Investigation of the Best Available Diagnostic Method of Intestinal Parasites in Stool Samples to Use in Hospital's Routine Exam in Baghdad

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Abstract
The study was conducted to determine the best diagnostic method which can use for daily routine stool examination in our hospitals and health centers, an attributive evaluation of three conventional diagnostic methods: direct wet mounts, formalin-ethyl acetate sedimentation, and zinc sulfate floatation, beside acid-fast staining technique was conducted for randomly-chosen 250 stool samples obtained from people who attended AL-Shaheed AL-Sadr hospital in Al-Sader City in Baghdad during Jan. 2011-Dec. 2012. The results illustrated that 52.4% (131/250) stool samples were contained at least one parasite by all evaluated diagnostic methods. Ova and cysts were detected by all used methods, floatation showed the highest detection rate 60% (90/150), followed by sedimentation 58.7% (88/150), while direct wet mount exam showed the lowest detection rate 39.3% (59/150). On the other hand, direct wet mount exam was exclusively responsible for detection of all trophozoites, whereas AF staining was responsible for all detected oocysts. Thus, the present study suggested multiple diagnostic methods for stool samples by examine all received stool samples by direct wet mount microscopic exam. Of these, any sample give -ve result then must be submit to floatation method and acid-fast staining so as to give more accurate results in suitable time, cost, and efforts.

Keyword: diagnostic methods, stool samples, intestinal parasites

التحري عن أفضل طريقة تشخيصية للطفيليات المعوية في عينات الغائط

لسلاسل الفحص الروتيني في المستشفيات في بغداد

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الخلاصة:
أجري البحث لتحديد أفضل طريقة تشخيصية يمكن استعمالها في الفحص الروتيني اليومي لعينات الغائط في المستشفيات والمراكز الصحية، تم إجراء تقييم وصف تخفيض، تقليدي، التصوير، الترتيب واستعمال القوائم - خلاصات الأولى والتفصيل باستخدام كربات الزرك فضلاً عن تقنيات التصويص الصادمة لفحص 250 عينة غائط مختارة عشوائياً لجمع المراجعين للمستشفى الشهير الصدر في مدينة الصدر بيرت النتائج أن 52.4% (131/250) عينة كانت تحتوي على طفيلي واحد على الأقل

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Introduction:

Intestinal parasitic infections are amongst the most common infections worldwide. About 3.5 billion people are affected resulting in 450 million cases of illness with the majority being children. These infections cause iron deficiency anemia, growth retardation in children and other physical and mental health problems so they represent as serious public health problem [1]. Till now, intestinal parasites are among the major contributors to the global infection burden as they are prevalent in different parts of the world such as *Ascaris lumbricoides*, *Entamoeba histolytica*, *Cyclospora cayetanensis*, *Giardia lamblia*, and *Cryptosporidium* spp. [2]. Despite its wide prevalence, controversy still surrounds the best means of intestinal protozoans diagnosis. Microscopy of direct fecal smears or smears prepared following formol-ether concentration and iodine staining has been reported to reach 97% sensitivity if three stool samples are examined [3].

Conventional microscopy of three stool samples (with or without concentration techniques) is still being recommended as the reference standard (gold standard) [4]. However, studies have been conflicting with some reports finding microscopy of direct smears without preservation as low as 50% sensitive [5], while others suggest that there is little diagnostic gain from more invasive and expensive testing [3, 6-9]. Last years, a remarkable development in sensitivity and specificity of diagnostic methods in virology, bacteriology, physiological have been observed in our health institutions. Unfortunately, such a development did not include the laboratories of parasitology which are until now depends only the old conventional direct wet saline mount may be because of the time-consume and high-cost of other diagnostic methods. Thus, the goal of the present study is to determine the best diagnostic method which is applicable for daily routine stool examination in laboratories to improve the detection rate of intestinal parasites.

