Scope and aims

Libyan Journal of Veterinary and Medical Sciences (Libyan J. Vet. Med. Sci.) is a biannually journal that publishes peer-reviewed papers on the fields of veterinary and basic medical sciences. Contributions may be in the form of original research or review articles and case reports. The goal of this journal is to provide a platform for scientists to promote, share and discuss various new issues and developments in all aspects of veterinary and basic medical sciences.

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Preparation of the manuscript

1. General guidelines

All manuscripts including references, tables and figure legends should be typed double-spaced on one side of the paper with 12 font size and Times New Roman format, with at least 25mm margin. The manuscript should be numbered consecutively beginning from the title page followed by the abstract, text, acknowledgments, references, tables and legends. Lines should be numbered in all sections of the manuscript.

All manuscripts should be accompanied by a cover letter from the corresponding author which specifies the following:

i. The type of submission.

ii. The name of corresponding author and his/her complete mailing address, telephone and fax numbers.

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2. Title page

The title page of the manuscript should be in a separate page and include the following:

i. A concise and informative title of fewer than 200 characters.

ii. Full names of authors with first name, middle initial and last name of each author.

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v. Superscript numbers should be used to link the author with institution and an asterisk (*) should be used to refer to the corresponding author.

3. Abstract

An abstract with up to 250 words in one paragraph for original and review articles should be submitted. The abstract should contain the background, purpose(s) of the study, procedure(s), results and conclusions. For review articles, the abstracts should summarize the contents of the review. Up to six key words should also be provided with the abstract.

4. Introduction

This section should provide a clear and concise justification of the study including its relevance. The number of citations should be kept to the minimum. The final paragraph should state the hypothesis and the aims of the study.

5. Materials and methods

This section should include the design and the methodology of the study and should be presented in sufficient details. However, previously published methods should not be discussed in details but cited with appropriate references instead.

6. Results

The content of this section should be informative and accompanied by self-explanatory tables, figures or other illustrations if necessary.
7. Discussion

Its contents should be interpretative and based on the study results only. The discussion can be in a single section or it can be presented together with the results and conclusion.

8. Tables

Tables should be typed double spaced each on a separate page. Pages should be numbered consecutively with text. The table number and title should be provided above the table. Footnote for each table should be provided for identifying all used abbreviations.

9. Figure legends

Must be self explanatory, placed under the figure and all abbreviations should be identified.

10. Figures

The maximum allowed number is up to 10 for all types of publications and should be in a good level of resolution.

11. Acknowledgement

12. References

References in the text must be written in parentheses and provided as name and year of the publication. They should be written in chronological order, then in alphabetical order, in parentheses; for example: (Albrecht 1983; Meyer 1983; Muller and Frank 1985; Schmitz et al. 1988). If the author's name is mentioned in the text directly, then the year of publication is to be written in parentheses; e.g. according to Smith (2007). If there are more than two authors, then the first is named and the others are summarized under "et al.". All the sources cited in the text should be in the reference list. It is the entire responsibility of the author(s) to cross-check the references and citations carefully.

The list of references should be organized alphabetically based on the first letter of the first author surname.

The following citation method is to be used in the list of references:

**Journal article**

The title of the journal should be abbreviated according to the official abbreviations given in Medline (www.ncbi.nlm.nih.gov). The journal name should be abbreviated without any full stops and in italic format (e.g. *Vet Med*); the issue numbers should NOT be mentioned.


**Book**


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**Conference proceedings**


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**Online materials**

Only authentic online materials should be used.


13. Case report

Case report will be considered for publication only if it concerns unrecognized condition or offers new insight into pathophysiology, diagnosis or treatment of a disease. The abstract of case reports should not exceed 100 words and overall text not exceeds 2000 words including tables, figures and references.
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Abstract
Several molecular techniques that are commercially described in the literature for early detection of blood stream infection in attempting to overcome the limitations of the gold standard blood culture. SepsiTSTM is an CE marked commercial platform that has been described in the literature. However, there were no studies addressing the accuracy of the test as a diagnostic platform for detection bacteraemia in whole blood samples. The study, conducted to investigate and discuss the strategy of SepsiTSTM in comparison to our previously validated real-time PCR based assays (BactScreen™TM) and the previously published “in-house” minor groove binder (MGB)-based all bacteria assay. The three assays showed different sensitivity patterns for detecting bacteria in blood stream pathogens in favour of using BactScreen™ test. SepsiTSTM could be valuable but their lowest sensitivity in addition to their use of the unspecific SYBR Green fluoresce dye that question its diagnostic accuracy could be used as a last choice molecular diagnostic technique for detection of bacteraemia in whole blood samples. However, SepsiTSTM strategy rather, may provide useful diagnostic tool for detecting live pathogens in food technology.

Keywords: SepsiTSTM, BactScreen™, PCR, Bloodstream pathogens

Introduction
Current infection diagnosis is based on standard blood culture techniques. However, microbiological culture has several limitations, not least that it takes several days to confirm infection and is therefore not useful in directing the early treatment with antibiotics. New techniques based on detection of pathogen DNA using real-time polymerase chain reaction (PCR) technology have the potential to address these limitations but their clinical utility is still to be proved. Studies have suggested that levels of bacteria in the bloodstream of patients with sepsis can be as little as 3-10 CFU/ml (Arpi et al., 1989; Beekmann et al., 2005; Peters et al., 2004). Therefore, maximising assay sensitivity is extremely important when developing effective pathogen DNA assays. The effectiveness of pathogen DNA extraction from blood is also an important determinant of assay sensitivity (Millar et al., 2000).

Septi Fast was reported by Lehmann, at al to have a detection limit between 3-100 CFU/ml depending on the pathogen (Lehmann et al., 2008). Septi Fast of which is so far the most studied platform proved to be valuable and highly sensitive for ruling in of 25 blood stream pathogens (Dark et al., 2015), but are expensive, laborious and complex platform for daily routine use (Leggieri et al., 2010). Therefore, finding simple cost less and highly sensitive pathogen detection system continued to be requisite. SepsiTSTM is an alternative commercial available PCR assays described in the literature for its ability to detect broad range of bacterial and fungal pathogens. Although, the assay has gained CE marked for European use but no proper study has addressed their accuracy as a diagnostic platform for detecting of bacteraemia from whole blood samples (Stevenson et al., 2016). SepsiTSTM technique is a broad range PCR using universal 16S Ribosomal DNA primer for bacteria and universal 18S ribosomal DNA primers for yeast, coupled with fluorescent detection of the products by SYBR® Green.

The presence of yeast or bacterial DNA is confirmed by melting curve analysis, although the technique does not allow species identification. “in-house” all-bacteria assay is a probe-based system that uses primers targeting the 16S Ribosomal DNA of bacteria and a minor groove binding (MGB) probe for detecting of the PCR products described originally by Ott and colleges (Ott et al., 2004). MGB-based tests are believed to be more sensitive than the SYBR Green detection based assays and may also produce less non-specific PCR products. Unfortunately, the MGB assay is unsuitable for melting curve analysis due to the hydrolysis nature of the probe during the reaction. The BactScreen ToolSet is hybridisation probe–based assay specifically adapted for amplification of eubacterial 16S rRNA and distinction of Gram-Negative and Gram-Positive species by Light Cycler PCR with melting curve analysis (Al-Griw, 2011). Our study was aimed to investigate the feasibility and the accuracy of
Sepsitest™ assay for detection of bacterial pathogens from blood samples compared to the “in-house” all-bacteria assay that utilised hydrolysis probe and BactScreen™ assay that utilise hybridised probe.

