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Whole-Cell Vaccine Versus Toxoid Vaccine

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To compare the efficacy of a whole-cell vaccine prepared out of *Escherichia coli* O157:H7 strain and a toxoid vaccine prepared from modified gene of shiga-like toxin of *Escherichia coli* O157:H7, for having lost its pathogenicity, using rats as animal models. Samples such as ground beef, chicken intestine, raw milk and pasteurized milk was collected and analysed for the isolation and identification of *Escherichia coli* strain by standard microbiological techniques including antimicrobial resistance profiles. Conventional PCR amplification confirmed the presence of five selected virulence genes. A protein was produced out of *stx1* gene variant from an isolate strain of *Escherichia coli* O157:H7 and used as a toxoid vaccine. Prepared vaccine was subjected to its efficacy test using rat models after obtaining proper ethical clearance. After challenging with ATCC and isolates, screening methods emphasised on studying the histopathology effects in liver and kidney on test rats as well as on control rats. Normal histology was seen in liver and kidney specimens of vaccinated and challenged rat groups as well as control un-inoculated unvaccinated rat groups with very few exceptional lesions. However, severe toxic evidences were observed in liver as well as kidneys of rat groups which were unvaccinated and challenged with pathogenic strains. Vaccine is believed to have lost its pathogenicity to a greater extent as validated by animal studies. When comparison was made between whole cell vaccine and toxoid, whole cell vaccine had greater extent of efficiency than toxoid.

Key words: *Escherichia coli* O157:H7, Toxoid Vaccine, Whole-cell vaccine, Haemolytic Uremic Syndrome.

Vaccine regimens which mimic actual infection offer the best opportunity for successful long-term immunoprotection against many bacterial diseases. Preclinical as well as clinical studies and testing strategies have the purpose of limiting risks, evaluating safety and immunogenicity, whenever a vaccine is to be used in humans. This *Escherichia coli O157:H7* is an enterohemorrhagic strain of the bacterium *Escherichia coli* and a cause of food-borne illness. Most illness has been associated with eating undercooked, contaminated ground beef, drinking unpasteurized milk, swimming in or drinking contaminated water, and eating contaminated vegetables. Young children and females had an increased risk of Haemolytic uremic syndrome (HUS) after Shiga-like Toxic producing Escherichia coli (STEC) O157 infection. With or without HUS, elderly persons had the highest proportion of deaths associated with STEC O157 infection. These data support recommendations for aggressive supportive care of young children and the elderly early during illness due to STEC O157[1]. HUS is characterised by three features-acute renal failure, microangiopathic haemolytic anaemia and thrombocytopenia[2]. A close association between

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VTEC and HUS was first reported by Karmali M. A., *et al.* in 1983[3] and studies since then have established that VTEC are a major cause of the typical form of HUS[4, 5]. *Escherichia coli O157:H7* was the causative agent of many out-breaks worldwide. Outbreaks of HC caused by VTEC occurred in different areas of the USA in 1982[6] and since then outbreaks and sporadic cases have been reported in several other countries including Canada, Britain and Japan[7, 8, 9]. Incidence of sporadic cases of hemorrhagic colitis due to *Escherichia coliO157:H7* may be higher than suspected[10]. Hence, an epidemiological outbreak of *Escherichia coli* O157:H7 can be expected in near future and a constant prevalent study has to be conducted throughout the world.

METHODS

Isolation and Identification of Strains

Around 500 samples were collected in a 2 years' time from five different sources such as small intestine of chicken, ground beef, cattle faeces, raw milk and pasteurized milk, and brought to the laboratory with standard care. ATCC strain was obtained from Madras Veterinary College, Chennai, India. Identification of *Escherichia coli O157* was done by performing the routine microbiological tests, such as Gram staining, motility, catalase test, oxidase test, carbohydrate fermentation and culture characterization. Also, latex agglutination test for confirmation of O157 strain was done by using "LK13 HiE.coli™ 157 Latex Test Kit". Apart from general characterization, antibiotic sensitivity tests were performed to determine antimicrobial resistance profiles.

