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HELICOBACTER PULLORUM MODEL ON BALB/cA MICE

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ABSTRACT

Helicobacter pullorum is the bacterium found in the poultry and is an infective intestinal pathogen of man. 40 BALB/cA mice were used for the experiment to investigate whether the mice get infected with *H. pullorum* or not. We analysed stomach, intestine, caecum, liver and faeces of the mice after infection at every two weeks interval and found to be culture and PCR positive. The controls showed negative results. Immunoblotting of serum samples from infected mice showed positive bands of antigen-antibodies reacting with the anti-IgG/IgM antibodies. Therefore, it can be concluded from this study that mice infected with H. pullorum get the infection in the their body which is comparable to human being which is also a mammal.

KEYWORDS: *H. pullorum*, Mice, Liver, Stomach, Intestine, Faeces, PCR

Short Running Title: *H. pullorum* Model

INTRODUCTION

Helicobacter pullorum is associated with poultry and is also an infective intestinal pathogen of man. Sixteen strains of *H. pullorum* have been isolated from poultry and human. It is oxidase positive, reduce nitrate, grow at both 37^{o}C and 42^{o}C under microaerobic conditions. None of the strains produce alkaline phosphatase, DNase or urease. The colonies of all 16 strains on 5% horse blood agar are pinpoint sized, watery, translucent and α-haemolytic. No pigments are produced. The standard strain of *H. pullorum* CCUG 33838 is gently curved rod, 3-4µm in length and 0.3-0.5µm in width. Flagella are single and unipolar; flagellar sheaths are not detected[1]. *H. pylori* and *H. pullorum* has been found in patients with cholelithiasis by immunoblot and PCR from their bile samples[2].

Serum antibodies to enterohepatic *Helicobacter* spp. in patients with chronic liver diseases have been found[3]. Also immunogenic proteins have been found in *H. pullorum*, *H. bilis* and *H. hepaticus* by two-dimensional gel electrophoresis and immunoblotting[4]. The *H. pullorum* strains tested stimulated interleukin 8 (IL8) secretion by the three cell lines[5]. There has been increased prevalence of seropositivity for non-gastric *Helicobacter* species in patients with autoimmune liver disease[6]. A mouse model C57BL/6NTac and C3H/HCNTac has been developed for *H. pullorum* colonization [7,8]. *H. pylori* infection has been studied in BALB/cA mice[9]. *H. pullorum* is found among patients with gastrointestinal disease and clinically healthy persons by PCR giving a 447 bp fragment of the 16SrRNA gene sequence[10].

We infected mice orally, intravenous and orally as well as intravenous with *H. pullorum* bacteria. At intervals of 2 weeks we sacrificed 2 mice from each set along with controls. We did culture, PCR, immunoblot from the mice organs. Culture was positive in almost all the mice. PCR was mainly positive in case of stomach, liver and faeces. Immunoblotting of the blood samples from the mice showed bands for *H. pullorum*, i.e., reaction with anti-IgG/IgM antibodies.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

H. pullorum CCUG 33838 standard strain was grown on 10 plates of Brucella blood agar with charcoal, hemin, isovitalex. The growth from all the plates was scrapped off with swab stick and was suspended in sterile PBS. It was vortexed for a few seconds, washed with PBS once by centrifuging the cells at 3000xg for 15 minutes. Cells were suspended in PBS and O.D. was adjusted at 1.8. Cells were transported to the animal house on ice.

Infection Strategy

40 BALB/cA female mice were purchased from the animal house laboratory (Lund University, Sweden). The mice were feeded with ad libitum and sterile water. The food and water was changed every week for the mice. In order to follow infection strategy the mice were grouped into four. In group number one, ten mice were included and they were orally infected *H. pullorum* bacterial suspension through a gavage tube. Another group which consisted of 10 mice were intravenously infected with bacterial suspension through the vein passing through the tail. Group third included ten mice which were orally as well as intravenously infected with bacteria. Group four included 10 mice and five injected with 500µl of sterile PBS orally and five injected with 500µl of sterile PBS intravenously. Infection was done for 3 times to give interval for proper infection in between 2 days gap was given.

Sacrification of Mice

At 20 days interval from the day of inoculation, 2 mice from each infected group and 1 mice from each uninfected group were sacrificed with sterile instruments in sterile conditions. Mice were anaesthesised with CO_2 gas for scarification. After scarification, the mice organs like stomach, intestine, caecum, liver were collected. Blood was collected from the heart. Faeces were also collected. All the samples were preserved for culture, PCR and immunoblot assays.

Culture

The organs and faeces were cultured after scarification on Brucella blood agar incorporating charcoal, hemin, isovitalex. Plates were incubated at 37^{o}C for 48 hours to 72 hours in a CO_2 incubater.

Polymerase Chain Reaction

For PCR 22 mice samples were analysed. 10 were oral and intravenous controls. Remaining 12 were 4 oral inoculation, 4 intravenous inoculation, and 4 were oral as well as intravenous inoculation samples.