The present study addressed the quality and quantity of isolated DNA from blood samples spiked with purified bacterial DNA and/or intact organisms using two extraction protocols, namely MolYsis and modified High pure PCR template preparation techniques. The two methods were then used to assess, the performance of the commercially available Sepsitest™ platform the LightCycler®2.0in comparison to the previously evaluated BactScreen™ (Al-Griw, 2011) and “in house” all bacteria primers and MGB probe on the LightCycler®480.

Materials and Methods

Spiking of Blood Samples with Intact Bacterial Cells

Blood samples were spiked with known amounts of intact bacterial. These were achieved by re-suspending 10⁶ CFU bacterial pellets obtained from culture with 1 ml EDTA treated blood samples proved to be clear from infection using Septi Fast assay. Using EDTA blood, 10-fold serial dilutions were prepared for each target bacterial and Genomic DNA was then extracted using different DNA extraction protocols as described below.

DNA extraction

All samples handling and genomic DNA extraction was performed under HEPA bio-safety cabinets class II using aseptic techniques for minimise the risk of contamination. Using MolYsis technique, Blood samples were processed according to the manufacturer’s instructions of the Sepsitest™ blood pathogen Detection Kit (Molzym GmbH, Bremen, and Germany). Briefly, 1ml of each spiked whole blood samples were subjected to human DNA depletion protocol following by microbial DNA extraction. The adsorbed pathogenic DNA is then eluted into 1.5ml DNA-free reagent tubes with 300 µl of preheated (70°C) DNA-free deionised water. DNA from the same spiked blood samples were extracted using high Pure PCR Template Preparation Kit combined with Red Cell Lysis Buffer (HP-RCL).

This extraction involves lysis and removal of red blood cells before pathogen DNA extraction. Briefly, 1 ml of each spiked whole blood samples were added to 2ml of red cell lysis buffer in a 15ml sterile Falcon tube. The contents were mixed by inverting the tubes several times, placed on a rocking platform for 10 min at room temperature and centrifuged at 2,500 rpm for 5 min. The clear red supernatant was then removed by pipetting and the lysis steps were repeated for another time when required. This washing step removes any remaining lysate, leaving pellets that contain white blood cells and any intact pathogen cells. RCL pellets were then re-suspended into 200µl PBS and transferred into nuclease-free 1.5 ml micro-centrifuge tubes and processed according to the manufacturer’s instructions of High Pure PCR template preparation kit(Roche Diagnostics GmbH) with the following modifications, use of 500µl of binding buffer, 80µl of proteinase K and 200µl of isopropanol. The isolated DNA from each tube was eluted in 200µl of pre-warmed elution buffer and stored at -80°C for further use.

Molecular diagnostics

The three PCR assays Sepsitest™, Bactscreen™ and “in-house” MGB-based all bacteria assay were carried out using the reagents provided with their kits. Sepsitest™ uses universal primers specific for 16sRNA bacterial sequences covering gram negative and gram-positive bacteria. Each reaction mixture contained; 8 µl DNA-free water, 8 µl 2.5x Mastermix, 2 µl 10x DNA staining solution, 0.8 µl MoTaq 16S enzyme and 2 µl of template DNA. After centrifugation; the PCR amplification was carried out in 20 µl glass capillary tubes in the LightCycler®2.0 instrument according to the following parameters: Pre- incubation cycle at 95°C for 1min, followed by 40 amplification cycles (denaturation at 95°C for 5 seconds, annealing at 55°C for 5 sec and extension at 72°C for 25 sec), a Tm analysis program for one cycle (95 °C, 65°C, 95 °C continuous and then a cooling period to 40 °C for 30 sec.

The methodology of “in-house” MGB-based all bacteria assay was described by Ott and co-workers using universal primers combined with minor groove binder (MGB) fluorescent probes to increase specificity and sensitivity (Ott et al., 2004). PCR amplification was carried out in 96-well PCR plates on the Light Cycler® 480 instrument. In this reaction, conserved primers that bind and amplify the full length of 16SrRNA gene were used (forward TPU1 5'-AGAGTTTGATCMTGGCG TCAG; reverse RTU8 5'-AAGGAGGTGATCCANCCRCA).

The reaction mix contained 5 µM of each primer, 2 µM VIC-labeled MGB universal probe (ACTGAGACACGGTCCA), 10 µl ready to use PCR Master mix (Applied Biosystems) and 2 µl of template DNA in a final volume of 20 µl. The run protocol on the Light Cycler 480 was adapted to the following parameters: pre-incubation program for activation of Taq polymerase (95°C for 10 min), 45 amplification cycles (denaturation at 95°C for 30 secs, annealing at 60°C for 1 min, extension at 72°C for 1.5 min with single fluorescence measurement), Followed by a period of 1 min for cooling the reaction block to 40°C. Fluorescence was detected at 528-564 nm. Bactscreen real-time PCR was carried out in 20 µl reactions on the LightCycler®480 instrument. The reaction mix and PCR parameters was prepared as described by Al-griw (2011).

Results

The limit of detection (LOD) of Sepsitest™ assay was determined using purified DNA from Gram-negative (Escherichia coli) and Gram-positive (Staphylococcus aureus) bacterial strains. The assay detected from 50ng to 25fg S.aureus DNA/µl with Ct...
values increasing as DNA levels decreased. Negative controls containing DNA-free water in place of *S. Aureus* DNA also generate a significant fluorescence signal. However, in melting curve analysis, the lowest concentration showed expected T_m values (87.63 ±0.1°C) for a Gram-positive species was 500fg/µl only whereas, reactions; 50 fg/µl, 25 fg/µl of gram positive *S.aureus* DNA and the negative control were observed under T_m values (77.82 ± 0.72 °C) indicating primer dimer or unspecific amplification. Similarly, the fluorescence amplification curves from *E.coli* DNA in the range 1ng/µl to 10fg/µl was also detected by SepsiTest™.

Typical T_m values (88.89± 0.1) were obtained in melting curve analysis for DNA samples for the concentrations down to 500fg/µl -0.001ng/µl though reactions; 100 fg/µl, 50 fg/µl, 25 fg/µl, 10 fg/µl of gram negative *E.coli* DNA and the negative control were observed under T_m values (78.17 ± 0.28 °C) indicating primer dimer or unspecific amplification. The performance of “in house” all bacteria primers and MGB probe on the LightCycler480 in detecting a range of concentrations of purified *E. coli* DNA and *S.aureus* DNA showed a great sensitivity compared than SepsiTest™ and was able to detect down to at least 25fg/µl (50fg/PCR) of *E. coli* DNA or *S.aureus* DNA without any significant amplification of the negative control. Limit of Detection (LOD) of BacScreen™ for DNA from Gram Negative and Gram-Positive Species has been previously evaluated (Al-Griw, 2011). The minimum analytic sensitivity of the assay as confirmed with the melting curve analysis was comparable to the “in house” all bacteria primers and MGB probe assay - 25fg/µl (50fg/PCR) of bacterial DNA.