Materials & Methods

Stool Samples Collection

Two hundred fifty stool samples of ≥2 gram weight were collected randomly from patients with different ages who came to the Parasitology Laboratory in AL-Shaheed AL-Sadr hospital located in AL-Sader City in Baghdad, suffering from gastrointestinal complaints with or without diarrhea, and workers who came to the hospital for annual examination during Jan. 2011- Dec. 2012. Samples were received in 60 ml clean wide-mouth plastic cups. The patient’s name, gender, age were fixed on each cup and diagnosed immediately.

Laboratory Diagnostic methods

All stool samples were submitted to the following:

1- Macroscopic examination by notice the consistency, color, odor and presence of blood and mucus.

2- Microscopic examination by direct wet (saline and iodine) mount

3- Microscopic examination after concentration methods:

A- Formalin-ethyl acetate sedimentation [10]. When the sediment was not examined immediately because of hospital’s laboratories work, it was transferred with a pipette to another tube and 5ml of normal saline was added. The suspension was centrifuged for 2min. at 1500rpm. After supernatant discarding, the sediment resuspended in 1ml normal saline. This step was performed to ensure more slide clearance and enable examination to be for a relatively long time.

B- Zinc sulfate floatation [11]. Minor modification was used to collect the concentrate from the surface; about 1-1.5 ml of the solution’s surface was carefully transferred with Pasteur pipette to another tube. Then normal saline was added till the suspension reached 5 ml; the tube was shaken well

And in this manner several aliquots were taken for microscopic examination.

Results and Discussion

Table 1 shows the sensitivity of different microscopy methods applied in the present study. The microscopic examination of direct smears without preservation showed 50% sensitivity, while microscopic examination after concentration techniques showed higher sensitivity reaching 91% and 92% for zinc sulfate floatation and formol-ether concentration, respectively. These results are in agreement with available literature [3, 4] that conventional microscopy of three stool samples (with or without concentration techniques) is still being recommended as the reference standard (gold standard) [4]. However, studies have been conflicting with some reports finding microscopy of direct smears without preservation as low as 50% sensitive [5], while others suggest that there is little diagnostic gain from more invasive and expensive testing [3, 6-9].

In conclusion, the present study confirms the importance of applying concentration techniques in order to increase the sensitivity of microscopic examination of direct smears. This study recommends the implementation of formalin-ethyl acetate sedimentation method as the most sensitive and time-efficient technique for daily routine stool examination in laboratories.
then centrifuged for 2 min at 1500 rpm, then the supernatant was discarded and the sediment resuspended in 1 ml normal saline. This step was performed to prevent cysts from collapsing under the high osmotic pressure of the zinc sulfate solution when they are left longer than 10-15 min before microscopic examination. Besides, this step ensures good quality and facility of staining procedure in the following item.

4-Microscopic examination after concentration method and staining with modified Ziehl-Neelsen (acid-fast stain) [12].

**Attributive Evaluation of Diagnostic Methods**

An attributive comparison between the diagnostic methods used in this study was done on a basis of significant attributes, which are:

1) Effectiveness in recovery of parasites' species and numbers.
2) Cost of materials.
3) Time of work.
4) Ease of use.
5) Clarity of parasite morphology.
6) Quality of smear background.
7) Batch testing which refer to the ability to process a large number of samples at a time [13]

**Statistical Analysis**

Chi-square test, under $P$ value $\leq 0.05$, was performed in order to investigate the presence of significant differences between direct wet mount, sedimentation, and floatation methods in ova/cysts detection.

