Cytopathic effect of coccoid forms of *Helicobacter pylori* in Albino rats and Swiss mice

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ABSTRACT: The effect of coccoid forms of a vaculating cytotoxin A positive *Helicobacter pylori* strain on *Mus musculus* (Swiss mice) and *Rattus norvegicus* (Albino rats) was studied. Two groups of Swiss mice and Albino rats were used. The first group of mice and rats was orally inoculated with coccoid forms of *Helicobacter pylori*, whereas, the second group of mice and rats were untreated and used as a control. The animals that had been orally inoculated with a total count of $7.0x10^5$ bacterial cell / ml died after 5-7 days of inoculation (mice), whereas, rats died after 7-9 days. Symptoms of mouth ulcers, darkening of the ventral part and peripheries and loss of weight were apparent, in addition to slow movement and general weakness compared to the control group. Histological examination of the stomach of the inoculated animals showed marked degeneration of the epithelial lining membrane and dark dense bodies - most probably - coccoid forms inside gastric glands. In addition dialated blood vessel, degenerated, vacuolated fused cells with coccoid forms and intravascular haemolysis were observed. These findings may indicate the toxicity of coccoid forms of *Helicobacter pylori* and hence their possible pathogenic role in humans.

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Key words: *Helicobacter pylori,* Albino rats, Swiss mice, coccoid forms, toxicity, cytopathic effect.

1.Introduction:

Helicobacter pylori (*H. pylori*) is a microaerophylic, Gram negative, motile bacterium that is known to exist in two morphological forms: the helical and the coccoid form. It has been reported that spiral forms may transform to the coccoid shape after two hours of exposure to oxygen (Borriello, *et al.* 2005). However, other researchers found that it converts into the coccoid form when it is exposed to detrimental environmental conditions (Andersen and Wadstrom, 2001; Nilsson, *et al*. 2002). The role of *H. pylori* spiral forms in many types of gastritis, gastric and duodenal ulcers, gastric carcinoma and MALT lymphoma have been documented (Uemura, *et al.* 2001; Blaser and Atherton, (2004). Furthermore, its implication in the development of cardiovascular, dermatological diseases and lately in the development of Parkinson's disease has been suggested, Wedi and Kapp (2002);Aceti, *et al*. (2004).Hernando-Harder, *et al*. (2009);Testerman, *et al., (*2011). Above all, Scientists estimated that more than 50% of the world's population harbor *H. pylori* in their stomach ,Talarico, *et al.,(* 2009) that can persist for life.

Interestingly, what makes infection of this bacterium a complicated dilemma, is that treatment with antibiotics induces the transformation of some spiral forms into the coccoid forms which consequently leads to failure of treatment ,Kusters, *et al*., (1997l) ; Brenciaglia, *et al*. (2000).

Although many authorities in the field used to believe that coccoid forms are manifestation of bacterial cell death ,Kusters, *et al*., (1997), yet many recent researchers started to prove the opposite of this concept ,Mizoguchi, *et al. (*1999);Azevedo, *et al., (*2007). In fact, different reports recorded the ability of coccoid forms to cause gastritis in experimental mice and to convert in-vivo into the spiral form Wang, *et al*., (1997); She, *et al*., (2003). Moreover, documented research proved that coccoid forms coexist with spiral forms in the stomach, and by semi-quantitative analysis the number of coccoid forms was found to be significantly greater in adenocarcinoma than that in benign peptic ulcers and were observed in more severely damaged regions of the gastric mucosa ,Chan, *et al*., (1994);Saito, *et al.*, (2003). The fact that –so far- coccoid forms are said to be unculturable ,Azevedo, *et al*., (2007), does not exclude their ability to cause disease or in transmitting infection, since an increasing number of studies support the potential role of coccoid forms in *H. pylori* diseases ,Figueroa, *et al*., (2002).

The aim of the present study was to investigate the ability of *H. pylori* coccoid forms to cause pathological changes in laboratory animals and hence their possible role in the transmission of infection.

