mm^{-3}, respectively.

However, the values of PCV, haemoglobin concentration and RBC count were lowest in the control animals with 47%, 11.6 g dL^{-1} and 2.2×10^3 mm^{-3}, respectively. Singh *et al*. (2005) indicated that a low value of RBC result from the tissue destructive potential of gelatinase and its cytotoxic nature. Hence, it could be involved in the lysis of the RBC leading to the anaemic condition observed in the gel$^+$ treated animals group (Cheesbrough, 2003).

Both gel$^+$ and gel$^-$ *E. faecalis* strains recorded a very low MCV and MCH in the 1st 2 weeks of the study, indicating microcytic. The values of the parameters latter increased with time. For MCHC there was an initial increase after which there was a decrease. The control group was lower than the value in the experimental groups.

Reduction in the MCHC level indicates hypocromia resulting from decreased heamoglobin concentration (EMDEX, 2007).

Table 3 shows the levels of various enzymes assay in the blood of the experimental animals. The four enzymes AST (GOT), ALT (GPT), ACP and ALP investigated had 105, 43, 39.39 and 102.63 IU L^{-1}, respectively for gel$^+$ treated animals, 108, 57, 164.6 and 428.94 IU L^{-1}, respectively for gel$^-$ and 108, 67, 77.77 and 202.63 IU L^{-1}, respectively for control animal group. ALT is found principally in the liver and when liver cell damage occurs, the serum or plasma levels of the enzyme are raised. AST test is to investigate liver diseases and

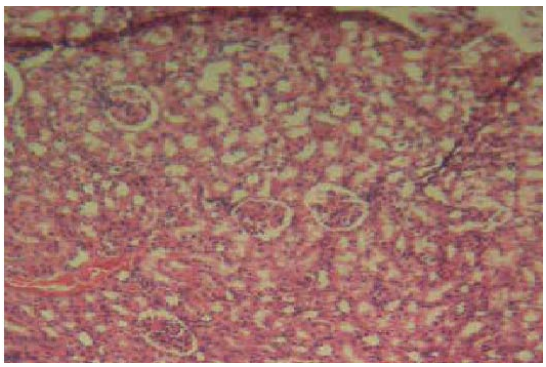

Fig. 1: Section showing well preserved tissue morphology with no areas of haemorrhagic or inflammatory changes in control group (X400)

myocardiac infarction. It is also a useful indicator of myocardiac damage (Adanlawo, 2003). Other causes of raised AST levels include diseases involving cellular injury such as severe bacterial infections (Worobetz *et al*., 2005). The results of the bilirubin test carried out showed TB 18.5 and CB 7.38 mg dL^{-1} for the gel$^+$ and gel$^-$ treated animals, respectively. High deconjugated bilirubin usually results following bacterial infection. Partial or complete stopping of bile flow as a result of the accumulation of CB in the blood initiates obstructive jaundice. *Enterococcus faecalis* has been reported to grow in the bile (Franz *et al*., 1999). Increase in both total and deconjugated bilirubin could lead to cell damage and affect the bile.

Histopathological effect on the liver, heart, spleen and kidney specimens of experimental animals are shown in Fig. 1-12. The plates were examined because histopathological changes within the individual groups varied and overall changes were less extensive than those observed with gel$^+$ *E. faecalis* or more virulent organisms

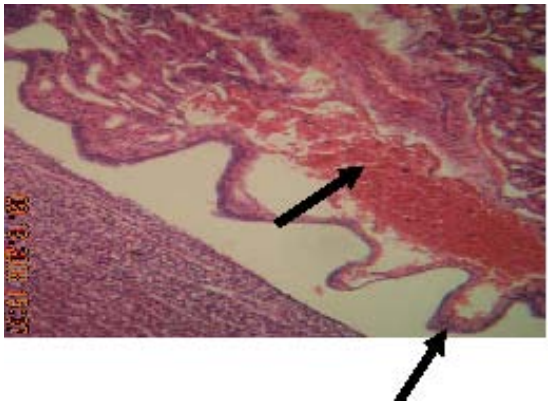

Fig. 2: Kidney tissue of albino rats injected with gel$^+$ strain showing areas of pronounced haemorrhage and distortion of the tissue morphology (X400)

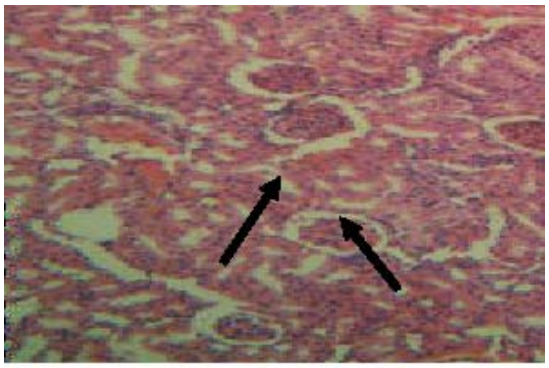

Fig. 3: Kidney tissue of albino rats injected with gel$^-$ strain showing areas of pronounced haemorrhage and distortion of the tissue morphology (X400)