**Results & Discussion**

The diagnostic methods in the present study were chosen particularly as they are the most conventional low-cost diagnostic methods that used in surveys and epidemiological research especially in developing world [14-20] Cysts & ova were detected by the three methods while trophozites were detected exclusively by direct wet mount, and oocysts were detected by acid-fast staining method. Diagnosis by the all used methods showed that 131/250 (52.4%) examined stool samples gave + ve results for at least one parasite, 122 (61%) belong to patients group, while 9 cases (18%) belong to workers group. Table 1 demonstrates the overall detected parasites.

| Table 1- The total detected parasites by all the diagnostic methods used in the present study |
|---------------------------------------------------------------|-----------------|------|
| Parasite(s)                                                 | No. of cases    | %    |
| *worker samples                                             |                 |      |
| * Giardia lamblia                                           | 4               | 8%   |
| * Entamoeba histolytica/Entamoeba dispar                     | 2               | 4%   |
| * Enteromonas hominis                                       | 1               | 2%   |
| * Blastocystis hominis                                      | 1               | 2%   |
| * Iodamoeba butchilii+ G. lamblia +E. histolytica            | 1               | 2%   |
| * Overall infections                                         | 9               | 18%  |
| Free of parasites                                           | 41              | 82%  |
| Total number of workers                                      | 50              | 100% |

* Patients samples

<table>
<thead>
<tr>
<th>Parasite(s)</th>
<th>No. of cases</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. histolytica</td>
<td>12</td>
<td>6%</td>
</tr>
<tr>
<td>G. lamblia</td>
<td>41</td>
<td>20.5%</td>
</tr>
<tr>
<td>E. histolytica/E. dispar</td>
<td>33</td>
<td>16.5%</td>
</tr>
<tr>
<td>B. hominis</td>
<td>14</td>
<td>7%</td>
</tr>
<tr>
<td>Trichomonas hominis</td>
<td>2</td>
<td>1%</td>
</tr>
<tr>
<td>Chilomastix misnilli</td>
<td>1</td>
<td>0.5%</td>
</tr>
<tr>
<td>Dientamoeba fragillis</td>
<td>1</td>
<td>0.5%</td>
</tr>
<tr>
<td>Cryptosporidium spp.</td>
<td>11</td>
<td>5.5%</td>
</tr>
<tr>
<td>E. histolytica +T. hominis+ B. hominis</td>
<td>1</td>
<td>0.5%</td>
</tr>
<tr>
<td>G. lamblia + Cryptosporidium sp.</td>
<td>1</td>
<td>0.5%</td>
</tr>
<tr>
<td>G. lamblia + E. histolytica</td>
<td>1</td>
<td>0.5%</td>
</tr>
<tr>
<td>G. lamblia + B. hominis</td>
<td>1</td>
<td>0.5%</td>
</tr>
<tr>
<td>G. lamblia + E. histolytica/E. dispar</td>
<td>1</td>
<td>0.5%</td>
</tr>
</tbody>
</table>
Within the 131 samples, there are 141 detected parasites (as some samples were co-infected) and 150 detected parasite stages as follow:-

<table>
<thead>
<tr>
<th>Parasite Combination</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. histolytica/E. dispar + T. hominis</td>
<td>1</td>
<td>0.5%</td>
</tr>
<tr>
<td>G. lamblia + Hymenolepis nana</td>
<td>1</td>
<td>0.5%</td>
</tr>
<tr>
<td>Overall infections</td>
<td>122</td>
<td>61%</td>
</tr>
<tr>
<td>free of parasites</td>
<td>78</td>
<td>39%</td>
</tr>
<tr>
<td>Total number of patients</td>
<td>200</td>
<td>100%</td>
</tr>
<tr>
<td>Total infection rate (worker &amp; patients)</td>
<td>131</td>
<td>52.4%</td>
</tr>
</tbody>
</table>

* Bloody diarrhea

For cyst/ova detection, the result showed that the highest detection rate of cysts /ova was obtained by floatation with 90/150 detected cases (60%), sedimentation came secondly with 88/150 detected cases (58.7%), while the lowest detection rate of cysts/ova was obtained by direct wet mount exam with only 59/150 detected cases (39.3%). Statistically, there was a significant difference between the diagnostic methods with the priority to floatation and sedimentation. The following figure demonstrates the species and number of detected parasites with each diagnostic method figure. 1.