2.Materials and Methods:

2.1. Materials:

2.1.1.Bacterial strain:

H. pylori was isolated from a gastric biopsy of a patient complaining of chronic superficial gastritis, the clinical specimen was provided by the Gastroenterology department at King Khalid National Guard Hospital, Jeddah City, Saudi Arabia.

2.1.2.**Laboratory animals:**

20 healthy male *Mus musculus* strain MF1(Swiss mice) and 20 healthy male *Rattus norvegicus* (Albino rats) aged three months and weighing 33-35 g and 230-250 g respectively were obtained from King Fahad research centre, king Abdul Aziz University (KAU). Each group of both mice and rats was divided into three subgroups: the first contained 5 inoculated animals with 1ml of $7.0x10^6$ Bacterial cell /ml, the second contained 10 inoculated animals with 1ml of $7.0x10^5$ Bacterial cell /ml and the third was five untreated animals as a control. The mentioned inoculum (infectious dose) was given as one dose/ day.

Each animal was housed in a separate stainless steel cage containing soft wood chips. A basal diet composed of 60% ground corn meal, 15% ground beans, 10% wheat bran, 10 % corn oil, 30 % casein, and 1 % minerals mixture was given. Water was supplied daily. Ghanem, and Aly, (2003). Animals were kept at 21° C air-conditioned room.

2.1.3.**Oral inoculation of animals:**

The treated animals (15 mice and 15 rats) were mildly anaesthetized –only before inoculation - using chloroform saturated cotton which was placed in the middle of each cage. For daily inoculation, the mouse or rat was held firmly by the scruff of the neck in a vertical position (Lee and Megraud, 1996) and orally inoculated using a disposable sterile 1 ml syringe (JMC, Korea) for five days (mice) and seven days (rats).The control group of animals was un treated.

2.2.Methods:

2.2.1.Culture and Identification:

Gastric biopsy was cultured on Blood and Chocolate agar using the rubbing technique and the plates were incubated under microaerophylic conditions at 37°C for five days (Milyani and Barhameen, 2004). Identification was by morphological studies, urease, catalase and oxidase tests in addition to motility (Lee and Megraud, 1996). The colonies were subcultured on five blood agar plates and after five days incubation, the harvested colonies from each plate were transferred to a separate Cryovile filled with 0.5 ml Thioglycolate broth with 15% glucose (five Cryovile), and stored at - 20° C as stock culture for further studies. Five years later, a 10 µl diameter sterile disposable loop (Sara Med. Saudi Arabia) was dipped in the stock culture and a loopfull was streaked on both Blood and Chocolate agar and incubated under the appropriate conditions as mentioned above.

Molecular identification for the isolated strain, using 16S rRNA and VacA genes, was carried out by Professor Osama El Sayed (National Research Centre, Cairo, Egypt).

2.1.2.**Preparation of the inoculums**:

Two inocula were prepared from the stock culture (a total count of $7.0x10^6$ and $7.0x10^5$ Bacterial cell /ml) by using counting chamber; in addition, a drop of each inoculum was examined by phase contrast microscope.

2.1.3.**Preparation of histological sections:**

Treated and untreated mice and rats were dissected and the stomach was removed and preserved in Boin's solution for histological studies. Cross sections were done and stained with haematoxylin and eosin dyes as described by Lee and Megraud, 1996 at King Fahad research centre (KAU).

3.Results:

3.1.Culture and Identification:

Culture of gastric biopsy revealed typical morphology of *H. pylori* colonies, proven by positive urease, catalase and oxidase tests. Gram stain also showed Gram negative S-shaped bacteria, in addition, using phase contrast microscope, the well recognized motility of *H. pylori* was recorded. However, culturing from the stock culture after five years gave undetectable viable counts, though, examining drops of both inocula using phase contrast microscope showed complete conversion to coccoid forms some of which appeared motile.