Genotypic confirmation:

Conventional PCR amplification confirmed the presence of five selected virulence genes, namely Intimin (*eae*), Shigatoxin1 (*stx*)1, Shigatoxin2 (*stx2*), Hemolysin (*hlyA*) and Flagellar antigen (*fliCh7*) genes. Oligonucleotide primer sequences used for PCR amplification was derived from a study conducted by EL-Jakee J. *et al.,* in Egypt in 2009[11].

Toxoid Vaccine Preparation and In-Vitro Studies:

A protein vaccine was produced from genetically modified gene of *Escherichia coli* O157:H7. Stx1 gene from *Escherichia coli* O157:H7 was PCR amplified and sequenced. Cloned in *E.coli* BL21 strain using TA vector. Cloned in Expression vector using LIC alicator kit and proteins were expressed. Expressed proteins were purified using Ni-Nta column and confirmed by SDS-PAGE. Cultured vero cell lines were infected with this purified protein (stx1 toxin) and CPE observed. Inactivated toxin produced by altering stx1 gene (restriction digested by selected duet restriction site oriented enzyme, namely Xho1 enzyme), which was also cloned and expressed similar to cloned *stx1* gene. Hence, purified and infected in vero cells too. Cytopathic effects shown by stx1 toxin when infected in vero cell lines were shown to have lost its pathogenicity when compared with stx1 toxoid infected vero cell lines[12]. Further, modelling the genes (before and after alterations) using swiss-model software and validation by Saves v4 software tools proved the inactivation of active site of toxin at in-vitro level[13]. Prepared vaccine was further subjected to its in-vivo efficacy test using laboratory animals.

Whole-Cell Vaccine Preparation:

A whole cell formalin killed vaccine was produced out of *Escherichia coli* O157:H7. Jan Holmgren *et al*., 2003[14], disclosed a method for producing a formalin-killed *E. coli* bacterial strain for use in a vaccine against enteric infection caused by *E. coli* bacteria in humans. *E. coli* bacterial strain was grown in liquid culture medium with vigorous agitation to a predetermined density, *E. coli* bacterial strain was harvested, and harvested *E. coli* bacterial strain was resuspended in saline. Formalin added to harvested, resuspended bacterial strain to a final concentration of 0.2M formaldehyde. Incubated at 37 C under conditions of continuous agitation for about 2 hours, further incubated formalin-treated bacterial strain at 4 C for about 24-48 hours, thereby obtaining a formalin-killed *E. coli* bacterial strain and, collecting said formalin-killed *E. coli* bacterial strain. Prepared vaccine was subjected to its efficacy test.

Clinical Validation of Toxoid and Whole-Cell Vaccine by Animal Studies:

Ethical clearance was obtained for performing clinical trials using animal models. Ethical clearance numbered KMCRET/PhD/07/2013-14 was obtained from KMCH College of Pharmacy, Coimbatore, as approved on 5th April 2013. Experimental design for clinical studies involving wistar rat models is depicted in Figure 1,

which included a total of seven groups as: Three control groups of wistar rats (1 group for complete uninoculated control, 1 group for ATCC and 1 group animal for isolate stain administration) and two test group rats (1 group for ATCC strain challenging after whole cell vaccination and 1 group for isolated strain challenging after whole cell vaccinating) and two test group rats (1 group for ATCC strain challenging after toxoid vaccination and 1 group for isolated strain challenging after toxoid vaccinating). Vaccine doses were given by oral route as per protocol of Evans D. J. *et al.*, 1988[15]. Screening methods emphasised on studying the effects on test rats as well as on control rats (mortality as well as morbidity in liver and kidney with the help of histopathology).