MolYsis extraction was included as a part of SepsiTest™ protocol. To address whether the sensitivity of SepsiTest™ could be affected with the use of MolYsis extraction protocol for samples prepare, a second extraction technique namely high pure purification kit in conjugation to red cell lysis buffer was used. Both extraction techniques were used for extraction of *E. coli* DNA from spiked blood sample with different concentration of *E. coli* cells. All DNA samples extracted by both methods then assayed using the three real-time PCR assays; SepsiTest™, Hybridized probe-based 16Sr DNA assay (Bactscreen™), and “in house” 16Sr DNA all bacteria and MBG probe. Figure (1) shows summary of the lower detection limits obtained by the three real-time PCR assays. When a MolYsis extraction technique was used, the lower detection limit of samples amplifying using SepsiTest™ assay as confirmed by the correct melting point (T_m values = 88.89 ± 0.1) in the melting curve analysis was only 10^3 CFU/ml Figure (2) (MB). The lower detection limit was raised to 10^3 – 10^4 CFU/ml (MB) (3) (A). When the same spiked samples were assayed using “in house” all bacteria primers and MGB probe on the LightCycler480, while hybridisation probe-based 16S rDNA assay (Bactscreen) for the same samples gave better detection limit (10^3 CFU/ml) figure (4) (MB). By using the second extraction protocol for the same spiked samples, similar detection limit were obtained for SepsiTest™ (10^3 CFU/ml) Fig.1, while samples assayed using all bacteria primers and MGB probe on the LightCycler480 revealed a detection limit of 10^3 CFU/ml amplifying the same samples with hybridisation probe-based 16S rDNA assay (BactScreen) gave even better detection limit (10^3- 10^4 CFU/ml) Figure (1).

**Figure 1.** Shows the lower detection limit of the three real-time PCR assays, SepsiTest™, Hybridized probe-based 16Sr DNA assay (Bactscreen™), and “in house” 16Sr DNA all bacteria and MBG probe using either MolYsis or high pure purification technique for extraction *E. coli* DNA from spiked blood sample with different concentration of *E. coli* cells.
Figure 2. Analysis of spiking blood sample with different concentration of *E. coli* cells extracted either with MolYsis extraction technology (MA& MB) or High pure DNA purification technique (HA and HB), using Sepsitest™. Data shows representative results of real-time Sepsitest™ PCR. Amplification curves (Panel A) and melting curves (Panel B) are shown for spiking with (10^6-10^1CFU/ml) of *E. coli* un-spiked blood sample (NB) and Negative control (NC).

Figure 3. Analysis of spiking blood sample with different concentration of *E. coli* cells using either Sepsitest™™ extraction technique (MolYsis) panel (A) or High pure purification DNA template in conjunction with the use of red cell lysis buffer Panel (B) and assayed using all bacteria primer and probes in house assay on LC480. (NC) Negative control, (NB) none spiked blood sample and (CP) control positive.
Figure 4. Analysis of spiking blood sample with different concentration of *E. coli* cells extracted with MolYsis (MA and MB) or High pure DNA purification technique (HA and HB) using BactScreen toolset. Data shows representative results of real-time BactScreen PCR. Amplification curves (Panel A) and melting curves (Panel B) are shown for spiking with (10^8 - 10^1 CFU/ml) of *E. coli*, Negative control (NC), Gram positive control (GP) and Gram negative control (GN).

**Discussion**

The objective of this study is to address the performance of the commercially available SepsiTest™ against another two real time PCR assays; “in house” all bacteria primer and MGB probe (hydrolysis probe) and Bactscreen (hybridized probe). SepsiTest™ is a complete kit, it uses a MolYsis technology for DNA extraction and use a 16 S Ribosomal RNA primer and a SYBR Green as a florescence dye for the PCR. In the first step of assessment, we aimed to investigate the PCR of SepsiTest™ assay, excluding the effect the extraction technique use to prepare the DNA templet for the assay, meaning to check whether the primer was designed to amplifies the target region as was intended without amplifying of any non-specific products. SepsiTest™ was able to detect only upto500fg/µl to 0.001ng/µl or 0.001-0.002ng/PCR of purified *E. coli* DNA and *S. aureus* DNA equivalent to 2-4 x 10^2 CFU/PCR according to Nadkarni and colleagues(Nadkarni et al., 2002).When the same samples assayed using “in-house” all-bacteria assay, we obtained better detection limit(25fg/µl or 50fg/PCR equivalent to 10 CFU/PCR for both gram negative and positive bacterial DNA with constant increase to the Ct values as DNA level decreased.

These results have point out that the Real-time PCR is affected by the type of dye or florescence probes used. “In house” all bacteria primers and MGB probe utilize Taq man probe a hydrolysis probe which can monitor the reaction much specifically than SYBR Green dye. However, as it is mechanism of detection relays on the amplification curves while unsuitable for melting curve analysis, any inhibition in the florescence could result in inhibition of the assay itself. The detection limit of BactScreen was previously studied and reported as 62fg/PCR or 12CFU/PCR of purified DNA(AI-Griw, 2011). This result is important because bacteremia is often associated with low numbers of circulating organisms. Kreger et al (1980) Showed that 73% of patients with gram-negative clinical significance bacteremia had less than 10 CFU per ml of blood culture (Kreger et al., 1980).

The second step aimed to examine the proposed extraction methods for purified pathogenic DNA and removing the inhibitory substance thus increases the assays sensitivity. These was done by determine the efficiency of the three assays for amplifying DNA that has been extracted from spiking blood samples (thus mimicking true clinical samples) using different methods of DNA extraction. We could only achieve positive results from spiking blood samples with as little
as $10^5$ CFU per ml of *E. coli* cells that have been extracted using MolYsis technique and assayed using SepsiTSTM. Smaller amounts, $10^3$- $10^4$ and $10^5$CFU per ml could be detect when the same DNA samples were assayed either using "in house" All bacteria primers - MGB probe or BactScreen respectively Fig. 2 (A and B). These results suggested that MolYsis technique was not efficient as would expect for removing PCR inhibitors thus increasing assays sensitivity. For more investigation, another extraction protocols named High pure template purification technique was used. These techniques are not intended to remove human or free DNA as suggested by MolYsis. The results indicated that SepsiTSTM PCR continued to have low sensitivity ($10^3$CFU/ml) even with substituting it is extraction protocol (MolYsis) with high pure template purification technique Fig.3 (HM). The lower sensitivity of SepsiTSTM may attributed to other blood component rather than human DNA that caused such inhibition specially when using SYBR Green1 as fluorescence detection dye. Moreover, when the same samples assayed using “in house” all bacteria assay,10-foldxtra sensitivity was gained with the use of high pure purification technique. However, the inhibition was more prominent with the use of high pure than when MolYsis where used. BactScreen gave sensitivity of $10^4$ CFU/ml when MolYsis extraction technique was used Fig.4 (MB) and much more with High pure DNA purification technique ($10^3$–$10^5$CFU/ml) Figure (4) (HB).