3.2. **Identification of 16S rRNA gene and VacA gene:**

PCR amplifications of the *H. pylori* isolate revealed the fragments with expected sizes of 163 bp that represented the 16S rRNA gene and 750 bp that represented the VacA gene.

3.3.Laboratory animals:

The animals in the preliminarily study that were orally inoculated with $7.0x10^6$ bacterial cell/ml, died after 24 hours. On the other hand, both orally inoculated mice and rats with $7.0x10^5$ *H. pylori* cell/ml, showed different abnormalities and pathological manifestations. Symptoms started to appear from the third day of inoculation as ulcers around the mouth, general weakness with slow movement, loss of appetite, darkening of the ventral part and terminals with some atrophy and loss of weight (fig. 1 and fig. 2).

The group of mice died after 5-7 days whereas, the group of rats died after 7-9 days, and the final average weight was decreased to 17.2 g for mice and 215 g for rats. No pathological changes were obtained in the control group that only drank sterile tap water.

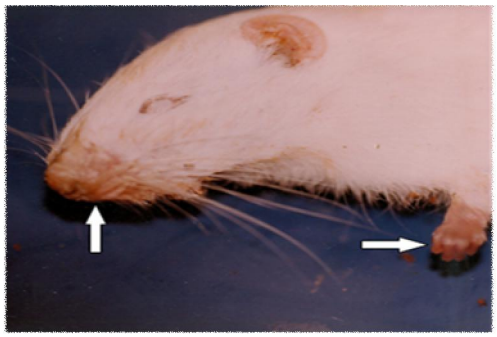

Fig. 1 Albino rat after oral ingestion with coccoid forms of *Helicobacter pylori* showing ulcer of the mouth and dark atrophied terminals.

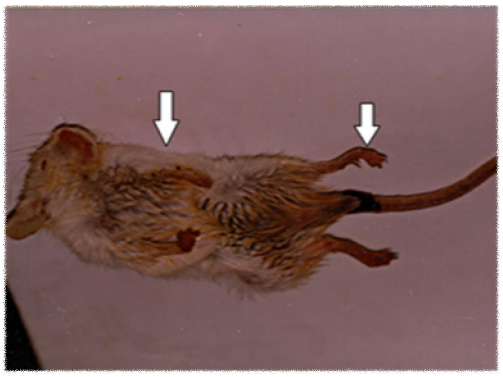

Fig. 2 Swiss mouse after oral ingestion with coccoid forms of *Helicobacter pylori* showing ulcer of the mouth, dark ventral part and atrophied terminals.

3.4.Histological sections:

Figures 3 – 5 show different Sections of mucosal layer of Albino rat's stomach that were orally inoculated with *H. pylori* coccoid forms. Degeneration of the epithelial lining membrane and dark dense bodies- most probably- coccoid forms inside gastric glands were apparent in fig. 3 (A) compared to the normal section of control rat fig. 3 (B). In addition fig. 4 (A) shows degeneration of epithelial lining membrane with no goblet cells and many vacuolated cells, while fig. (B) shows dilated blood vessel, degenerated, vacuolated fused cells with dark dense bodies. Moreover, in Fig. 5, intravascular haemolysis was seen.

On the other hand, figures 6 -7- show different Sections of mucosal layer of Swiss mice stomach that were orally inoculated with *H. pylori* coccoid forms in which fig. 6 shows highly damaged epithelial lining membrane with degenerated cells in the inner layers and many scattered dark dense bodies (coccoid forms), in contrast to fig. 6 (B) which shows normal stomach mouse wall. However, many coccoid forms in the mucosa and sub mucosa and connective tissue were seen in Fig. 7 (A) whereas, wavy fibres in the submucosal layer on which many dark bodies were deposited.

