Isolation of Campylobacter bacteria that causing diarrhea in patients of Tikrit city and diagnose it by polymerase chain reaction PCR

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Wahran Khudhair Abbas Al-douri
Tikrit University-Education College

Dr. Osama Nadhom Nijris
Samaraa University-Practical Science College

Summary

This study has been included collection of one hundred fifty 150 stool samples which were 120 watery and 30 once bloody, collected from children that affected by diarrhea there age range is 1-5 years, who admitted to Tikrit teaching hospital, at time from September 2012 till March 2013. Campylobacter isolated from 5 samples by using classical methods of diagnosis which include phenotypic tests represented by studying culturing feature of growing groups by noting the shape, color and size of these groups, in addition to studying of microscopic features of these groups throughout preparation of gram stain and doing of biochemical and physiological tests as oxidase, catalase, urease, hippurate hydrolysis test, discovery of H₂S, their ability to grow in 27, 37, 42 centigrade degree of temperature, nitrate reduction test, their ability to tolerate salt, growing on macConky solid media, motility test and sensitivity to the antibiotics as nalidixic acid and cephalothin. All these tests reveals that's all the causative agents of diarrhea belonging to species of C.jejuni. Isolation was been achieved by two methods; the first one by use of selective media (Campylobacter agar), and the second by use of Millipore filters of 0.45 μm diameter, this percentage represents just 3.3% of total number of samples, divided as 3 watery stool samples and 2 of bloody stool samples. Comparison was made between results of classical diagnosis methods and advanced methods of diagnosis by use of PCR, PCR diagnosis methods did by using primers that’s antagonize the DNA chain which carry Vac gene, the gene responsible for production of C. jejuni special toxin (Vacuolating cytotoxin), and SADC gene that’s functioning as serine amino acid transporter protein producer. The results obtained by use of PCR are showing presence of Vac and SADC genes in 4 samples only, while the 5th sample did not give any result, which was one of watery stool samples. For that the diagnosis by PCR more accurate than the diagnosis by classical methods.

Introduction

Diarrhea and it's Complication considered as a major cause of death among children especially in developing countries, it is commonest second cause of death in children below 5 years of age in the world [1], it's cause due to different types of pathogens that differ from developing to developed countries. These pathogens may be viral, bacterial, protozoal or mixed infections of these pathogens [2].

Campylobacter bacteria is considered as an important cause of acute diarrhea in human, which increases in the last decades of 20th century [3]. In addition to infection of domestic animal that is widely spread [4]. Despite that this increase can help in the best investigation and diagnoses of bacteria, it decreased

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since the beginning of 21st century tiu 2003 [3].Campylobacter genus contains several species include C. jejuni, C. coli, C. fetus, C. lari, C. upsaliensis and C. hyointestinalis. All these species are pathogenic. It's source is contaminated food causing diarrhea in addition to extraintestinal symptoms and other diseases [5].

C. jejuni is the commonest cause of diarrhea in human. Alone it cause about 400 millions case of diarrhea annually. Thus most of studies directed toward C. jejuni [6]. Symptoms of gastroenteritis caused by C. jejuni vary from simple, that heal spontaneously like watery diarrhea to acute characterized by acute abdominal pain, vomiting and bloody diarrhea which necessitate use of antibiotics and replacement of lost fluids [7].

C. jejuni may spread directly from the intestine to cause infections outside the intestine, like bacteremia, septicemia, pancreatitis, cholecystitis, peritonitis, pericarditis, meningitis, in addition to hepatitis [8]. It can cause autoimmune diseases like reactive arthritis and Guillian-Barre syndrome [9,10].

Campylobacter bacteria lives as coexistence in intestines of several warm blooded animals like birds that considered as natural reservoir of C. jejuni, because it's normal body temperature about 42 C°, an optimal temperature for growth of bacteria. So birds are considered as amine source of it's transmission by it's contaminated meat and other products.

Stool of reservoirs animals or infected animals by this bacteria like cattels, sheeps, pigs, monkeys, rabbets, cats and dogs is major source of environmental contamination through contamination of foods and water [11,12].