**Figure 1** - Number of detected cysts/ova by each of direct wet mount, sedimentation, and flotation methods.

This result is in line with previous studies which confirmed that the concentration methods were more efficient in intestinal parasitic detection [17-23] all these studies showed that there was a significant increase in the number of detected parasites by concentration methods against direct exam. The efficacy of concentration methods in this study and studies above-mentioned due to ability of detect parasites even if they were in small number, also when larger sample's volume had been examined by concentration methods, they detected more than one parasitic species in the same sample. For trophozoites detection, the morphology and motility of trophozoites are important diagnostic characters that help in the identification of protozoa [24].

In the present study all reported trophozoites were detected by direct wet mount exam. For increasing efficacy of direct microscopic exam, iodine wet mount. It enhances the detail of protozoan cysts especially the glycogen vacuoles and nuclei [25-26]. Stool samples containing trophozoites were also diagnosed by concentration methods. There was no any trophozoite detected neither by sedimentation nor floatation expect in some cases trophozoites appeared but distorted and not typical and thus could not be relied upon it in the diagnosis process. Trophozoites are not detected using concentration methods because they cannot withstand the specific gravity of the concentration solution [27] besides centrifugation steps may be disintegrated them.

Oguoma & Ekwunife [22] found in their study that direct smear was effective in detecting for intestinal parasites in watery stools than formal ether concentration. This is in a line with results in the
present study because direct microscope exam is the only method that the trophozoites could be detected especially their motility beside cysts and ova in heavy infections. Based on the forgoing it is recommended to exam watery and loose stool samples before other soft and formed samples when they reach to the laboratory as soon as possible in order to detect the motile living trophozoites and the typical morphology of parasite, this view is consist with [26] who also recommended that liquid stool should be examined within 30 minutes of passage as it trophozoites are usually found in liquid stool. Semi-formed stool which may yield a mixture of protozoan cysts and trophozoites should be examined within 1 hour of passage while formed stool can be held for 24 hours following collection. Other advantages of direct microscopic exam compared to other diagnosis methods it is the cheapest exam, fast in preparation time, easy and simple for daily routine use, parasites appeared in clear and typical morphology especially in fresh stool, other important findings could be detected like RBCs, WBCs, Charcot-Lyden crystal, fungi .... etc, large number of samples could be process at a time but it is preferred to process 6-8 slide as a maximum especially in summer, because slides dry rapidly. In addition, for increasing efficacy of direct microscopic exam, iodine wet mount was used to detect intestinal parasites. It enhances the detail of protozoan cysts especially the glycogen vacuoles and nuclei [25, 26].

In the light of obtained result in the present study, we concluded that direct wet mount is the basic diagnosis methods in hospital work as it diagnosed cysts, and trophozoites (especially in loose and wetary stool samples) in 107/150 parasitic cases with detection rate 71.3% this rate was a good base to be increased by the other diagnostic methods. On the other hand, direct microscope exam was less sensitive than floatation and sedimentation and it could not detected parasites from light infections. This evaluation is similar to that of [28] who found that microscope examination may be represent a suitable method for detecting low levels of parasite infections and he suggested to exam 3 samples on different days to increase the sensitivity and use RealTime-PCR method as an alternative method for detecting pathogenic protozoa figure 2.

Figure 2- Number and percentage of detected cysts/ova, trophozoites, and oocysts by direct, sedimentation, floatation, acid fast stain among 150 parasitic stages in 131 fecal samples.

Advantage of sedimentation beside the higher sensitivity than direct microscope exam were included the clean smear background (especially after the washing step). Disadvantages of sedimentation in the present study were included the high cost compared with other method, time-consuming, many of the steps were involved and process a sample set at a time need more time than that in direct wet mount and floatation method. Other details were observed in application of sedimentation method, we found that this method need the largest fecal volume compared with floatation to produce sufficient sediment to exam. Moreover, remaining ethyl acetate droplets might be remixed with sediment and became troublesome as they obscured reading especially with iodine, also debris might be remixed with the sediment leading to dirty background. However, these problems have been solved by washing step.

