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HISTOPATHOLOGICAL CHANGES INDUCED BY STAPHYLOCOCCAL ENTEROTOXIN PRODUCED IN YOGHURT

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ABSTRACT

In this study, six Staphylococcus aureus strains isolated from contaminated yoghurt were evaluated for enterotoxigenicity. Two of the strains were enterotoxigenic and caused fluid accumulation in rabbit ileal loops. Fluid aspirated from the loops was bloody and histopathological changes in sections collected from rabbit ileum, inoculated with crude enterotoxin, were characterized by circulatory disturbances, degenerative/ necrotic and inflammatory changes, including hyperaemia, fibrinous exudation and necrosis of villi epithelial cells. These findings showed that although SE are typically associated with vomiting and diarrhoea, which often abate within 24 hours, there was potential for more serious disturbances such as inflammation, tissue damage and toxic shock. Moreover, the production of potent SE by strains isolated from commonly consumed products such as yoghurt emphasizes the need for complete elimination of staphylococcal contaminants from foods in order to protect consumers.

Keywords: *Staphylococcus aureus,* Enterotoxins, Hyperaemia, Histopathological changes, Necrosis of villi

INTRODUCTION

Staphylococcal foodborne intoxication has been reported to be one of the most common bacterial foodborne outbreaks in many countries (Balaban and Rasooly, 2000; Adwan *et al*., 2005). A survey of sixteen European countries implicated *Staphylococcus aureus* in 1 – 13% of all reported foodborne disease outbreaks and in the United States there were 42 documented outbreaks due to *S. aureus* between 1993 and 1997 (Mead *et* al., 1999; ECHCPD, 2003). The entrotoxicosis typically occurs 30 min to 8 h after ingestion of heat-stable staphylococcal enterotoxins (SEs) synthesized in food by enterotoxigenic strains of coagulase-positive staphylococci (CPS), mainly *S. aureus* (Holeckova *et al*., 2002; Le Loir *et al*., 2003).

The staphylococcal enterotoxins are a group of pyrogenic, heat-stable exotoxins with molecular weights of 25 to 29 KDa (Holeckova *et* al., 2002; ECHCPD, 2003; van Gessel *et al*., 2004). Currently, there are about 14 serotypes of SE described, which are named sequentially by letter (SEA – SEO), in order of discovery (Jarraud *et al*., 2001; ECHCPD, 2003; Le Loir *et al*., 2003). There are no established patterns of SEs production in foods. However, studies have shown a predominance of SEB and SEC producing strains in raw milk, particularly from mastitic cows (ECHCPD, 2003; Kuroishi *et al*., 2003; van Gessel *et al*., 2004; Adwan *et al*., 2005). *S. aureus* are ubiquitous in nature, existing in dust, sewage, water, environmental surfaces, humans and animals body surfaces and may contaminate foods through raw materials or handling during the preparation or manufacturing process. Not all strains produce enterotoxin. Growth and enterotoxin production are subject to a number of genetic and environmental factors. However, once the enterotoxins are produced, they are normally resistant to both heat and digestive enzymes (ECHCPD, 2003; Le Loir *et al*., 2003).

Exposure to SE has been shown to cause a range of clinical abnormalities from gastrointestinal upset to lethal toxic shock syndrome (TSS) (Ellis *et* al., 2003; van Gessel *et al*., 2004).

In this study, six strains of *S. aureus* isolated from contaminated yoghurt products were evaluated for their enterotoxin-producing ability by the ligated rabbit ileal loop assay and histopathological evaluation conducted on the rabbit intestines.

MATERIALS AND METHODS

Isolation and Maintenance of Bacterial Strains: Six *S.* aureus isolates were recovered from contaminated yoghurt samples during a study evaluating the microbiological quality of various yoghurt products. Colonies were selected following morphological and biochemical characterization of colonies resulting from nutrient agar plates inoculated with various yoghurt samples. The *S. aureus* strains were routinely maintained in Nutrient broth and stocks were maintained on nutrient agar slants at 4^{o}C.

Culture of *S. aureus* for Enterotoxin Production: Cultures for enterotoxin production were initially prepared using nutrient broth. Ten milliliter aliquots of sterile nutrient broth, in sterile Bijou bottles, were inoculated each with approximately 10^5 cells per ml and incubated at 37^{o}C for 48 h.

Subsequently, the *S. aureus* strains were cultured in 10 ml of yoghurt, pasteurized by heating to 80°C for 30 min and cultures were incubated as with nutrient broth cultures.

Preparation of Enterotoxin: Following 48 h incubation of the nutrient broth and yoghurt cultures, cell free culture supernatants were collected by centrifugation at 5000 rpm, followed by filtration through 0.22 μm Millex syringe filters. The cell free filtrates were then used as crude toxin preparation.