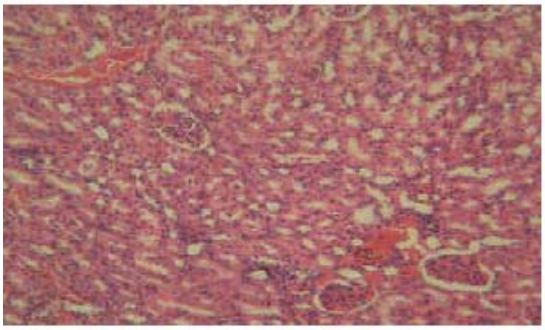

Fig. 4: Liver tissue of albino rats (Control group) showing normal tissue appearance (X400)

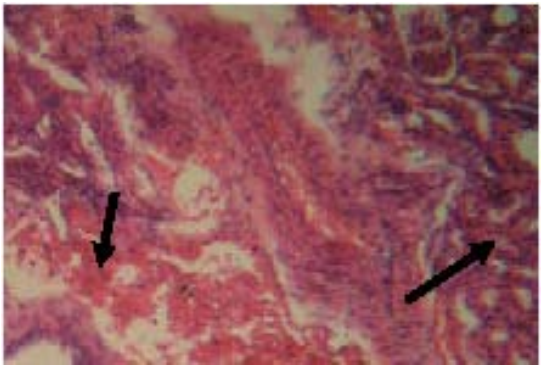

Fig. 5: Liver tissue of albino rats, injected with gel$^+$ strai of *E. faecalis* showing pronounced renal corpuscles and areas of inflammatory changes (X400)

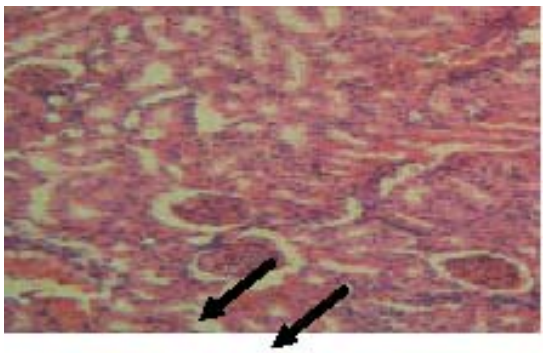

Fig. 6: Liver tissue of albino rats, injected with gel$^-$ strain of *E. faecalis* showing well preserved renal corpuscles and less pronounced areas of inflammatory changes (X400)

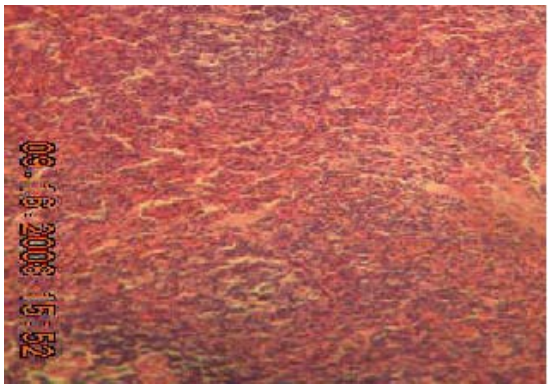

Fig. 7: Section shows normal morphological appearance of spleen of control animal with no areas of haemorrhagic features (X400)

such as *S. aureus* (Antalek *et al*., 1995; Sifri *et al*., 2003). Thin-section histopathology of the specimen showed some extra-cellular matrix and inflammatory cells which clearly discerned infection with *E. faecalis* showed marked decreased in the density of the inner and outer nuclear layers and mild polymorphonuclear. This may be

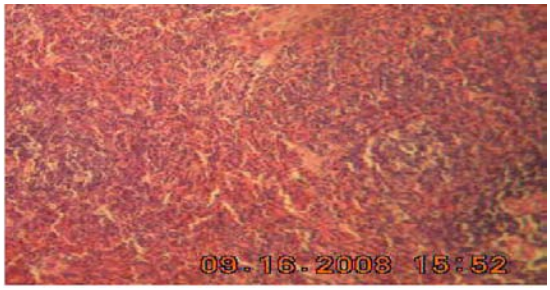

Fig. 8: Section shows pronounced areas of inflammation and the distortion of the morphological appearance of spleen of rat injected with gel$^+$ strain (X400)