RESULTS

The morphological (gram staining and motility test), sugar fermentation (glucose, sucrose, lactose, and maltose), biochemical (indole, methyl red, voges-proskauer, citrate, urease, catalase, oxidase) characterization of *Escherichia coli O157:H7* isolate as well as ATCC were performed. The colony morphology of *Escherichia coli O157:H7* isolates as well as ATCC strain, when grown on various medium like Nutrient agar, Eosin methylene blue agar, MacConkey agar and MacConkey Sorbitol agar base with added tellurite, was observed. Latex agglutination test was also done for confirmation of the strain using "LK13 HiE.coli™ 157 Latex Test Kit". It was observed that more than 90% of samples were positive for *Escherichia coli* but only 5 samples were positive for the bacteria *Escherichia coli O157:H7*. The antibiotic patterns, the multiple drug resistance and the frequency of antibiotic resistance exhibited by the five isolates of *Escherichia coli O157:H7* and ATCC strain were also observed and compared. A cent percent resistance was observed for Ampicillin, Amoxicillin, and Tetracycline. Genotypic confirmation was done with conventional PCR amplification in all five isolates and prevalence of 5 different virulence genes was determined.

Histopathology for Vaccine Testing

Reports revealed the following

1. In Rat Group No. 1 (Neither vaccine nor strain administered), Group No. 4 (Vaccinated with prepared toxoid vaccine and challenged with ATCC strain), Group No. 5 (Vaccinated with prepared toxoid vaccine and challenged with Isolate strain), Group No. 6 (Vaccinated with prepared whole-cell vaccine and challenged with ATCC strain), and Group No. 7 (Vaccinated with prepared whole-cell vaccine and challenged with Isolate strain): Liver and Kidney Specimen [As shown in Figure 2]: Normal lobular architecture, portal tracts, central vein and sinusoids. The hepatic parenchyma is unremarkable. No inflammation, toxic changes or fibrosis. Normal cortex, medulla, pelvicalyceal system and distal convoluted tubules. Cortex shows normal glomeruli, proximal convoluted tubules. The interstitium is unremarkable. No evidence of toxic changes.

EXPERIMENTAL DESIGN

TOTAL NUMBER OF ANIMALS: Rats, 42 in number

Group I : Control Rats, 3 groups (18 nos)

Group II : Test Rats, 4 groups (24 nos)

		Control Rats			Test Rats			
		RAT GROUP NO.1	RAT GROUP NO.2	RAT GROUP NO.3	RAT GROUP NO.4	RAT GROUP NO.5	RAT GROUP NO.6	RAT GROUP NO.7
STEP I	→	***	***	***	Vaccine A given	Vaccine A given	Vaccine B given	Vaccine B given
STEP II	→	***	***	***	Booster Dose of Vaccine A given	Booster Dose of Vaccine A given	Booster Dose of Vaccine B given	Booster Dose of Vaccine B given
STEP III	→	***	Standard ATCC strain administered	Isolate strain administered	Challenged with ATCC strain	Challenged with isolate strain.	Challenged with ATCC strain	Challenged with isolate strain.

Key:

*** : Nothing administered into rats

Vaccine A : Toxoid vaccine

Vaccine B : Whole-cell vaccine

Fig. 1. Experimental Design of Animal Studies Work

2. In Rat Group No. 2 (Unvaccinated and ATCC administered) and Group No.3 (Unvaccinated and Isolate strain administered): Liver and Kidney Specimen [As shown in Figure 3]: Intact architecture with lobular inflammation. The portal tracts show acute inflammation and mild lymphocytic infiltration. The hepatic parenchyma shows lobular inflammation with focal granuloma is noted. The central vein and sinusoids are mildly dilated. Some of the glomeruli with loss of shape with increased in interstitium indicating questionable fibrosis. The proximal convoluted tubules show dilatation and shedding of epithelium indicating tubular necrosis, in some areas. The distal convoluted tubules are markedly dilated at places. Interstitium shows lymphocytic inflammation. Few congested blood vessels also noted.