These results emphasise that with MolYsis extraction technique we not only lose human DNA but a reasonable amount of bacterial DNA could be also removed while the extraction. Moreover, if the primer designed to be highly sensitive, present or absent of human DNA would not affect the sensitivity of the assay. In a comparative study between 5 primer pairs to determine the most sensitive primer for diagnosis of *Brucella* from blood in present or absent of human DNA, one primer pair was found to be most sensitive and promising tool for diagnosis of *Brucella* although it is sensitivity was affected with the present of human DNA (Navarro et al., 2002).

The MolYsis DNA isolation kit as a part of SepsiTSTM kit, proved to be effective in removing PCR inhibitors, this was obvious when DNA extracted from spiked blood samples assayed using the all bacteria primers and MGB probe on the LightCycler480 while DNA extracted from spiked blood samples using other extraction technique such high pure purification fail to amplify with the same efficiency Figure (2) (A and B). Gebert et al., 2008 compared the efficiency of using MolYsis complete DNA isolation kit with QIAamp DNA Mini Kit (Qiagen) and High Pure PCR Template Preparation Kit (Roche) in conjunction with a universal Gram-differential -PCR to monitor the time to positive signalling in spiked blood cultures. He confirmed the efficiency of MolYsis DNA isolation kit for removing the PCR inhibitors from blood cultures comparing with the other studied techniques. However, they reported a lower detection limit of 10-20 CFU/ml for Gram-positive bacteria and 200-400 CFU/ml for Gram-negative bacteria(Gebert et al., 2008).

Similarly, MÜhln (2010) reported a lower detection limit of110-460CFU/ml for Gram-negative and 20-40CFU/ml for Gram-positive using the same extraction technique (Muhl et al., 2010). Although, the PCR of those two studies were different from each other and from our study, the detection limit difference between Gram-positive and Gram negative suggested that the sensitivity of those assays has been dramatically affected by the extraction techniques.

Gram-positive bacteria have rigid cell walls, which to some extant can stand the lysis action of MolDNase enzyme, in contrast to Gram-negative bacteria that have relatively weaker cell wall and higher fat content, making it more susceptible to the diffusion of MolDNase inside the cells and fragile to the effect of residual DNAase. Indeed, the application of MolDNase as a first step to remove human DNA can cause loss of some bacterial cells specially those affected with the treatment of antibiotic. The loss of bacterial DNA in the extraction could be related to three points. First, diffusion of the MolDNase through the thin labile bacterial cell wall (e.g. genus *treponema*) or fragile bacterial cell wall such as those affected with antibiotic or attacked with body defense mechanism (Horz et al., 2008). Second, the protocol of the extraction lacks the heat inactivation step thus the effect of residual DNAase would be continued on the finally released template DNA following the washing step. Residual DNAase activity has been documented even with incubation on 95 °C for 50 min of heat treatment(Hanaki et al., 2000),(Silkie et al., 2008). Finally, our previous study suggested that at least 90% of free DNA could be removed with the supernatant following the centrifugation step without of application of MolDNase (Al-Griw, 2011). This is particularly important in case of low grad bacteremia where the amount of bacteria DNA fall under the detection limit of the assay.

The lower sensitivity of SepsiTSTM especially for gram-negative bacteria raises some concern and uncertainty about the effectiveness of using this test for diagnosis of gram-negative sepsis. Horz et al (2008) raise very important question about how much removing of interfering human DNA is necessary and how much reduction in bacterial DNA would be acceptable(Horz et al., 2008).Moreover, It has been reported that the 16S rDNA gene codes for the 16S ribosomal is found only in bacteria phylum and therefore it is unlikely that 16S ribosomal PCR-based assay could be affected by mammalian DNA contamination(Sleigh et al., 2001), the more likely is that other blood components are responsible of such interfering. In fact, losing of 90% of bacterial DNA could be compensate by addition of 3 or 4 cycles or concentrate the bacterial DNA by reducing the finally amount of elution buffer (e.g. 30µl).

Unfortunately, SepsiTSTM uses SYBR Green as fluorescence detection that would gave unspecific
amplification and primer dimer particularly with low copies of pathogenic DNA.

In fact, MolYsis DNA extraction technique for elimination of human DNA may become useful if larger volume of blood samples is used i.e. ≥ 5ml, although the efficiency of the extraction may still be affected when high amount of free DNA in the sample. In normal blood sample the amount of human DNA ~ 15ng/µl (Al-Griw, 2011) and using 5ml of blood sample could raise the amount of human DNA to 75ng/µl and even more in septic patient, thus remaining of 90% of human DNA meaning at least 7.5ng/µl would be remaining.

The strategy of removing free DNA using molYsis extraction techniques incorporated in SepsiTest™ may improve the PCR amplification but would be more useful to be used in food technology. Similar strategy were developed by Mukhopadhyay et al., 2002, to eliminate the free extraneous DNA released in the media following death and lysis of pathogenic cells to predict the possible live pathogen present in the food (Mukhopadhyay and Mukhopadhyay, 2002).

Although SepsiTest™ has gained CE mark for European use; no comprehensive evaluation studies are available in the literature up to date. Wellinghausen et al., (2009) evaluated SepsiTest™ performance for rapid detection of blood stream infection compared to blood culture on 342 clinical blood sample taken from 187 patients whom exhibit SIRS, sepsis or neutropenic fever (Wellinghausen et al., 2009). Although, the sensitivity and specificity of the assay was 87% and 85% in respectively, the result was rather difficult to interpret. Considering 41 samples in which PCR was positive and blood culture negative, only 11 samples who classified as probable to true bacteremia were confirmed from other body cultures in that, 5 samples were Coagulase-negative staphylococci (CNS). Moreover, in at least three episodes of PCR positive in which the result classified as indeterminate PCR results, non-pathogenic soil and water bacterial species were found.

These results raise a big concern about the accuracy of the sampling methods particularly that most blood samples taken from peripheral catheter systems. CNS in most cases represent contaminations from the skin flora although they can be aetologically relevant (Beekmann et al., 2005). Contaminant bacteria DNA may get access into PCR reactions from plastic ware, anti-coagulant, or skin flora itself (Sontakke et al., 2009). Unfortunately, SepsiTest™ has not adjusted for acceptable level of contamination with CNS as was done with Septi Fast (Haag et al., 2013). The problem of contamination and PCR-culture discrepant results was reported by Haag H et al., (2013). In their study SepsiTest™ was evaluated for their use in routine diagnostics. Typical 96 specimens comprised tissue from the heart in case of infective endocarditis (IE), synovial tissue from suspected prosthetic joint infections, or blood and blood cultures from patients with suspected blood stream infection were PCR analysed and compared to culture results. Focusing on the 14-blood sample among the samples studied, two whole blood (WB) samples were invalid and thus excluded from the analyses due to sample cross contamination. Four cases where concordant negative and concordant true positive approved infections in two cases both with S. aureus pathogens. Among four cases that classified as culture-negative while PCR analyses was positive, one case was considered to be false positive because the relevance of the PCR finding is not clear. Interestingly, three blood cultures were taken from the same patient, two-yielded growth of Enterococcus faecium while the third remained negative.