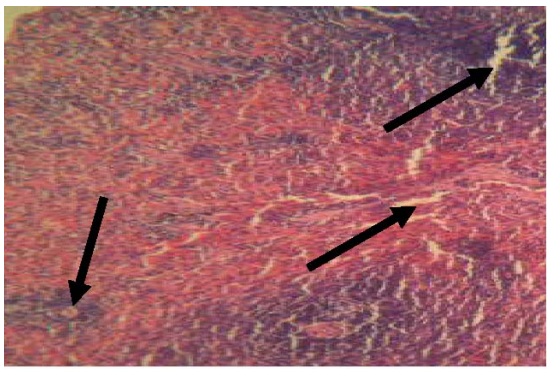

Fig. 9: Section shows areas of inflammation and the distortion of the morphological appearance of spleen of rat injected with gel$^-$ strain (X400)

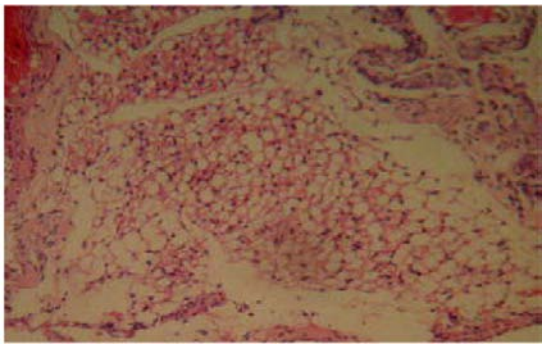

Fig. 10: Heart tissue of albino rats (control group) showing normal tissue appearance (X400)

as a result of infiltrate and overall loss of structural integrity (Adanlawo, 2003; Kawalec *et al*., 2005). The histopathological results showed areas of pronounced haemorrhage, necrosis and V distortion in morphology of the organs' tissue which was more pronounced in the gel$^+$ than the gel$^-$ animal group. Liver sections from gel$^+$

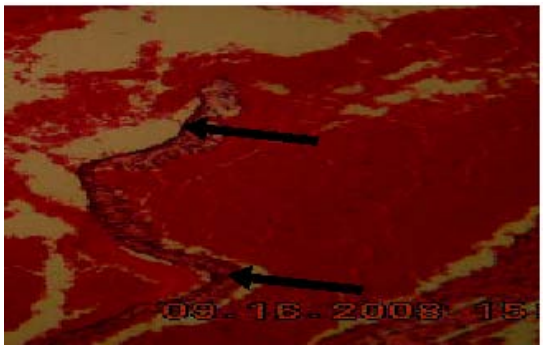

Fig. 11: Heart tissue of albino rats injected with gel$^-$ strain showing areas of pronounced haemorrhage and distortion of the tissue morphology (X400)

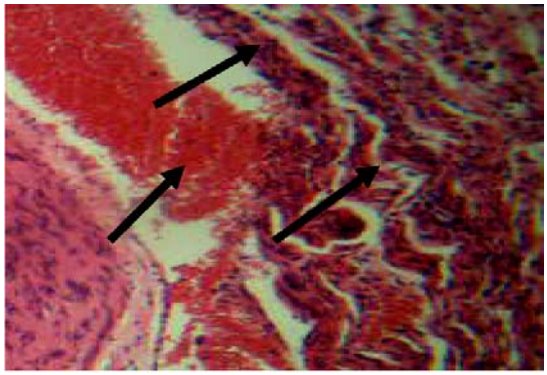

Fig. 12: Heart tissue of albino rats injected with gel$^+$ strain showing section of less haemorrhage and tissue distortion (X400)

rat after infection with *E. faecalis* produced numerous multifocal coalescing cells due to aggregates of leukocytes and caseous which may be a result of the assault on the kupffer cell (Nakayama *et al*., 2002). Histopathological lesions observed in the kidneys in the severity between gel$^-$ group and gel$^+$ treated animals group. Infection of the kidneys is associated with rat morbidity but is not a prognostic indicator of relative mortality (Akanji, 1986; Jha *et al*., 2005). The major differences in pathological lesions were observed between gel$^+$ and gel$^-$ infected rats which agree with earlier reports by Maadani *et al*. (2007). Bacterial clusters were on the internal limiting membrane. This may be responsible for the overall loss of structural integrity (Bourgogne *et al*., 2007). In contrast, most of the animals and heart of the infected rats showed a striking difference injected with gel$^-$ showed only a mild polymorphonuclear infiltrate, preserved structure of all

layers and inflammatory infiltrate; features which were similar to those of normal saline-injected control rats, this may be as a result of pathogenic factor, gelatinase (Worobetz *et al*., 2005; Arias *et al*., 2007).

CONCLUSION

This study shows that infection with gel^- resulted in less severe histopathological changes after 21 days of infection than those observed in the animals infected with gel^+, showing that gelatinase has contributed greatly to the severity of enterococcal infection.

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