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The Correlation Between *Helicobacter pylori* SeroPositive Patients and Throat Infection

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Abstract: *Heliocobacter pylori* besides being the cause of chronic gastritis it was shown be implicated in various extra gastric manifestation. Gastric juice infected with *H. pylori* might have important role in upper respiratory tract. However it has not been elucidated. Therefore this study aim to clarify whether *H. pylori* play a role in the etiology and pathogenesis of throat infection. 307 volunteers were participated in this study. Throat swabs from tonsils and oropharynx were collected, conventional bacteriological methods were applied for identification of pathogenic bacteria, followed by PCR confirmation. Along with, serum sample were collected for determination of anti-*H. pylori* IgG titer by quantitative ELISA method. Prior ICT antibody-based screening test were performed. Simultaneously questionnaire data form from our participants were collected for statistical analysis. This study was designed as case control study. From throat swabs *Streptococcus pyogenes* was the most common in our isolates. The mean of IgG antibody titer was 95.21 RU/ml, the positive samples for IgG was quite 88.2%. Clinical respiratory symptoms were observed only in 9.7% of participants in this study 89% of them confirmed with *H. pylori*. Statistical data showed very high significant correlation between pathogenic bacteria *S. pyogenes* and *H. pylori* infection.

Keywords: *H. pylori*, Anti-Helicibacter Pylori IgG Titer, Throat Swabs, Oropharynx, Tonsil, URT, *Streptococcus pyogenes*

1. Introduction

The most common complaint among patients in outpatients' clinics is sore throat, and the most frequent diagnosis is chronic nonspecific pharyngitis. Chronic pharyngitis, a chronic inflammation of the pharyngeal mucosa and underlying etiopathogenesis is still controversial [1]. Throat infection includes tonsillitis and pharyngitis may be caused by a wide variety of microbial agents, but the most common bacterial cause is group A beta-hemolytic streptococci. Other streptococcal (group C & G) and non-streptococcal bacteria are rarely cause and in certain conditions [2-6]. Treatment is usually difficult and based on reducing the symptoms by medical or behavioral methods [7]. However, the causative microorganisms in many cases remain unclear without laboratory diagnosis [8, 9].

Helicobacter pylori (*H. pylori*) is a major cause of chronic gastritis and gastric ulcers and associated with gastric malignancy in addition to various other conditions including pulmonary, vascular and autoimmune disorders [10]. Socioeconomic status is clearly the most important determinant for the development of *H. pylori* infection, with lower social classes exhibiting much higher prevalence [11]. Factor encompasses conditions such as levels of hygiene, sanitation and educational opportunities, which have all been individually identified as markers of the bacterium presence [12]. Gastric juice infected with *H. pylori* might play an important role in upper respiratory tract infection. Although direct and/or indirect mechanisms might be involved in the association between *H. pylori* and upper respiratory tract diseases, the etiological role of *H. pylori* in upper respiratory tract disorders has not yet been fully elucidated [10]. Epidemiological studies have shown that the prevalence of carrying *H. pylori* ranges from 10%-20% to 80%-90% in developed and developing countries, respectively, and most carriers are asymptomatic [13, 14]. The findings of published studies on the impact of *H. pylori* on the upper respiratory tract are inconsistent, so that, more studies are required [10].

This study aimed to determine the relationship between *H. pylori* infection and throat infection to clarify whether it may play role in the etiology and pathogenesis of bacterial pharyngitis and tonsillitis.

2. Materials and Methods

Study design: case control study

This study was conducted under the ethical consideration and Approval from Khartoum Ministry of Health, research department. The research purpose and objectives were explained to participant in clear simple words. Participants had right voluntary informed consent and the right to withdraw at any time, right to benefit, to no harm, to privacy and confidentiality.

Study area and population:

This study was performed in the state of Khartoum with the collaboration of the private clinical labs and hospitals. The study subjects were selected from patients who are admitted in internal out-patient's clinics, patients who had *H. pylori* positive results to the screening Ab-based ICT were considered the cases criteria, and patients who had *H. pylori* negative results were considered the control.