For the PCR DNA was extracted by KTET extraction procedure as well as Dynabeads extraction procedure in order to see the efficiency and accuracy of the two methods of extraction. The organs were homogenized with the sterile plastic homogenizer and were mixed with 200µl of KTET buffer (75mM KCl, 150mM Tris-HCl, 3mM EDTA, 0.75% Tween-20). Incubate for 10 minutes at 90^{o}C, cool for 2 minutes with ice. Centrifuge for 10 minutes at 20,000xg at 4^{o}C for the DNA extraction. Supernatant is transferred to another tube for PCR amplification.

In case of Dynabeads experiment, the samples were homogenized in PBS-GAB (0.1%) buffer to make approximate volume of 5ml. Vortex it to mix for 60 seconds. Centrifuge at 200g for 1 minute. Remove the supernatant into eppendroff tubes in 1 ml aliquots.

Centrifuge at 12000xg for 10 minutes. Supernatant was discarded and 1 ml of PBS- 0.1% was added. Tubes were vortexed to mix for 30-60 seconds. Bead-antibody mixture was added to each of the 5 tubes in 10µl, 20µl, 30µl, 40µl, 50µl volumes. Mixture was incubated at 4^{o}C for 60 minutes on Dynal mixer. Magnetic particles are separated from the

suspension using a magnetic device and washed 3 times for 10 minutes with PBS, 0.1% BSA. The beads are then resuspended in 30µl d/w containing 0.1% Tween 20, boiled for 10 minutes, briefly chilled on ice, centrifuge for 7.5 minutes at12,000xg. Supernatant transferred to fresh microcentrifuge tubes and frozen until analysed by PCR.

PCR conditions were the same as explained in Nilsson et al [11]. The primers used were 16SrRNA H. pullF and H. pullR. The positive control used was CCUG 33838 and negative control used was double distilled water.

Immunoblot Assay

For immunoblots first of all the *H. pullorum* cell surface proteins extractions were made by 0.2% CHAPS method. The bacteria were grown in culture plates for 2-3 days under microaerophilic conditions at 37^{o}C. One tube of 50ml was taken and weighed. Then 15ml PBS (pH 7.2) was added to the tube. The cells were harvested and added to the tube. Tube was vortexed softly and left on vortex for 10 minutes at room temperature and centrifuged for 15 minutes, 5000 rpm at 8^{o}C. Supernatant was discarded and the cells were weighed. The cells were resuspended and the pellet was dissolved in 0.2% CHAPS and vortexed for 10 minutes. Protease inhibitor was added to the suspension and stirred magnetically for 15 minutes. The tube is centrifuged at 8^{o}C for 15 minutes at 5000 rpm. The supernatant is collected and neutralized with NaOH to pH-6. It is centrifuged for 20 minutes at 8^{o}C at 8000 rpm. It is dialysed overnight with PBS-H_2O (1:10); the buffer is changed after 1 hour. The protein is concentrated in PEG. The protein concentration is determined by using BioRad assay.

Immunoblotted membranes made already (3mm wide strips) were placed in an incubation tray. For this the *H. pullorum* water extracts were already run on two-dimensional SDS-PAGE to separate the antigens. These antigens were immunoblotted on the nitrocellulose membrane filter pappers and the strips were cut 3 mm wide and were used for the antibody reaction with the sera obtained from mice.

Strips were washed for 10 minutes in $NN_8$1x on a shaker/rotor. The strips were overlayed with 1:50 diluted serum sample in $NN_8$1x-buffer and incubated on a shaker at room temperature for 4-5 hours. After incubation strips are rinsed for 5 minutes twice in $NN_8$1x. Peroxidase conjugated anti-IgG/IgM antibodies (Dakopatt P214) dil. 1/600 in $NN_8$1x is used and incubate for minimum 2 hours and maximum 3 hours in coldroom on shaking table or at room temperature for 1 hour. Strips are rinsed 3 times for 5 minutes with $NN_8$1x. Aspirate the washing buffer to absolute dryness. Substrate buffer (dye solution, acetate buffer, 30% H_2O_2) is added. The strips are incubated at room temperature in darkness until the color is optimized (20-30 minutes). Stop the reaction by rinsing the strips in water. Dry the strips between filter papers.

RESULTS

Culture

At first scarification from almost all the groups two mice were found culture negative except mice number 1 of oral plus intravenous infection which showed Gram negative curved rods for intestine and faeces. One oral control mouse and one intravenous control mouse were also found culture negative. At second scarification, only caecum and faeces showed Gram negative curved bacteria from mice number one of intravenous plus oral infection and one orally infected mouse, liver was found to have Gram negative curved bacteria. One oral control mouse and one intravenous control mouse were also found culture negative. The colonies were translucent, grey and pinpoint. Later on at every 2 weeks of interval the mice were sacrificed and organs and faeces were cultured. Most of the samples showed pinpoint colonies which were found to be urease negative and Gram negative curved rods. The oral and intravenous controls were found to be culture negative.