Assay for Enterotoxin Activity: Assay for enterotoxin was by ligated rabbit ileal loop method (Ike *et al.*, 2005). Briefly, six to nine week old rabbits were starved for 24 h with water supplied *ad libitum*. Each rabbit was anaesthesized with 2 ml of ketamin injection and secured in dorsal recumbency. Following a midline incision, starting from the rectal end, the ileum was divided into segments of 5 cm in length with string ligatures. The crude toxin preparation (0.5 ml) was injected into different segments. Uninoculated broth and sterile saline were injected into some segments to serve as negative controls. The incisions were then sutured and the animals allowed to recover from anaesthesia. After 7 h, the animals were sacrificed and the segments examined for fluid accumulation (dilatation).

For positive loops, the volume of fluid recovered by aspiration was used to determine the dilatation index (DI) estimated as the ratio of volume of fluid to length of ileal segment. A DI $\geq$ 0.2 was taken as positive. The test was done in triplicate animals.

Histopathology: Sections of both normal and enterotoxin-inoculated rabbit ileum were fixed by immersing the cut pieces in 10 % formol saline for 24 – 48 h. Following fixation, the tissues were dehydrated in a series of ascending ethanol concentrations (70 %, 80 %, 90 %, 95 %, and absolute), and then embedded in paraffin. Sections were thereafter cut with a microtome at 5-6 μm. Cut tissues were stained with haematoxylin and eosin and examined microscopically. Photomicrographs were taken.

RESULTS AND DISCUSSION

Of six *S. aureus* isolates tested, two isolates were found to be enterotoxigenic. Cell-free culture supernatants (crude toxin preparations) of the *S. aureus* strains caused fluid accumulation when injected into rabbit ileal segments, indicating enterotoxin activity. Dilatation index (DI) values ranged from 0.2 to 0.57. Moreover, there was a dark-reddish colouration of the positive ileal loops (Figure 1) and the aspirated fluid from such segments appeared bloody. Histopathologic changes in sections collected from the rabbit ileum were characterized by circulatory disturbances, degenerative/necrotic, and inflammatory changes. Sections of the intestine collected from untreated (control) rabbits showed mucosae (including glands) and submucosae with

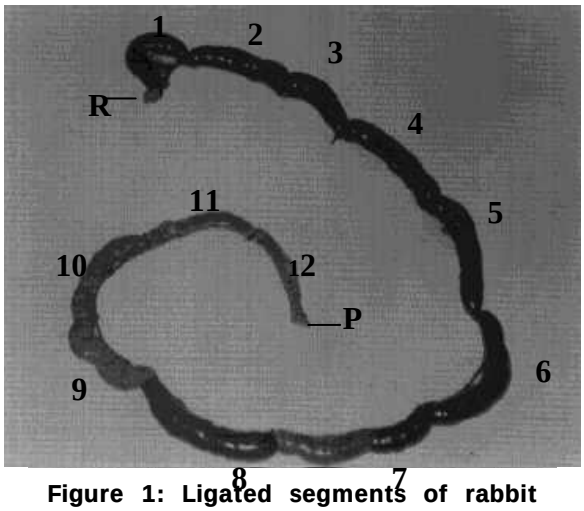

Figure 1: Ligated segments of rabbit ileal loop after injection with crude preparations of staphylococcal enterotoxin (SE) produced under different growth conditions. 1 – 8, SE produced at pH 5; 9 and 10, SE produced at pH 4 – there was change to a brownish colouration but no fluid accumulation; 11, segment inoculated with sterile saline and 12, uninoculated control. R and P, represent the rectal and pyloric ends respectively.

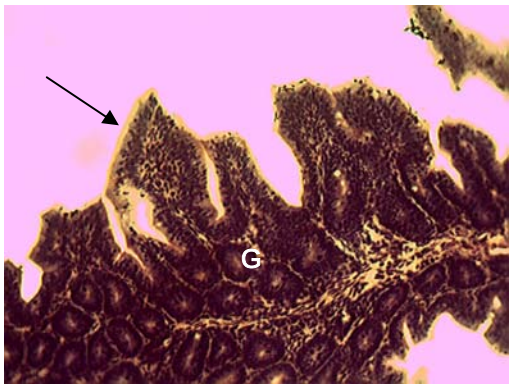

Figure 2: Section of control rabbit ileum showing normal villus (arrow) and intestinal gland (G). H and E stain, x 200.