Sample size:

307 throat swabs (from tonsils and posterior auropharynx) and 307 blood samples was obtained collectively. Sample size was calculated according to the following formula.

$$\text{Calculation: } n = 1.96 \times \frac{Pexp^2(1-Pexp)^2}{d^2}$$

n: required sample size

P_{exp}: expected prevalence

d: desired absolute precision=0.05

Expected prevalence is 70% as recorded by using confidence level 95%.

Data collection tools:

Questionnaire form is included. Patient's demographic data should be retrieved from medical records on enrollment or by asking, including: Age, Gender, Smoking history, Body mass index. Hypertension, Diabetes mellitus, Questionnaires about general manifestations and symptoms.

Data analysis obtained by IBM SPSS platform.

Sterilization and disinfection techniques include autoclaving, dry heat oven sanitation and UV light in addition to chemical disinfection; were held prior to each experiment. The media was prepared according to Barrow and Feltham [4].

Control and transportation of collected samples

The throat swabs were coated with sheep serum broth aseptically at the clinical lab and all collected samples were preserved with quality control and transported in ice bags container to the research lab.

Conventional Isolation and Identification from throat swabs:

The specimens were inoculated on sheep blood agar and was preferred to be incubated anaerobically in carbon dioxide enriched atmosphere at 37°C for 24 hours.

Different primary and secondary keys were followed in Barrow and Feltham identification manual (2003) including cultural characteristics, microscopic, sensitivity and biochemical features.

PCR confirmation of isolated bacteria:

In our preliminary experiments we estimated that the alkaline lysis manual protocol modified from "plasmid DNA extraction" involved alkaline cell lysis yields high concentration of extracted DNA compared with bacterial DNA extraction kit (GF-1 BA-100, Vivant^{TM}) protocol, using nanodrop apparatus (Thermofischer).

Polymerase Chain Reaction (PCR): iTaq DNA polymerase kits (intron) containing mastermix pre-mix tubes were used for DNA amplification:

Detection of *Streptococcus sp.:* based on the use of degenerate (dprimer 480bp) that provide a PCR product that representing approximately 85% of the *sodA* gene found in 29 type strains of streptococci. F: 5¯-CCITAYICITAYGAYGCIYTIGARCC-3¯, R: 5¯ARRTARTAIGCRTGYTCCCAIACRTC-3¯ [15].

Table 1. *PCR cycles setting used to detect Streptococcus sp.*

cycle		Program temperature	Time
Initial denatutation		95°C	3min.
	denaturation	95°C	30sec.
35 cycle	annealing	37°C	90sec.
	Extension	72°C	90sec.
Final extension		72°C	10min.

Detection of Streptococcus pyogenes:

In the course of experiment PCR played an important role for confirmation of *S. pyogenes* a spy 1258 (407 bp) had been used as primer, although this primer can be found in 13 potential *S. pyogenes* strains (out of more than 200 strains discovered so far), it considered as the most ubiquitous and common primer for molecular detection of *S. pyogenes*. F (AAAGACCGCCTTAACCACCT) and R (TGGCAAGGTAAACTTCTAAAGCA) [16].

Table 2. *PCR cycles setting used to detect S. pyogenes strains.*

cycle		Program temperature	Time
Initial denaturation		95°C	5min.
	denaturation	95°C	30sec.
35 cycle	annealing	64°C	30sec.
	Extension	72°C	45sec.
Final extension		72°C	2min.

Gel documentation system: PCR products were resolved by electrophoresis in 1.5% agarose gel stained by ethedium bromide. 5x TBE (Tris-borate EDTA) was used as running buffer and diluted to 1x by adding1ml of 5x TBE to 99ml d.w. Addition of 2µl of ethedium bromide resin each 100ml of TBE was recommended for clear band results.

ICT screening of *H. pylori* seroprevalence among participants:

The *H. pylori* rapid test cassette (serum/plasma) is a qualitative membrane based immunoassay was used for the detection of *H. pylori* antibodies in serum samples.

Determination of Anti-*Helicobacter pylori* (IgG) titer

Anti-*Helicobacter pylori* ELISA (IgG) test kits (EUROIMUM) were used for measurement of a Anti-*Helicobacter pylori* IgG titer in patients serum. The ELISA was perform according to manufacture (EUROIMUM). The ELISA kit provide quantitative assay for human IgG antibody. The serum sample concentration obtained by point-to-point plotting of the extinction values measured for three calibration sera against the corresponding units (linear/linear). Point-to-point plotting for calculation of the standard curve by computer.