Campylobacter bacteria needs special selective media and special aerobic conditions in addition to temperature differ from that used to incubate another bacteria, So the classical methods used in laboratories not enough to culture and growth of this bacteria [13].

Studies rehered to the possibility of rapid diagnosis of Campylobacter in stool specimens through the direct microscopy examination it by simple dye like carbol foxin in concentration of 1%, this method investigates for alive and dead bacteria [14], and to confirm the diagnosis, biochemical, surgical and genetic tests are used [15].

Genetic methods are advanced and capasel of detecting the species that can't detected by serological methods because genetic methods depend on the difference in the fixed number of chromosomes in compared with serological methods that depend on the antigenic properties which not fixed, from these genetic methods is polymerase chain reaction [16].

Current study done to achieve the following objects:

1- Isolation of Campylobacter bacteria from stool samples of children affected by watery and bloody diarrhea.

2- Diagnosis of bacteria species belong to this genus by using the classical phenotypic, biochemical and physiological methods.

3- Using the molecular studies by polymerase chain reaction technique to confirm the diagnosis of isolated species.

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**Methods of Work**

**Collection of samples**

One hundred and fifty stools samples were collected from children affected with diarrhea aged from 1-5 years in Tikrit teaching hospital in saladin government. Through the period of September 2012-March 2013, part of these samples was collected by swab and put in tubes containing transmitted media which is peptone water, transported to the lab in period not exceed 3-6 hours [17].

**Laboratory diagnosis**

1- Macrosopic examination of stool.
2- Direct staining.
3- Culture of samples on selected media like campylobacter agar and chocolate agar.
4- Morphological and cultural characteristics.

**Biochemical tests**

1- Oxidase test.
2- Catalase test.
3- Urease tast.
4- Hippuret hydrolysis test.
5- Production of hydrogen sulfate test.
6- Growth in 25 C°,37 C° and 42 C° test.
7- Nitrate reaction test.
8- Salt tolerat test.
9- Growth on MacConkey agar.
10- Motility test.
11- Sensitivity to Nalidixic acid and Cephalothin test.

**DNA extraction**

Extraction of DNA was performed from 5 stool samples of affected children, previously diagnosed by classical biochemical methods as *Campylobacter* bacteria this extraction was done by using previously prepared solutions by OMEGA/USA 2012 company and steps of work followed as mention by the leaflet accompanied the extraction tooles.

**Primers**

The extracted bacterial DNA from bacterial isolations was used as template in polymerase chain reaction technique to investigate about the Vac gene which is responsible for production of vacuolating cytotoxin specific for *C. jejuni* and SADC gene responsible for production of transporter protein of serine amino acid and this investigation is considered as qualitative.

Qualitative primers were used in sequences as in table (1) to detect specific consequence of Vac and SADC genes according to Bin Jasass and Parker (2009) which provided by Bio Basic/Texas-USA company [18].

Polymerase chain reaction used to magnify genetic pieces of Vac and SADC by mentioned primers that provided as powder (Lyophilized) and dissolved in volume of 1000 micro liter of distil water to achieve the desired concentration in Pom/µL unit, so the concentration were prepared to achieve a stored solution for each primer.

These primers stored as lyophilized in test tubes specific for this purpose in room temperature, then 1 milliliter of distil water was add to dissolve all the powder of primers to prepare stock solution and this solution stored in -20 C°.
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<table>
<thead>
<tr>
<th>Component</th>
<th>20 µl/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA polymerase</td>
<td>1 U</td>
</tr>
<tr>
<td>Each:(dATP, dCTP,dGTP,Dttp)</td>
<td>250µM</td>
</tr>
<tr>
<td>Tris-HCl (pH 9.0)</td>
<td>10Mm</td>
</tr>
<tr>
<td>KCl</td>
<td>30mM</td>
</tr>
<tr>
<td>Mg Cl₂</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>D.W</td>
<td>14µl</td>
</tr>
<tr>
<td>Primer F</td>
<td>1 µl</td>
</tr>
<tr>
<td>Primer R</td>
<td>1 µl</td>
</tr>
<tr>
<td>DNA template</td>
<td>2 µl</td>
</tr>
</tbody>
</table>

Table (3) conditions in PCR

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
</table>

Table (1) used primers and it’s characteristics

F: forward.; R: Reverse (the final concentration after adding 1 mililiter of distil water).
Bp: Base pair.
Tm: melting temperature (temperature according to characteristic mentioned by the company)

Procedure of polymerase chain reaction technique

1st: preparation of polymerase chain reaction

The procedure done in volume of 20 microliter depends on the leaflet of PCR premix provided by BioNEER/coria company to find the DNA by using primers designed for this purpose which produce bands in certain volumes. This procedure is done by using PCR micro tubes in volume of 0.2 mililiter according to table (2).