The PCR analyses of the third blood culture identified A. johnsonii and Corynebacterium spp. This PCR was classified as likely contaminant and false negative. Presence of A. johnsonii and Corynebacterium reflects presumably a contamination event during blood drawing or during routine culture processing in the bacteriology unit (Haag et al., 2013). In general, the promising sensitivity and specificity of SepsiTest™ assays reported by Wellinghausen et al., 2009 and the study results of Haag et al., (2013) did not reflect the quality of the test. Detection of soil, water or irrelevant bacteria species confirming the unspecific amplification as indicates by the use of SYBR Green dyeand raise important question of whether the melting curve analysis were used in these studies as a primary analysis for positive cases otherwise sequencing of such samples are misleading, costly and time consuming. Keeping in mind the overall actual cost for each specimen (180€), hands-on time and the highly demand for molecular diagnostics skilled technician to perform the test, we believe that SepsiTest™ assay was not robust enough to be used alongside blood culture for identification of infectious agent present in the blood.

References


**Original article**

**Soil contamination with Toxocara spp. Eggs in the Public Parks of Tripoli City, Libya**

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\(\text{\(^2\)Department of Preventive Medicine, Faculty of Veterinary Medicine, University of Tripoli} \)

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**Abstract**

Toxocara spp. is a highly prevalent nematode that parasitizes the gastrointestinal tract of dogs and other canids. The eggs shed in the host’s feces can survive for a very long time in the environment and infect other canids and humans. Little information is available on the prevalence of and the risk factors associated with toxocariasis in Libya. To assess soil contamination with Toxocara spp., one hundred and five soil samples from public parks in different localities in Tripoli were examined by Dunsmore modified technique. The overall prevalence of Toxocara spp. in soil from public parks was 59.0% (62/105 samples). Our findings show a widespread soil contamination in public parks with Toxocara spp. eggs. Combined, these are a potential public health risk.

**Keywords**: Toxocara spp; Public parks; Soil; Libya

**Introduction**

Dogs and other canids are the definitive hosts for Toxocara canis. The mature worms in the intestines shed large numbers of unembryonated eggs into the feces, and the eggs become embryonated in the external environment. Therefore, the infection is commonly acquired after ingestion of embryonated T. canis eggs that are present in soil contaminated with dog feces. Children are the most susceptible to infection because they are often playing in the places which are easily contaminated, moreover, of their habits of pica (Oversaasuw 1997; Despommier 2003). Several studies from all over the world demonstrated high rates (10-30%) of soil contamination with Toxocara eggs in parks, playgrounds, sandpits and other public places (Tavassoli et al. 2008). T. canis eggs are reported to be found in soil more commonly than other parasites or other Toxocara spp. (Glickman and Magnaval 1993). The study of the prevalence and zoonotic importance of T. canis remains a major concern for scientists in both developed and under-developed countries (Oversaasuw et al. 2009; Klimpel et al. 2010).

In Libya, data on the prevalence and epidemiology of pet parasitic infections and zoonosis are sparse. We hope that this study will highlight the importance of the prevalence of soil contamination of public parks by Toxocara spp. for the benefit of the animals and humans in contact with them. This study aimed to investigate the prevalence of Toxocara spp. in soil from public parks in Tripoli.

**Materials and Methods**

**Study area**

This study was done in the metropolitan area of Tripoli, the largest city and capital of Libya. It has a population of about 1.1 million people and a population density of 4,500/km\(^2\). Tripoli is located in the northwestern part of Libya at 32° 54’ north and 20° 4’ east. It has a hot subtropical semi-arid climate with long, hot, dry summers and relatively wet, mild winters with a Mediterranean rainfall pattern. In summer, temperatures often exceed 38°C and can reach into the forties. In December, temperatures can reach as low as 0°C, but the average is between 9 and 18°C. The average annual rainfall is less than 400 mm, but it can be very erratic.

**Soil analysis for Toxocara eggs**

In the Tripoli area there are about 35 parks (green area) with a total area of approximately 1.7 km\(^2\). Twenty-one public parks were selected randomly from the four areas of Tripoli, and five soil samples were taken from each park (Table 1). The specific parks covered by the study are shown in Table 2.

**Table 1. Number of parks sampled from each of the four regions of Tripoli.**

<table>
<thead>
<tr>
<th>Regions</th>
<th>No. of parks (samples/per park)</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center</td>
<td>10 (5)</td>
<td>50</td>
</tr>
<tr>
<td>West</td>
<td>5 (5)</td>
<td>25</td>
</tr>
<tr>
<td>South</td>
<td>4 (5)</td>
<td>20</td>
</tr>
<tr>
<td>East</td>
<td>2 (5)</td>
<td>10</td>
</tr>
</tbody>
</table>

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Table 2. Number, names and locations of the public parks included in the study.

<table>
<thead>
<tr>
<th>Center</th>
<th>West</th>
<th>South</th>
<th>East</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saraya park(1)</td>
<td>Saraj</td>
<td>Abu Salim</td>
<td>Shat Alhinsher</td>
</tr>
<tr>
<td>Saraya park(2)</td>
<td>Hay Alandalus</td>
<td>Tripoli National</td>
<td>Al Tarsana</td>
</tr>
<tr>
<td>Al-shat (1)</td>
<td>Gurji</td>
<td>Tripoli National</td>
<td></td>
</tr>
<tr>
<td>Al-shat (2)</td>
<td>Gurji</td>
<td>Tripoli National</td>
<td></td>
</tr>
<tr>
<td>Bab benghashir</td>
<td>Sports city</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sooq althalha</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sooq althalha</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Al jalaa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fashloum</td>
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<td></td>
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<tr>
<td>Al dahra</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

The selection of samples from each park focused on the places where children play. Each sample consisted of 250–300 g soil from an area of 20 cm² to a depth of 8 cm because *Toxocara* eggs are more abundant in the top soil (Uga et al. 1989). Samples were obtained using a cylindrical soil sampling tool, and the samples were placed in polyethylene bags. The sampling was done from late May to early June.

Parasitological examination of the soil samples was done according to (Dunsmore et al. 1984). The soil samples were sieved through a 4-mm² mesh sieve to remove stones and large pieces of organic matter. A sample of 30 g of soil was soaked overnight in 100 ml of distilled water containing a few drops of Tween-80. The mixture was then homogenized using an electric mixer (Multimixer and Creamer) for 10 min and allowed to stand for 5 min. Two 15-ml centrifuge tubes were filled with the mixture and centrifuged for 10 min at 2000 rpm. The supernatant was discarded, 3.98 M aqueous NaNO₃ solution (specific gravity 1.18–1.20) was added to the tube, and the sediment were suspended. The tubes were filled to the top with NaNO₃ solution and a slide was placed on the meniscus and left for 25 min. and observed under a microscope.