To summarise the results, vaccinated rats showed normal pathology as that of unchallenged control group and unvaccinated rat models showed abnormal lesions. However, negligible exceptional lesions were seen in toxoid vaccinated rats after challenging with pathogen whereas whole-cell vaccinated rats showed cent percent protection when challenged with pathogen.

DISCUSSION

So far, no toxoid vaccine has been prepared worldwide for Escherichia coli O157:H7, hence no comparison could be made with literature studies. When comparison was made with efficiency of two types of prepared vaccines in this study, namely toxoid and whole-cell vaccine, both were believed to have lost its pathogenicity

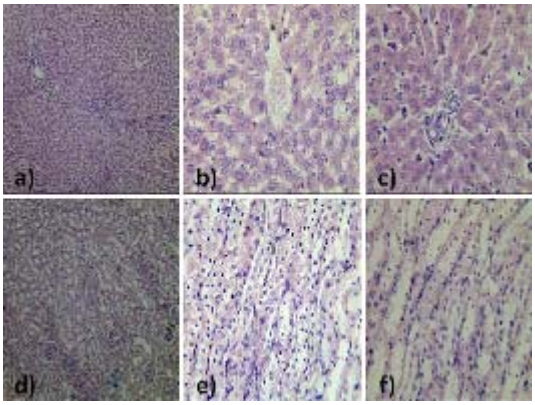

Fig. 2. Histology of Liver and Kidney Specimens of Group 1, 4, 5, 6 and 7: Normal lobular architecture, portal tracts, central vein and sinusoids. The hepatic parenchyma is unremarkable. No inflammation, toxic changes or fibrosis. a) 10X shows normal lobular architecture. b) 40X shows normal central vein. c) 40X shows normal portal tract. Normal cortex, medulla, pelvicalyceal system and distal convoluted tubules. Cortex shows normal glomeruli, proximal convoluted tubules. The interstitium is unremarkable. No evidence of toxic changes. d) 10X shows normal glomeruli and tubules. f) 40X shows normal interstitium. g) 40X shows normal tubules.

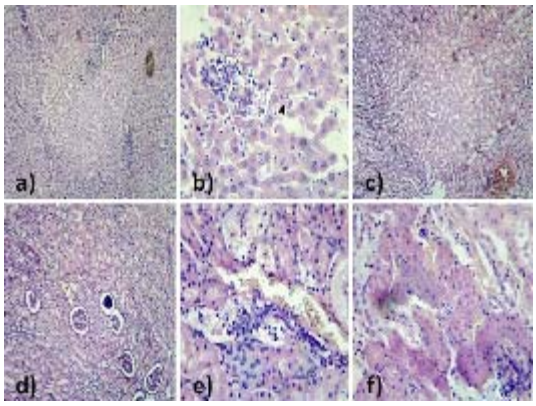

Fig. 3. Histology of Liver and Kidney Specimens of Group 2 and 3: Intact architecture with lobular inflammation. The portal tracts show acute inflammation and mild lymphocytic infiltration. The hepatic parenchyma shows lobular inflammation with focal granuloma is noted. The central vein and sinusoids are mildly dilated. a) 10 x shows normal lobular architecture with lobular inflammation. b) 40 x shows dilated sinusoids. Some of the glomeruli with loss of shape with increased in interstitium indicating questionable fibrosis. The proximal convoluted tubules show dilatation and shedding of epithelium indicating tubular necrosis, in some areas. The distal concoluted tubules are markedly dilated at places. Interstitium shows lymphocytic inflammation. Few congested blood vessels also noted. c) 10X shows dilated tubules with congested blood vessels. d) 10X shows glomeruli with degenerative changes and focal inflammation. e) 40X shows interstitium with congestion. f) 40X shows shedding of epithelium with dilated tubules.

as validated by animal studies but whole-cell vaccine has proved to show much higher efficiency than toxoid vaccine. However, this is just a preliminary confirmation before proceeding to human volunteer level studies.

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