3. Result

Various types of gram positive and gram-negative bacteria were isolated and identified from 556 individual throat swabs according to the preliminary biochemical method of bacterial identification, as described by Cowan' and Steel manual. *Streptococcus pyogenes* was the most common in our isolates. The frequency of *Streptococcus pyogenes* and others Streptococci isolates as they were dominant among our isolated bacteria was illustrated in figures 1 and 2.

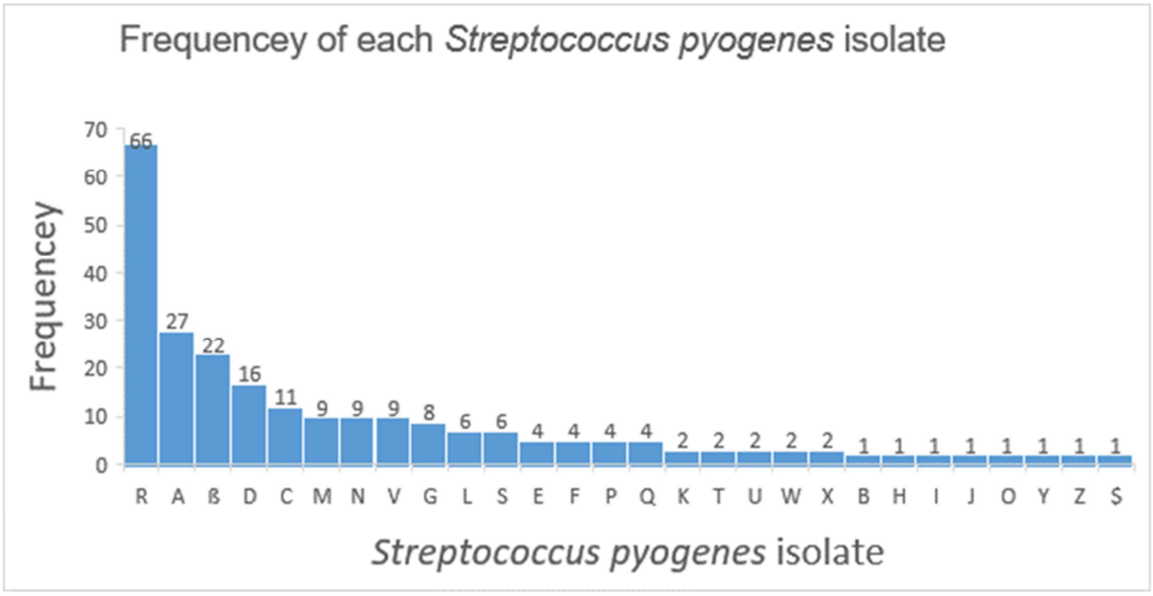

Figure 1. *The frequency of Streptococcus pyogenes.*

Key chart for the frequency figure above:

A	Confirmed *S.pyogenes* with biochemical tests and PCR
B	Starch –ve *Trhalose –ve* PCR-*confirmed S. pyogenes*
C	Arginine –ve PCR-confirmed *S. pyogenes*
D	Arginine –ve PCR-unconfirmed *S. pyogenes*
E	*Arginin –ve* Starch –ve PCR-unconfirmed *S. pyogenes*
F	*Arginin –ve* Starch –ve PCR-confirmed *S. pyogenes*
G	*arginine –ve sorbitol* + PCR-unconfirmed *S. pyogenes*
H	Arginine and lactose –ve PCR-unconfirmed *S. pyogenes*
I	Arginine and lactose –ve PCR-confirmed *S. pyogenes*
J	Arginine –ve ribose + Confirmed *S. pyogenes*
K	Arginine and trehalose –ve Confirmed *S. pyogenes*
L	trehalose –ve PCR-Confirmed *S. pyogenes*
M	trehalose –ve PCR-unConfirmed *S. pyogenes*
N	Lactose –ve PCR-unconfirmed *S.pyogenes*
O	Ripose +ve lactose –ve confirmed *S. pyogenes*
P	*Sorbitol + trehalose –ve* PCR-unconfirmed *S. pyogenes*
Q	*Starch –ve and sorbitol* + PCR-unconfirmed *S. pyogenes*
R	Sorbitol + PCR-unconfirmed *S. pyogenes*
S	Sorbitol + PCR-Confirmed S. pyogenes
T	Sorbitol+, ribose + PCR-Confirmed *S. pyogenes*
U	*Starch & lactose –ve* PCR-unconfirmed *S. pyogenes*
V	Starch and trehalose –ve PCR-unconfirmed *S. pyogenes*
W	vp+ Confirmed *S. pyogenes*
X	Vp+ and ribose+ PCR-Confirmed *S.pyogenes*
Y	Trehalose –ve and vp+ PCR-unconfirmed *S.pyogenes*
Z	Lactose –ve and vp+ PCR-unconfirmed *S.pyogenes*
ß	*Starch –ve* PCR-unconfirmed *S. pyogenes*
$	*Starch –ve* PCR-confirmed *S. pyogenes*