**Results**

**Soil infection with *T. canis***

From each of the 21 public parks that were sampled, five soil samples were examined. A public park was considered infected if one or more soil samples were infected. The prevalence within the public parks could vary from 0% (0/5) to 100% (5/5) positive. The highest frequency of positive samples were in eastern Tripoli (70%), followed by the center (64%), the south (55%) and finally the west (48%) (Table 3).

Table 3. Frequency and prevalence of *Toxocara* spp. in soil samples from public parks in Tripoli

<table>
<thead>
<tr>
<th>Tripoli region</th>
<th>Frequency of the proportion of infected garden per region</th>
<th>Number of gardens (number of samples)</th>
<th>Prevalence of infected samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center</td>
<td>1 1 5 1 2</td>
<td>10 (50)</td>
<td>64% (32/50)</td>
</tr>
<tr>
<td>West</td>
<td>1 1 3 0 0</td>
<td>5 (25)</td>
<td>48% (12/25)</td>
</tr>
<tr>
<td>South</td>
<td>0 1 3 0 0</td>
<td>4 (20)</td>
<td>55% (11/20)</td>
</tr>
<tr>
<td>East</td>
<td>0 1 0 0 1</td>
<td>2 (20)</td>
<td>70% (7/10)</td>
</tr>
<tr>
<td>Total</td>
<td>2 4 11 1 3</td>
<td>21 (105)</td>
<td>59% (62/105)</td>
</tr>
</tbody>
</table>
Discussion

Contamination with *Toxocara* eggs and zoonotic helminths is a matter of concern for public health workers worldwide, especially in developing countries (Alonso et al. 2001). In recent years, the number of pet owners in Tripoli has increased. In addition, the number of stray dogs and cats is increasing, and all these animals defecate in parks and other public areas (Abdi 2003). Therefore, examined the extent of contamination of soil with *Toxocara* spp. at sites of public health was importance in Tripoli.

We found a contamination rate with *Toxocara* spp. eggs of 59.0% (62/105 soil samples) in soil samples collected from public parks in different locations of Tripoli. Contamination of public parks was highest in eastern Tripoli (70%; 7/10), followed by Tripoli Center (64%; 32/50) and southern Tripoli (55%; 11/20). The lowest prevalence of contamination (48%; 12/25) was in western Tripoli. Therefore, contamination was high in all four areas of Tripoli. Reported contamination rates vary widely, and the prevalence rate we observed in Tripoli is not exceptional.

Public parks and playgrounds that provide free access to pets and stray animals are open to contamination that serves as a source of infection of uninfected animals. Further, street food vendors discard leftovers, which attract stray and scavenging animals. Surprisingly, pets are brought to such public places to defecate, thereby contaminating the soil. When children play on such contaminated soil, they are clearly open to infection.

Some physical properties of the soil, including humidity, oxygenation and compactness, can influence egg survival in the environment (Xavier et al. 2010). Importantly, the use primarily of sand in playgrounds, which does not retain water well, probably plays a role in the low contamination of playgrounds. *Toxocara* eggs are resistant to environmental conditions and can remain infectious for years in a favorable environment, but low humidity is lethal to *Toxocara* eggs (Martinez-Moreno et al. 2007; Teixeira et al. 2008). In Italy and Iran, it was noticed that public parks were more contaminated with *Toxocara* eggs than the playgrounds (Habluetzel et al. 2003; Zibaei et al. 2010). That is comparable with the results of Mizgajaska (2001) who reported that the most contaminated areas were city backyards, with 38–53% of soil samples positive for *Toxocara* eggs. The presence of puppies could increase the probability of finding fertile *Toxocara* spp. eggs in the soil surface, as puppies release more eggs in their feces than adult dogs.

Over the past two decades, many reports have documented contamination rates of *Toxocara* eggs in several countries (Habluetzel et al. 2003; Tinoco-Gracia et al. 2007; Zibaei et al. 2010). However, it is not possible to compare the present study directly with these surveys because of the different sampling and detection methods used in these studies. Nevertheless, the contamination rates observed here are relatively similar to those reported previously.

References:


The Effect of Pesticide Chlorpyrifos (Dursban$^R$) on Sperm Parameters and Testicular Tissue in Mice

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Abstract

Environmental pollution by the pesticide chlorpyrifos has been documented as the most commonly detected pesticide in food and water posing a potential hazard to human health. Therefore, the present study was conducted to determine the effect of pesticide chlorpyrifos on sperm parameters and testicular tissue in mice. Sperm count, sperm motility and sperm morphology parameters are very important semen characteristics and are strong predictors of male fertility. The reproductive toxicity of the pesticide chlorpyrifos was studied in adult male mice via intraperitoneal administration of chlorpyrifos (20 mg/kg) on alternate days for a period of 15 days. The results of this study showed a decrease in all sperm parameters, especially sperm motility and histological tests of testicular tissue. The histological lesions emphasize the positive correlation between cytogenetic damage and abnormal sperm parameters. These results support the hypothesis that exposure to pesticides may be associated with decreased semen quality and hence infertility.

Key words: Chlorpyrifos, Pesticide, Sperm analysis, Testes, Mice

Introduction

Several studies have reported an apparent decline in semen quality (Carleson et al. 1992; Auger et al., 1995; Schisterman et al. 2014) and an increase in male infertility (de Kretser, 1997; Schisterman et al. 2014) over the past 50 years. The reasons for these detrimental male reproductive changes appear to be environmental rather than genetic due to the short time period over which they have occurred. Therefore, scientific research has mainly focused on environmental chemical contaminants. These include: synthetic chemicals (e.g. trichloroethylene (TCE), polychlorinated biphenols (PCBs), dioxin), heavy metals (e.g. cadmium, lead, arsenic, nickel), plastics (e.g. bisphenol A (BPA), phthalates), and pesticides (e.g. vinclozolin, Diazinon (DZN), dichlorodiphenyltrichloroethane (DDT), methoxychlor) (Anway et al. 2005; Olea and Fernandez, 2007; Stouter and Paoloni-Giacobino, 2010; Dwivedi and Flora, 2011; Manikkam et al., 2012; Skinner et al. 2013; Al-Griw et al. 2015; Adamkovicova et al. 2016). In wildlife chemical pollution has frequently been associated with adverse male reproductive effects (e.g. damage to the testes and spermatozoa) (Colborn et al. 1993; Guillette and Gunderson, 2001; Multigner et al. 2008). Recently, Martenies and Perry (2013) suggested that exposure to pesticides environmentally or occupationally may be associated with decreased sperm count in men. Thus, the evaluation of reproductive toxicity of common pesticides is of great importance to public and environmental health.