Reaction contents are mixed by vortex mixes then centrifuged for 10 seconds in microcentrifuged to precipitate drops of solution that presented on the tube wall.

After completion of all additions, tubes preserved on thermocycles carefully to complete the reaction and by using the program of Bin Jasass and Park (2009) as shown on table (3) [18].

Table (2) components of PCR Premix mixture

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<table>
<thead>
<tr>
<th>Initial denaturation</th>
<th>94 C°</th>
<th>3 min</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>94 C°</td>
<td>45 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>50 C°</td>
<td>1min</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72 C°</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72 C°</td>
<td>10 min</td>
<td>1</td>
</tr>
</tbody>
</table>

After ending of reaction time, samples pulled fro the thermocycles and 0.4 microliter from cutter enzyme added and samples put in incubator at 37 C° for 15 minutes and then the doubled samples of nucleic acid pulled and this mixture put in agarose gel in 1.5 % concentration.

2nd: Electrical deportation(expulsion) on agarose gel

Sambrook and Rusell method (2001) was used for electrical deportation to examine the DNA to show PCR results, and the exportation results were examined by using gel documentation device and DNA bands are measured in compared with DNA ladder and pictured by using digital camera with high efficacy and saving program in gel documentation [18].

Results and Discussion

Isolation

This study includes 150 samples of stools of children affected with diarrhea range from 1-5 years of age in a period from September 2012-March 2013. These samples were as watery diarrhea (120) and bloody diarrhea (30). From these samples, only 4 were of C. jejuni represent about 2.66%, 2 of them (1.6%) were of watery diarrhea and the other 2 (6.6%) of bloody diarrhea.

These results were compatible with same local and national studies that depending of PCR technique in diagnosis, in Aljubori study (2012), C. jejuni isolation ratio was 2.35% in form of 8 isolations from 340 stool samples of children with diarrhea [19]. In Ali study (2006), C. jejuni was isolated in ratio of 3.8% from children with diarrhea [20].

In case of studies that depend on classical phenotypic and biochemical testes in diagnosis of C. jejuni, the results of isolation were more than the results of isolation of studies depending on PCR technique in the diagnosis, from these studies Witwit study (2010) it's results were about 9.6% from children with diarrhea [21]. Where the results of Salih and Al-saad (1994) were about 10% [22] and Rizal and his group (2010) obtained about 8.5% [23].

Diagnosis

1st: the diagnosis genus level

Campylobacter bacteria was diagnosed to genus level by using the classical diagnostic methods like direct staining of stool samples, examination of stained samples with carbol fuchsine stain 1% show that 12 stool samples were positive to staining which means that these samples contain Campylobacter bacilli of spiral shape or like gull bird wing.

These samples include 9 samples (7.5%) of watery diarrhea which equal to 6% of the total of samples of this study and 3 samples (10%) of bloody diarrhea which...
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equal to 2% of total number like the down diagram.

Another methods of classical diagnosis is the growth culture of selective primary isolation media of growth culture by using filter paper, the result of both types of growth culture is the appearance of bacterial colonies which like water drops, small transparent and pure, in addition to it's diagnosis by ordinary optical microscopic examination which show different shapes of Campylobacter bacteria as spiral, like S-letter, curved bacilli or gull bird wing.

Results of biochemical and physiological tests were showed in figure (4).