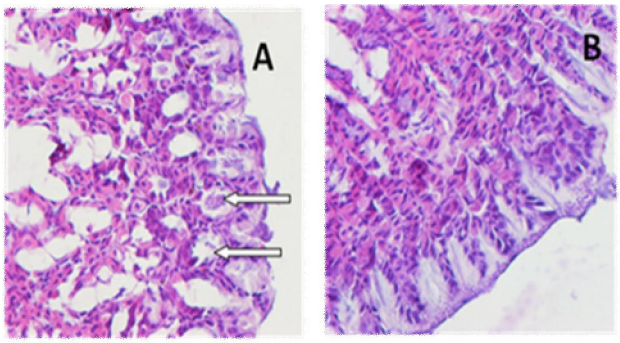

Fig3 Section of mucosal layer of Albino Rat stomach (A) orally inoculated with *H. pylori* coccoid forms showing degeneration of the epithelial lining membrane and dark dense bodies inside gastric glands (B) normal section of control (x 400).

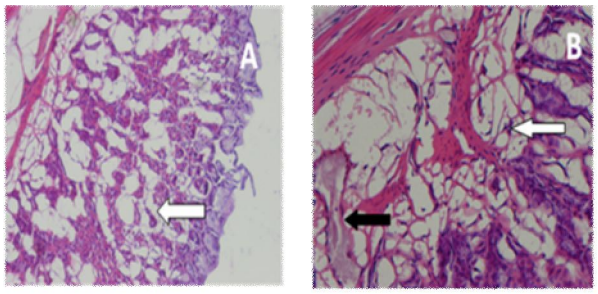

Fig.4 Section of mucosal layer of Albino Rat stomach that had been orally inoculated with *H. pylori* coccoid forms (A) showing degenerated epithelial lining membrane with no goblet cells and many vacuolated cells (B) showing dialated blood vessel (black arrow) and degenerated, vacuolated fused cells with dark dense bodies (x 400).

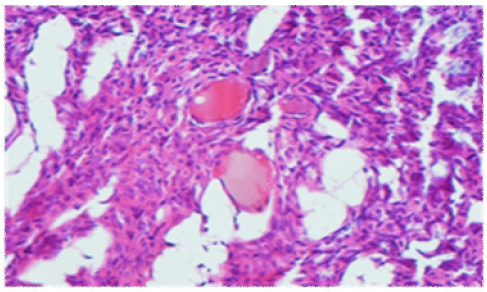

Fig.5 Section of Albino Rat stomach that had been orally inoculated with *H. pylori* coccoid forms showing intravascular haemolysis (x 400).

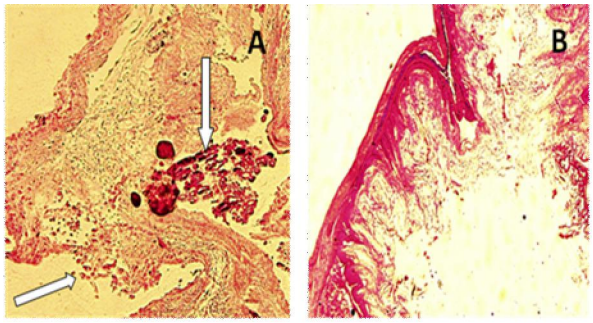

Fig.6 Section of mouse stomach that had been orally inoculated with *H. pylori* coccoid forms showing highly damaged epithelial lining membrane with degenerated cells in the inner layers and many scattered dark dense bodies (A), (x 630).
Section (B) show normal stomach mouse wall (x 250)

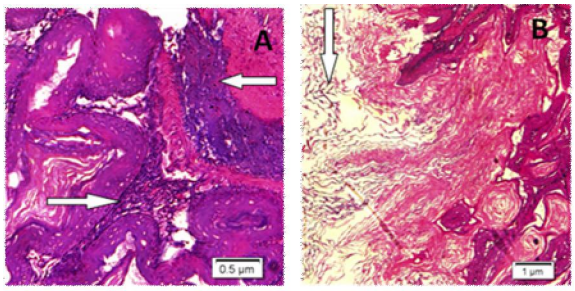

Fig.7 Section of stomach wall of Swiss mouse (A) shows many dark dense bodies in the mucosa, sub mucosa and connective tissue (x 260). (B) Section in the wall of mouse stomach showing many wavy fibers in the submucosal layer plus many dark dense bodies deposited on the fibers (x 630).