The pesticide chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate) is the most widely used organophosphate insecticide (Betancourt et al., 2006). Chlorpyrifos account for up to 50% of all insecticide application worldwide (Casida and Quistad, 2004). Chlorpyrifos is a broad spectrum organophosphate insecticide used in the control of agriculture pests and household insects such as fire ants, cockroaches, and fleas (Eaton et al. 2008). Commercially, it is available in different trade names such as Dursban, Lorsban, Agromil, Dhanwan, Suscon Green, Empire, Equity, Dorson, and Omexan (Watts, 2013; John and Shaika, 2015). Varying concentrations of chlorpyrifos pollution in the environment have been reported (George et al. 2014; Lari et al. 2014; Marasinghe et al. 2014; Gulati et al., 2015), posing a potential hazard to human health. It has been reported that chlorpyrifos is linked to brain deformities (Rauh et al., 2012), neural tube defects (Abdelmalek et al. 2016) and impaired fetal growth (Wyllt et al. 2004) in human fetuses. However, few studies have evaluated the impact of chlorpyrifos pesticide exposure on male reproductive health (Joshi et al. 2007; Mandal and Das, 2011; Sai et al. 2014). Therefore, the aim of this study was to evaluate the reproductive toxicity of the insecticide chlorpyrifos in male mice. Sperm parameters (sperm count, sperm motility and sperm morphology) and histology of testicular tissue were investigated.

Materials and Methods

Study area

The Pesticide chlorpyrifos (O,O-diethylO-3,5,6-trichloropyridin-2-ethyl phosphorothioate) under the trade name Dursban 4 EC (emulsifiable concentrate) manufactured by Dow Agrosciences was obtained from a local pesticide store, supplied by Libyan Agrochemicals & Agriculture Supplies Specialized Co.. It was emulsified in water immediately before use and
administrated to animals intraperitoneal (i/p) at a dose of 20 mg/Kg.

**Animals and Treatments**

Animals used in this study were mature male Swiss albino mice (*Mus musculus*) 6 to 8 weeks old, weighing 25 to 28 gm. They were bred in the animal house of the Zoology Department, Faculty of Science, University of Tripoli. The mice were housed in plastic cages containing wooden flakes under husbandry, and maintained at room temperature 22 ± 3°C under natural light/dark photoperiod. The mice were fed with a standard mouse diet and drinking water *ad libitum*. They were randomly divided into two groups: treated and control of five males each. The treated mice were administered intraperitoneal (i/p) injections of chloropyrifos 20 mg/kg body weight every alternate day for two weeks and the control mice did not receive any treatment. The mice were observed daily for survival and clinical signs of toxicity. At the end of the treatment period, all mice were sacrificed by cervical dislocation and sperms were isolated from vas deferens and sperm analysis was evaluated.

**Sperm count**

Sperm count was determined by Neubauer hemocytometer counting chamber following the method of Wang et al. (1995). Sperm samples were collected from vas deferens, the count was repeated at least three times to minimize error and mean value was taken for calculation. Each vas deferens was gently squeezed and thoroughly stripped in a clean small glass Petri dish containing 1 mL of physiological normal saline (0.9% NaCl). The sperm suspension was incubated for five minutes at 37 °C to allow sperm separation, after incubation sperm suspension was thoroughly mixed with a fine pipette, 100 uL of the diluted sperm suspension was placed on counting chamber. The number of motile and immotile sperms was counted under a light microscope at 400X magnification. The calculated results were expressed as percent motility or immotility (e.g. the number of motile sperm/total number of sperm x 100). For total sperm count, the number of motile and immotile sperms was added and multiplied by dilution factor and expressed as x10³/mL.

**Sperm Morphology**

Sperm morphology examination was done by making sperm smears from the sperm suspension. One drop of sperm suspension was placed on a clean microscopic slide and a sperm smear was made, allowed to air dry, and then stained with 1% eosin for ten minutes. These smears were observed at 400X magnification using a standard light microscope and the number of normal and abnormal sperms was determined. The calculated results were finally expressed as percent: the number of abnormal sperm/total number of sperm x 100. At least 500 sperms were counted from each animal to determine sperm morphology and abnormalities. The criteria for abnormal sperm morphology include the following: the shape of sperm head, the presence or absence of hook, and tail shape.

**Histological examination**

Testes were fixed in 10% buffered formalin, passed through ascending series of ethanol and then through xylene and embedded in paraffin wax. Tissues were sectioned at thickness of 7 uM and stained with hematoxylin and eosin (H and E) and microscopically analyzed and photographed.

**Statistical Analysis**

The data was analyzed by one way analysis of variance (ANOVA) using MSTATE-C version 4 software. The statistical significance of difference between the control and treated groups for sperm parameters: sperm count, sperm morphology, and sperm motility was determined with Duncan’s test; a probability value of $P \leq 0.05$ was considered statistically significant.

**Results**

The pesticide chlorpyrifos had toxic effect on all sperm parameters: sperm count, sperm motility, and sperm morphology analyzed in this study, as well as histopathological changes in testicular tissue.

**The effect of chlorpyrifos on sperm parameters**

There was a decrease in sperm count of treated mice ($22.4x10^6$) in comparison with control group ($25.4x10^6$); however the probability value ($P = 0.400$) indicate that this decrease was not significant (Table 1). The greatest toxic effect of chlorpyrifos was observed on sperm motility. There was very significant ($P=0.000$) decline in the mean percentage of sperm motility of treated mice (4.7%) verses control (94.2%) group (Table 1).

**Table1.** Sperm parameters in control and chlorpyrifos treated mice.

<table>
<thead>
<tr>
<th>Sperm parameter</th>
<th>Control group</th>
<th>Chlorpyrifos group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm count</td>
<td>25.4x10⁶/mL</td>
<td>22.4x10⁶/mL</td>
</tr>
<tr>
<td>Percent of motile sperms</td>
<td>94.2%</td>
<td>4.7%*</td>
</tr>
<tr>
<td>Percent of abnormal sperm morphology</td>
<td>4.8%</td>
<td>14.2%**</td>
</tr>
<tr>
<td>Percent of normal sperm morphology</td>
<td>95.2%</td>
<td>85.8%</td>
</tr>
</tbody>
</table>

* $P=0.000$, ** $P<0.022$

There was a significant increase ($P<0.022$) in the mean percentage of morphologically abnormal sperm shapes in treated mice (14.2%) with respect to control (4.8%) group (Table 1). The morphological different forms of sperm shape abnormalities in this study include banana head, coiled tail, thin tail, and ring tail (Figure 1).
The effect of chlorpyrifos on testicular tissue

Light microscopic examination of testicular tissue in the control group shows normal histological structure, normal pattern of seminiferous tubules with orderly arranged spermatogenic cells and high spermatozoa concentration in the lumen, and little interstitial space (Figure 2). Meanwhile, the testes of chlorpyrifos treated group revealed marked damage in histarchitecture and organization of seminiferous tubules (Figure 3) compared to control group (Figure 2). The histological lesions of testicular tissue include: distortion of seminiferous tubules, reduction in lumen width of seminiferous tubules, reduction in spermatozoa numbers in the lumen of the seminiferous tubules, derangement and sloughing of normal germinal epithelial cells (spermatogonia) lining seminiferous tubules, increased interstitial space, and vasodilatation of interstitial blood vessels (Figure 3).