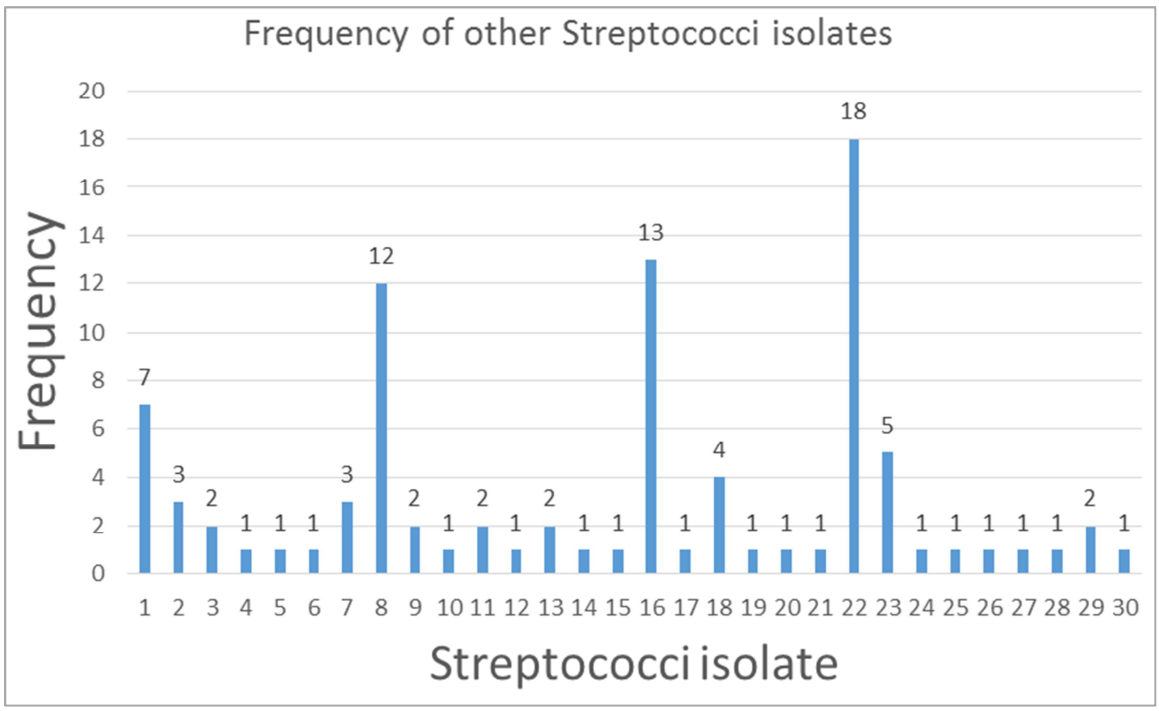

Figure 2. *The frequency of other Streptococcus sp. (more description).*

Key chart for the frequency figure above:

1	*Starch –ve strepto. Spp group L*
2	*Streptococcus. Spp group L*
3	*Mannitol + strepto. Spp group L*
4	*Starch & lactose –ve strepto. Spp group L*
5	*lactose –ve strepto. Spp group L*
6	*Starch -ve trehalose –ve S. spp. Group L*
7	*S. morbillorum*
8	*S. salivarius*
9	Trehalose –ve *S. pnemoniae*
10	Lactose –ve *S. pnemoniae*
11	*S.pnemonae*
12	*Mannitol +S. sanguis*
13	Ripose –ve mannitol +ve *s. dysagalactia*
14	Ripose –ve and lactose –ve *s. dysagalactia*
15	Ripose –ve trhalose –ve *s. dysagalactia*
16	Ripose –ve *s. dysagalactia*
17	Starch –ve *S. equi*
18	*S. equi* (group c)
19	*Mannitol + S. equi* (group c)
20	*Arginine –ve S. equi* (group c)
21	*S. zooepidemicus*
22	*Streptococcus sp.*
23	*S. suis*
24	*Arginine –ve S.suis*
25	*Mannitol + S. suis*
26	Sorbitol + *S. suis*
27	Starch –ve mannitol + *S. suis*
28	mannitol + *S. suis*
29	Trehalose – ve and arginine –ve *S. suis*
30	Starch and arginine –ve *S. suis*

Figure 3 shows some of eighty five samples of 556 samples (15.3%) were detected by PCR and confirmed as *Streptococci* on the size 480bp of the Dprimer, which represent 15.3% of the isolated *Streptococci*.

Statistical analysis results

Sore throat cases were found to be significantly (at 95% confident interval, 2 tailed) correlated with ICT results of this study, and also significantly correlated with symptoms of general body fatigue and respiratory signs. Moreover, sore throat increase the serum anti-*Helicobacter pylori* IgG 4 times more than normal throat individuals (chart 5). The linear Regression equation for the sore throat has been found to be:

$$Y=93.248 + 4.127\ X$$

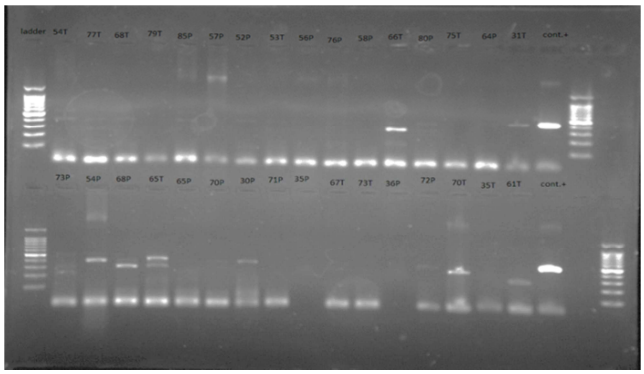

Figure 3. *Some of seventy two samples of 556 samples (13%) were detected by PCR and confirned as Streptococcus pyogenes on the size 407bp of spy 1258 primer. Which represent 17.8% of the isolated Streptococcus pyogenes.*

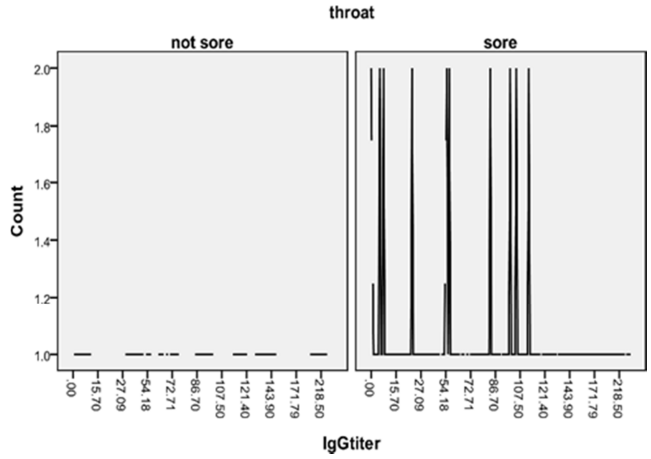

Figure 4. *This histogram compare the difference in Anti-Helicobacter pylori IgG titer between patients with sore throat and those with no sore throat. (more description)*

H. pylori positive (by ELISA or ICT) cases were found to be significantly (at 99% confident interval, 2 tailed) correlated with upper digestive tract signs (chart 6).