Figure (4) biochemical and physiological tests

<table>
<thead>
<tr>
<th>No.</th>
<th>Test</th>
<th>+ve/-ve</th>
<th>Result</th>
</tr>
</thead>
</table>

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<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oxidase</td>
<td>Changes of colonies color to purple</td>
</tr>
<tr>
<td>2</td>
<td>Catalase</td>
<td>Bubbles formation within 10-30 second</td>
</tr>
<tr>
<td>3</td>
<td>Urease</td>
<td>No urease enzyme production</td>
</tr>
<tr>
<td>4</td>
<td>Nitrate reduction</td>
<td>Chang of media from red to green</td>
</tr>
<tr>
<td>5</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;S production</td>
<td>No black precipitation</td>
</tr>
<tr>
<td>6</td>
<td>Growth at 25 C°</td>
<td>No growth</td>
</tr>
<tr>
<td>7</td>
<td>Growth at 37 C°</td>
<td>Moderate growth</td>
</tr>
<tr>
<td>8</td>
<td>Growth at 42 C°</td>
<td>Abundant growth</td>
</tr>
<tr>
<td>9</td>
<td>Motility</td>
<td>Rapid growth like dart</td>
</tr>
</tbody>
</table>

2<sup>nd</sup>: The diagnosis to species level

This diagnosis is done by biochemical and physiological tests to know the species belong to *Campylobacter* genus in samples of this study which show that all of them are *C. jejuni* as in figure (5).

**Figure (5) biochemical and physiological tests**

<table>
<thead>
<tr>
<th>No.</th>
<th>Test</th>
<th>+ve/-ve</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hippurate hydrolysis</td>
<td>+ve</td>
<td>Changes color of media from transported yellow to dark purple</td>
</tr>
<tr>
<td>2</td>
<td>Salt tolerant</td>
<td>-ve</td>
<td>No growth</td>
</tr>
<tr>
<td>3</td>
<td>Growth on MacConkey agar</td>
<td>-ve</td>
<td>No growth</td>
</tr>
</tbody>
</table>

Genomic DNA isolation

Genomic DNA isolation depends on multiple buffers solutions provided by OMEGA company which give an easy and accurate isolation of DNA that represented by chemical substances used for isolation solutions. Each substance acts to remove one of undesired cellular component and at same time protect the DNA as the target of extraction.

The amount of genomic DNA resulted from this method is abundant which about 200-500 microgram for each 3 mL of bacterial growth for each newly reactivated sample. The purity and concentration of DNA were assessed by adding 1 microliter of extracted sample in nano drop in which the purity ranges from 1.6-1.8 and the result was DNA in concentration of 115-160 Nanogram/milliliter [24]. Studies referred to if the purity more than 2, means contamination with RNA while if the ratio less than 1.6, means contamination with proteins.
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Polymerase chain reaction

PCR technique has high sensitivity and specificity make it's ability to diagnose C. jejuni in clinical samples [18]. Because there is no information about the presence and genetic recurrence in the clinical samples of C. jejuni that colonize hospitals in Iraq generally and Tikrit city specially so the current study considered C. jejuni investigation through discovering the presence of particular and specific genes by using PCR technique to compare between the accuracy and efficacy of this technique and other classical diagnostic methods.

The 5 samples previously diagnosed by classical methods as C. jejuni undergo an electrical translation and the result was appearance of bandas 705 bp resemble the based pair 700 of DNA ladder for only 4 isolations specific for Vac primer and the appearance of band as 1750 bp resemble the based pair 1517 of DNA ladder specific for SADC primer for same isolations that show Vac gene as shown in picture 1. From this we conclude that from 5 isolations diagnosed by classical methods, only 4 isolations are C. jejuni depending on mentioned genes.
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Results of current study are compatible with some studies depending on PCR technique in the diagnosis. Al-sibahee study (2010) had 4 isolations of C. jejuni in ratio of 2.1% from 186 stool samples while Andrew and his group (2003) had 2 isolations from 97 sample in ratio of 2.06%.

The result also was compatible to Scott (2005) where they isolate. From 129 sample only 5 isolations in ratio of 3.87% of C. jejuni from cases of diarrhea in adults while Ismael (2002) had this bacteria in ratio of 1.6%.

References
1) Forsberg, B. C.; Patzold M. G. and Cooke, M. (2007). Diarrhea case management in low and middle

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