Polymerase Chain Reaction

After amplification, the PCR-products were run on 1.5% agarose gel electrophoresis and observed under U.V. light. PCR-product was found to be 447 bp fragment for *H. pullorum* infection in mice organs and faeces. In the first week of infection, orally inoculated mice were negative for *H. pullorum* and so were the results of intravenously inoculated mice. After the second week of infection oral, intravenous and oral plus intravenous mice infected with *H. pullorum* showed positive results for samples like stomach, liver and faeces and intestine was found to be negative. All the oral and intravenous control mice were found to be negative by PCR for *H. pullorum* primers.

Immunoblotting

All blood samples which were collected during scarification and were tested by immunoblotting experiments to detect the antibodies produced during the infection. The serum was separated from the blood samples and was used for immunoblotting. The immunoblotted antigen-antibody reaction showed various size of bands on the immunoblot strips. They were ranging in size from 16, 20, 37, 48 kDa. We found generally these four bands in all the orally infected mice for *H. pullorum* after second week. Intravenously infected mice by *H. pullorum* showed the same bands after one week. Intravenous plus oral infection gave all these 4 band sizes of proteins. We compared these results with the standard molecular weight marker which ranges from 97.4, 66.2, 45.0, 31.0, 21.5, 14.4 kDa and also the water extracts of *H. pullorum*.

DISCUSSIONS

Helicobacter species have been involved in many diseases related to human and animal[1]. *H. pylori, H. pullorum* has been detected from patients with cholelithiasis[2]. *H. pullorum, H. bilis* and *H. hepaticus* has been identified by two-dimensional gel electrophoresis and immunoblotting[3]. *H. pullorum, H. bilis* and *H. hepaticus* were found to have causing autoimmunity in liver disease. The bile samples were seropositive for these bacteria[6]. *H. pullorum* has been isolated and detected by PCR from faeces of patients suffering from gastroenteritis disease and healthy persons also[10]. It has been found that enterohepatic *Helicobacter* spp. have been found in patients with chronic liver disease and in a population with high prevalence of *H. pylori* infection[3]. *H. pullorum* has been found to have proinflammatory properties on human epithelial cells *in vitro*[5]. *H. pylori* and enterohepatic *Helicobacter* spp. have been detected in the livers of some patients with ulcerative cholitis and concomitant liver disease, as well as in other children with liver diseases[12]. Recently, *H. pullorum* have been isolated from mouse strains C57BL/6NTac mice[8]. We have confirmed *H. pullorum* infection in BALB/cA mice by infecting them orally and intravenously to detect that *H. pullorum* colonises all the mice or not. We analysed stomach, jejunum, caecum, liver, faeces by culture and PCR for *H. pullorum*. We also collected blood samples and confirmed the infection by immunoblotting.

Liver disease is caused due to Primary Scleorising Cholangitis (PSC) charactierized by chronic and cholestatic liver by fibrosing inflammation of the extra- and intrahepatic bile ducts [13,14]. New species of *Helicobacter, H. muridarum, H. rappini, H. hepaticus* and *H. bilis* have been identified in gastrointestinal tracts of mice[15]. A new species of *H. pullorum* has been found and isolated from poultry and from human patients with gastroenteritis[1]. *H. pylori* gastric colonization model has been made in BALB/cA mice[9]. We therefore, used the same strain of mice i.e., BALB/cA to produce a model in them with *H. pullorum*.

Helicobacter is associated with liver disease in humans specially in pathogenesis of idiopathic hepatitis in humans. It is also associated with liver disease in animals[16]. *H. pylori* has been found to have high prevalence in liver cirrhosis [17,18]. In BALB/cA mice infection has been produced by spiral and coccoid forms of *H. pylori*[19]. The mucosal and

the systemic immune system plays a significant role in inflammatory bowel disease, whipple disease, autoimmune gastritis, *Helicobacter pylori* infection, immunoploriferative small intestinal disease, hepatitis A,B,C,D,E,F and G, autoimmune hepatitis, primary biliary cirrhosis (PBC) progressive sclerosing cholangitis and vanishing bile duct syndrome[20]. *Helicobacter* spp. have been identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. These bacteria play causative role in the development of gallbladder cancer[21]. There have been studies in which enterohepatic *Helicobacter*s have been naturally experimentally modeled[22]. *Helicobacter* spp. are associated with biliary tract disease of animals and humans[23]. Other kind of bacteria have also been found in bile duct in PSC patients[24]. *H. pylori* has been found in PSC or PBC patients by PCR, hybridization, sequencing in human liver samples[25]. The whole cell fatty acid extracts analysis has been done on *H. pullorum* and compared with other strains of *Helicobacter* and *Campylobacter*[26]. In our study we isolated *H. pullorum* after infection in different organs of gut of mice by culture and PCR. We also ran sera of the mice to confirm *H. pullorum* infection by immunoblotting. In the future this study will throw a light on the infections caused in mice naturally and experimentally. Other animal models like monkeys, guinea pigs, rats can be used as animal models as this infection is mostly found in mammals. Thus, *H. pullorum* infection can be related to gut and biliary tract diseases.

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