Floatation was the best diagnostic method in several aspects table 2: high sensitivity in cysts detection, acceptable moderate cost, suitable time of work, easier than sedimentation as it include less steps, parasite cysts were clear in morphology but not after 20 minute in zinc sulfate solution because cysts became non-typical as they prone to shrinkage. Also the process of a sample set is simpler than
sedimentation. The major disadvantage of floatation was the dirty smear background which was obscure reading, likewise in sedimentation washing step solved this problem.

**Table 2-** Ranking diagnostic methods on a basis of some attributes

<table>
<thead>
<tr>
<th>Attributes</th>
<th>Direct exam.</th>
<th>Floatation</th>
<th>Sedimentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-effective recovery of parasites spp. &amp; n.</td>
<td>3*</td>
<td>1*</td>
<td>2*</td>
</tr>
<tr>
<td>2-cost of materials</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>3-time of work**</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>4-ease of use</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>5-clarity of parasite morphology</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>6-quality of smear background***</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>7-batch testing</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

**Ranking scale:** 1, high; 2, moderate; 3, low level

**Time of microscopic reading was constant for all**

**With modified step, background quality became almost equal in floatation & sedimentation**

Other observations related with stool consistency, we found that number of cysts in formed stool increased remarkably by concentration methods, while in loose and watery samples especially mucoid samples, the number of cysts was nearly the same or less than direct wet mount. This might be because the un-strainability nature of these samples. Fortunately, these samples usually represent acute stage of disease and contain a large number of parasites particularly in trophozoite stage which can readily diagnosed by direct wet mount. Also this reveals the relevance in protozoan infections as the presence of active trophozoites is usually associated with the loose to watery stools while cysts are more likely to presence in formed stools [29]. Both sedimentation and floatation methods were only responsible for detected 31/150 cysts cases with detection rate 20.7%. This considerable value was important as a step forward in decreasing false-negative results. As concentration methods are more sensitive than direct we mount especially in formed and soft stool samples, the study suggest to get benefit from these methods in epidemiological researches and population based prevalence. These studies are not constrained by a time limit for the completion.

After concentration step by either sedimentation or floatation, acid-fast stain was used for the detection of *Cryptosporidium* spp. oocysts, result showed that 12/250 cases of *Cryptosporidium* was detected with infection rate 4.8%. 11 cases of single infection with *Cryptosporidium* sp, while single case was co-infected with *G. lamblia* cysts. This infection rate was clearly lower than other studies in Baghdad as that of [30] who found that the infection rate of cryptosporidiosis was 14.78%, similarly, Yaqoob et al. [31] found that the infection rate was 12.4%. This different results may reveals the difference in each study circumstances like study population, demographic status, number of examined samples and the staining procedure that use in each study.

Based on the results aforementioned and regarding to the specificity of work in health institution, the present study recommended to reliance on multiple diagnostic methods upon single received stool sample as follows figure. 3:

**First,** all received stool samples should be examined by direct wet mount, in two preparations at least: with normal saline and iodine stain.

**Second,** all samples that gave -ve result with direct wet mount must be submitted to:
1- Concentration by zinc sulfate floatation method.
2- After concentration, staining with acid-fast stain.
Figure 3- Illustrating diagram for the recommended protocol of daily exam of stool samples in hospitals and health centers.

Indeed, several conditions must be taken into consideration in order to evaluate diagnosis methods that fit the work of hospitals where the time is crucial factor like emergency cases, number of technicians in laboratory, their experiences, number of samples that they can handle, tools and equipments they have, in addition, the volume of fecal sample brought by the patient, the freshness of it, the number and species of parasite. We believe that this work procedure is suitable and necessary to apply in our hospitals and health centers as it reduce the false-negative result, give higher sensitivity, and reduce time lose as possible.

References