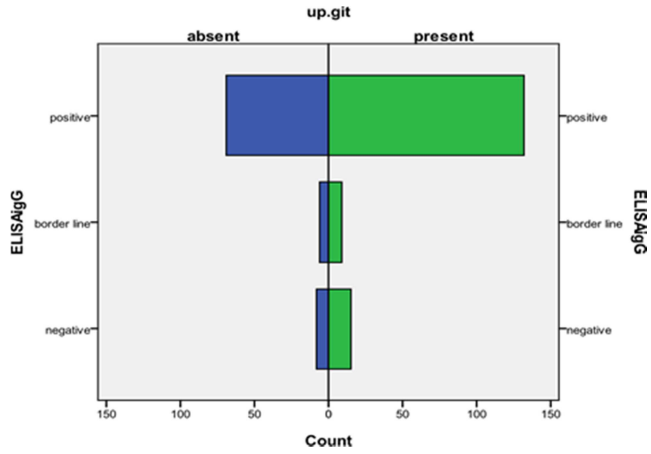

Figure 5. *The increase of IgG concentration in correlation with upper digestive tract signs.*

Contrary to upper digestive tract illnesses signs, the lower digestive tract signs have negative correlation with sore throat and *H. pylori* infection (at 95% confident interval, 2 tailed).

The mean of IgG antibody titer was 95.21 RU/ml and the maximum was 299.20 RU/ml while every sample above 22 RU/ml was considered positive according to manufacturer. The positive samples for IgG was quite 88.2%, 11.4% were Negative and 4.6% were border line. 28.1% were detected negative by ICT and 71.9% were positive.

Pathogenic sore throat causing bacteria were isolated from tonsils of 228 individual (74%) from whole samples, from pharynx 235 individual (76.3%) from whole samples while while 78.2% had pathogenic bacteria in the tonsils and pharynx.

Although sore throat has been observed to be very prevalent, but clinical respiratory symptoms were observed only in 9.7% of participants in this study, 89% of them were confirmed with *H. pylori* infection.

Among collected samples 11.3% individuals had been infected with *H. pylori* at least once before and 28% of them were complaining from respiratory signs.

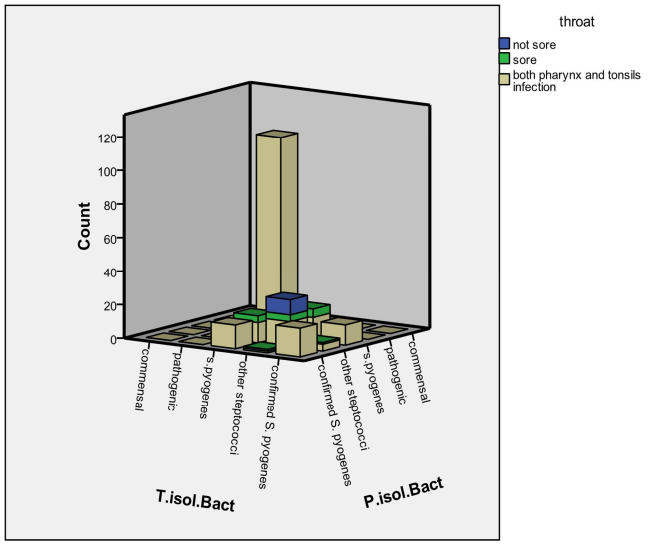

Figure 6. *The high frequency of isolation Streptococcus pyogenes among all other isolated bacteria. S. pyogenes represent about 71.2% among about 556 bacterial isolate, obtains and identified from throat in this study.*

Presence of previous *H. pylori* infection history was found to be significantly correlated with isolation of pathogenic bacteria particularly from tonsils. Tonsils can be a reservoir organ for throat pathogenic bacteria (i.e. *Streptococcus pyogenes*).