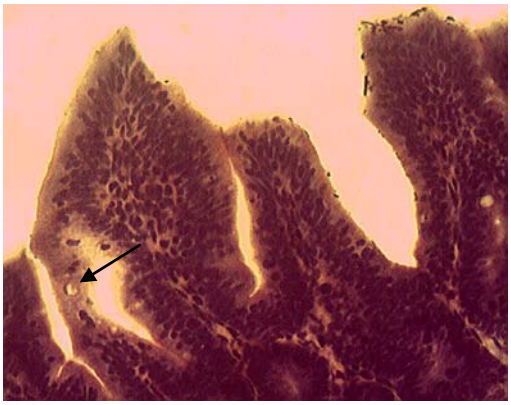

Figure 3: Section of control rabbit ileum showing columnar epithelium lining cells of villi and Goblet cell (arrow). H and E stain, x 400.

normal histomorphology (Figures 2 and 3), while sections from rabbit ileum inoculated with crude toxin preparations showed moderate to severe hyperaemia, with moderate fibrinous exudation onto the intestinal mucosae (Figure 4). In addition, there was distortion of villi epithelium at the tips and necrosis of epithelial lining cells. The intestinal glands were generally normal (Figure 5).

These findings are similar to those reported by other investigators in which various SEs induced varying degrees of inflammatory and degenerative changes. Kuroishi *et al*. (2003), elucidated mechanisms by which SEC induced inflammatory changes in bovine mammary glands. The SEC-inoculated mammary glands exhibited interstitial inflammation, with epithelial cell degeneration and migration of polymorphonuclear neutrophils.

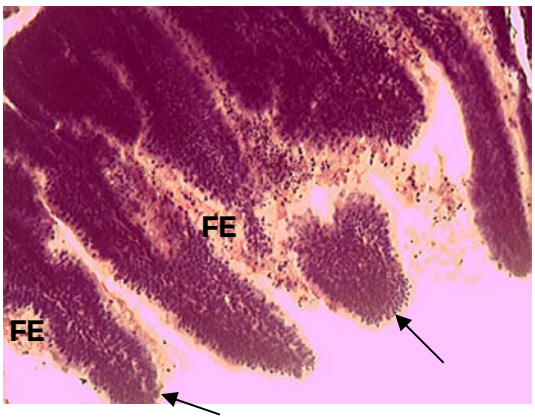

Figure 4: Section of rabbit ileum inoculated with crude staphylococcal enterotoxin, showing fibrinous exudate (FE) on mucosae with degeneration and necrosis of villi epithelial cells (arrow) epithelial cells. H and E stain, x 200.

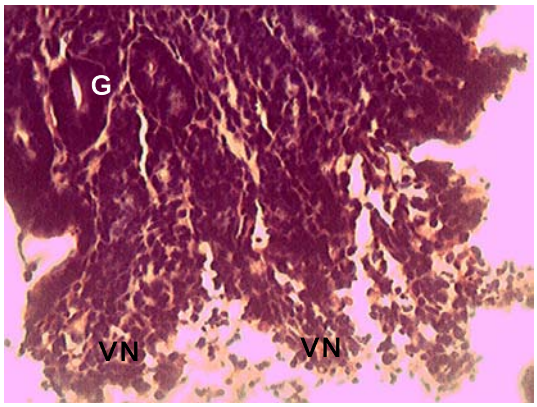

Figure 5: Section of rabbit ileum inoculated with crude staphylococcal enterotoxin, showing necrosis of villi (VN) and normal intestinal gland (G). H and E stain, x 400.

Other studies have shown that exposure to SE can cause a range of clinical abnormalities from gastrointestinal upset to lethal toxic shock syndrome (TSS) and once introduced into host tissues; these toxins have the ability to elicit pathology in different systems (Jett *et al.,* 1990; Greenfield *et al*., 2002). Van Gessel *et al*. (2004) working with piglet models demonstrated lethal SEB intoxication. Clinical signs observed in that study included pyrexia, vomiting and diarrhoea within 4 h, followed by terminal hypotension and shock by 96 h. There has even been reported involvement of SEA in a fatal case of endocarditis (Ellis *et al*., 2003).

The SEs have been reported to act as superantigens (Yokomizo *et* al., 1995; Johnson *et* al., 1996; Ferens *et al*., 1998; Kuroishi *et al*., 2003; van Gessel *et al*., 2004) and have the capacity to cause T-cell proliferation with massive inflammatory cytokine release. Much of the lethal effects of SEs have been attributed to this superantigenic activity (Miethke *et al*., 1992; Jardetzky *et al*., 1994; Johnson *et al*., 1996). However, the more common symptoms of intoxication due to SEs remain those of pyrexia, vomiting and diarrhoea, which often abate within 24 h (Jett *et* al., 1990; van Gessel *et al*., 2004). Consequently, there may be a tendency by the general population to underestimate the health risks posed by formation of SEs in food. However, the findings in this study, show that vomiting and diarrhoea may be only the least of possible health problems that could result from ingestion of preformed SEs with food, particularly in more sensitive individuals such as the very young and very old.

Although our study describes histological changes in a rabbit model, there is documented evidence that the clinical syndromes in some animal models simulate human enterotoxicosis (van Gessel *et* al., 2004). There is, therefore, a need to reassess the importance of preventing the growth of the coagulase-positive staphylococci in foods and their production of enterotoxins.

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