4.Discussion

Coccoid forms of *H. pylori* have for a long time been a debate between scholars. The fact that no one should ignore and has been documented is their existence in the stomach of patients together with spiral forms and their possible role in pathogenicity, Saito, *et al.*, (2003). Furthermore, what makes coccoid forms a dilemma to physicians, pharmacologists and scientists is that up to date, they are said to be unculturable ,Borriello, *et al.* (2005) and after eradication therapy a small amount of them revert to the unculturable state and remain in the form of coccoids ,Bardakhch'ian, (2003). Above all, *H. pylori* has been classified as type 1 carcinogen of gastric cancer by the WHO (Correa and Houghton, 2007). All the previous data was a strong stimulus for the present study which was to investigate the capability of coccoid forms to cause cytopathic effect in laboratory animals.

Wang *et al.*, (1997) demonstrated that both forms of *H. pylori* can infect mice with an inoculum size of 10^8 CFU whereas, at the present study, an infectious dose of $7x10^6$ and $7x10^5$ of coccoid cell /ml were used. The former only allowed 24 hours survival of both rats and mice, whereas, the latter ($7x10^5$) allowed survival of the tested animals for 5 – 9 days. It was surprising that although Wang and his colleagues used a higher infectious dose, yet their mice survived for a longer period. This could be attributed to the genotype of the *H. pylori* strain under study that might have been more virulent than Wang's strain and also than the strains used by She and colleagues, Wang *et al.*, (2001)'; She, *et al.*, (2003). The present studied strain revealed to be VacA positive which could have exerted a toxic lethal effect on the inoculated animals. In addition, observation of a drop of the stock culture by phase contrast microscope showed some motile coccoid forms indicating its viability. What's more, Milyani and Barhameen, (2003) studied the survival ability of seven *H. pylori* isolates in three different fluids aerobically, at room temperature and at 4° C and reported differences among the survival ability of the strains, since only one strain was able to maintain a detectable level of $3.88x10^4$ CFU after eight days in tap water at 4° C, after which viable count completely dropped. They concluded the possibility of *H. pylori* to stay viable but unculturable and thus might be a risk for transmitting infection. Also, the high diversity and variations among *H. pylori* strains and the role of VacA in causing epithelial cell damage with formation of large vacuoles within the cells have been well established, and could be another factor for the obvious toxicity at the present work ,Milyani (2011);Argent *et al.* (2008). On the other hand, the obtained results at the present work, when inoculation with sub lethal dose lead to different abnormalities and pathological manifestations such as mouth ulcers, atrophy of terminals and the apparent acute toxicity that ended by death, may prove the pathogenicity of coccoid forms to rats and mice. Moreover, the histological abnormalities that occurred in the stomach including marked degeneration of the epithelial lining

membrane and vacuolated fused cells with dark dense bodies which are most probably coccoid forms (Lee and Megraud, 1996) may indicate their potent detrimental effect and a risk factor for other pathological manifestations (Blaser and Atherton, (2004), Hernando-Harder *et al.* (2009), Testerman *et al.*, 2011). Nonetheless, we should bear in mind the immune response of the tested animals sine it could also play a role in the final outcome of infection Benaissa , *et al.* (1996); Zhao, *et al.,(*2007). Adhesion of coccoid forms to gastric mucosal cells and their role in gastro-duodenal disease has also been studied and recorded ,Liu, *et al., (*2006); Vijayakumari, *et al., (*1995).

In conclusion, the previous data strongly emphasize the pathogenic role of coccoid forms and recommend pursuing detailed research on this amazing phase of *H. pylori.* The clearly perceptible achievement and outcome of such research is: successful therapy, accurate diagnosis, extra biological data and the discovery of the source and precise mode of transmission which eventually will enable us to control and prevent different serious diseases caused by *H. pylori.*

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