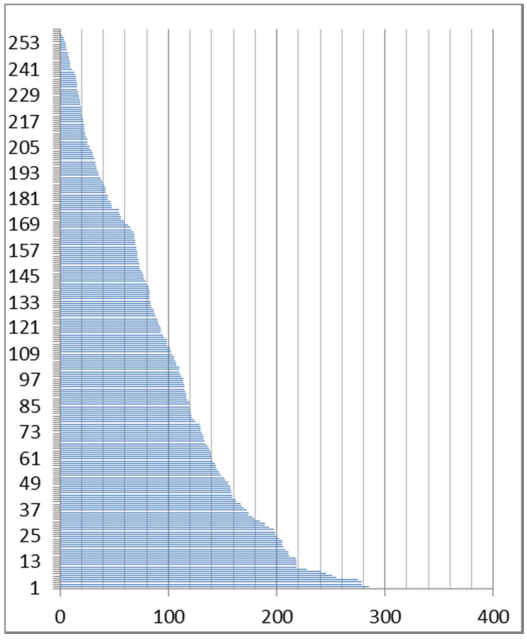

Figure 7. *IgG Cons. (Ru/ml) among patients, there is very high significant correlation between pathogenic bacteria (Strepto. pyogenes) and H. pylori infection.*

5. Discussion

Most confirmatory tests for bovine brucellosis are more

complicated and more expensive to perform. In this study, developed indirect ELISA using intact antigen for the first time, can reduce the cost of import ELISA kits. In addition to increase the sensitivity of the test; while whole Brucella obtained from vitro culture can provide a relatively stable source of a wide range of antigenic determinants, perhaps including some which are not available in the soluble antigen preparation.

In this study, reactivity of 50 serum samples collected from lactating cows in Shriq Al-neel locality (Kuku scheme), Khartoum State were tested by Rose Bengal Plate Test (RBPT); and the seroprevalence was 60%. It was higher than that reported in the previous study about 4 years ago, where the overall seroprevalence of bovine brucellosis within the milking cows was 32% and 38.8% using RBPT and sELISA, respectively. It was also higher than the seroprevalence of 27% by RBPT and 24.4% by sELISA reported by Salman and his colleagues [22].

The prevalence was also higher than that reported in the previous study among all localities of Khartoum State that estimate the average rate for the State was found to be 27.5%. and the weighted average was 25.1% prevalence of Brucellosis using Rose Bengal test [10]; suggesting that the prevalence of the disease is growing and the indirect ELISA and RBT of this study is growing. In 2009 the prevalence in Kuku scheme was 30.1% [23].

However using modified ELISA the detecting levels of Brucella was higher (46) than the detecting level using Rose Bengal. The obtained result also cope with the truth that although the ELISAs are more specific than the RBT, sometimes they do not detect infected animals which are RBPT positive

Rose Bengal false negative may be due to prozoning or because the antibody produced is only IgG isotype in acute infection cases, bearing in mind that IgG is less agglutinator than IgM. ELISA used to detect more positive sera since it can detect all isotypes.

Thus by intact brucella ELISA we can show the sensitivity of the ELISA to detect the positives and negatives samples. The cut-off point of intact brucella ELISA was determined using 5 negative controls and sera and it was 0.2 OD which is the cutoff point according to the kit from veterinary research laboratory; that means those samples results higher than 0.2 OD value will be considered as positives.

We have thus demonstrated that the use of intact Brucella in an ELISA to detect anti Brucella antibodies would be practical method and useful in epidemiological studies or for early diagnosis and we recommend to introduce and validate this method in massive bovine herds diagnosis programs in the field to establish its limits.

This study showed the ability of ELISA test using intact *Brucella abortus* to detect the titer of Antibodies on the serum of animals suspected with Brucellosis. We agree with Chachra and co-workers [24]; that in order to get a definite diagnosis of brucellosis, a combination of RBPT and Dot-ELISA should be used, especially in case of samples found negative by either RBPT or STAT used alone or in combination.

6. Conclusion

This is the first study in which intact brucella (whole cell) antigen to be used in ELISA, and it has been found to be significantly valid approach.

Intact ELISA technique is sensitive and specific which expected to detect only antibody of *B.abortus* so cross-reaction with other bacteria can be eliminated. The specificity and sensitivity of the new method make it quite promising for the future and therefore be useful for diagnosis and epidemiological surveys, and may reduce the dependence on imported, expensive commercial materials.

7. Recommendations

Sera from cattle infected with related microorganism (E.coli, Yersinia…) should be tested in this type of ELISA to investigate the cross reaction.

Local production of antibovine conjugate is needed for more minimizing ELISA cost and application.

This type of ELISA is recommended to be used for immunodiagnosis and epidemiological survey.

Study the stability of intact brucella antigen in the plate upon storage on -20°C, 4°C and at room temperature for long time and undder different conditions.

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