Discussion

In the present study, reproductive and histopathological studies were performed on the testes of adult male mice after i/p administration of pesticide chlorpyrifos at a concentration of 20 mg/Kg (which represent 1/3 of oral LD$_{50}$) over a period of two weeks. The results indicate decreased sperm count, significant decline in sperm motility and normal sperm morphology as well as testicular tissue damage of the mice exposed to chlorpyrifos. These findings are similar to those reported from experimental animal (Joshi et al. 2007; Zidan, 2009; Farag et al. 2010; El-Kashoury and Tag El-Din, 2010; Alaa-Eldin et al. 2017) and clinical human studies (Padungtod et al., 2000; Meeker et al. 2004; Perry et al. 2011; Marteniesa and Perry, 2013).

Joshi et al. (2007) studied the toxic effects of chlorpyrifos at dose levels of 7.5, 12.5 and 17.5 mg/kg i/d administered orally to male rats for 30 days on testicular histology, biochemistry, sperm dynamics and testosterone levels; their results indicated that Chlorpyrifos induces severe testicular damage, decreased testosterone levels, and reduction in sperm count (Joshi et al. 2007). Similarly, adult male mice were treated by oral gavage with chlorpyrifos at doses...
of 5, 15, and 25 mg/kg/d for 4 weeks resulted in decrease of the percent of morphologically normal spermatozoa in 15 and 25 mg/kg/d dose groups; however, sperm motility and count were decreased in all treated groups compared to the control (Farag et al., 2010). Exposure to three trade names of formulated chlorpyrifos from different Egyptian manufactures (chloroazon, pestpan, and pyriban) administrated orally to male rats at dose of 23.43, 21.40 and 17.43 mg/kg b.w. with 5 doses per week for 28 days resulted in decreased sperm motility and impairment of spermatogenesis (El-Kashoury and Tag El-Din, 2010). In addition, administration of chlorpyrifos to rats at a dose of 6.75 mg/kg b.w./daily by oral gavage for 12 weeks resulted in reduced testicular weight, decreased sperm count, motility and viability, significantly increased percent of morphologically abnormal spermatozoa, and significant increments in sperm DNA fragmentation index (DFI) with respect to control group (Alaa-Eldin et al., 2017).

In this study, the percentage of morphologically normal sperm was low due to large number of sperms with abnormal tail morphology, especially coiled tail. These results are consistent with Joshi et al. (2007), Zidan (2009), Farag et al. (2010), El-Kashoury and Tag El-Din (2010), and Alaa-Eldin et al. (2017). Abnormal tail morphology significantly reduces the fertilization capabilities of sperm because of spermatozoa movement dysfunction (Selvaraju et al., 2011).

Epidemiological human data reveal a potential association between exposure to chlorpyrifos and decreased semen quality. A survey on Chinese pesticide factory workers showed that exposure to organophosphate insecticides was associated with decreased sperm count and motility (Padungtod et al., 2000). Association between low sperm count and serum concentrations of 3,5,6-trichloropyridinol (a metabolite of chlorpyrifos and chlorpyrifosmethyl) has been reported in men (Meeker et al., 2004; Marteniesa and Perry, 2013). Measurable levels of urinary 3,5,6-trichloro-2-pyridinol was reported in more than 90% of males in the United States (CDC, 2003).

The changes in sperm parameters may be attributed to impairment of spermatogonia proliferation and maturation, which might be due to oxidative stress or to low levels of testosterone hormone (Saradha and Mathur, 2006). Testicular tissues are vulnerable to oxidative injury (Mendez-Alvarez et al., 2002) and defective sperm function is associated with an increase in lipid peroxidation derived free radicals and impaired antioxidant defense (Attia et al., 2012). Chlorpyrifos toxicity involves formation of reactive oxygen species (ROS) (El-Kashoury and Tag El-Din, 2010; Mandal and Das, 2011), inhibition of the activities of marker enzymes: alkaline and acid phosphates (ALP and ACP) and lactate dehydrogenase (LDH) (El-Kashoury and Tag El-Din, 2010), and significant decrease in activities of antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) which counteract the toxicity of ROS (Attia et al., 2012). ROS impede sperm motility by reducing mitochondrial membrane potential and decreasing ATP availability (Wang et al., 2003; Tremellen, 2008).

In this study, light microscopic examination of testes revealed that chlorpyrifos induced numerous histopathological changes in testicular tissues. Several previous studies confirm this result (Joshi et al., 2007; Akhtar et al., 2009; Zidan, 2009; Kalender et al., 2012; Sai et al., 2014; Alaa-Eldin et al., 2017); who found mild to severe histopathological changes in the testis of rodents (rats and mice) at various dose levels of chlorpyrifos. The histopathological changes included: degeneration and atrophy of seminiferous tubules with large interstitial space, decreasing number of spermatogenic cells in seminiferous tubules, sloughing of germinal epithelium into the lumen of seminiferous tubules and loss of sperms, edema, congested blood vessels, and exudate in interstitial tissue of the testis. The histological lesions induced by chlorpyrifos support the findings in this study and indicate that the exposure to chlorpyrifos caused a severe disturbance of spermatogenesis, with a marked decline in sperm quality; it also emphasize the positive correlation between cytogenetic damage and abnormal sperm parameters.

Conclusion

Sperm analysis is one of the end points in reproductive toxicology studies. Sperm count, sperm motility and sperm morphology parameters are very important semen characteristics and are strong predictors of male fertility (Perreault and Cancel, 2001; Buraaimuh et al., 2012). Small changes in sperm counts are known to have adverse effects on human fertility. Reduced motility and tail abnormalities are distinctive features observed in idiopathic asthenozoospermia and teratozoospermia in humans; these disorders are major causes of male infertility (Maruyama et al., 2016). Humans are exposed to environmental chemicals from numerous sources. Recently, the focus has been on adverse reproductive outcomes associated with widespread and permanent contamination by pesticides (Gulati et al., 2015).

Environmental pollution by the pesticide chlorpyrifos has been documented as the most commonly detected pesticide in food and water posing a potential hazard to human health (Marasinghe et al., 2014; John and Shiakle, 2015). Therefore, the present study was designed to determine the effects of pesticide chlorpyrifos on sperm parameters and testicular tissue in mice. The current study demonstrated adverse effects of chlorpyrifos exposure on sperm parameters at sub-lethal dose 20 mg/kg. The results presented in this study support the hypothesis that exposures to pesticides may be associated with decreased semen quality and hence infertility. Therefore, it is recommended to restrict the use of chlorpyrifos and to focus on alternative safe methods (e.g. biological control) to overcome the pollution and toxicity problems associated with chlorpyrifos usages.

Acknowledgement

The authors thank the Zoology Department, Faculty of Science, University of Tripoli, Tripoli - Libya for their support.
References


Incidence and Antimicrobial Susceptibility of *Salmonella* Carrier among Apparently Healthy Camels in Some Libyan Regions

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**Abstract**

Our study was planned to investigate the incidence of *salmonella* species among apparently healthy camels. One hundred and twenty one (121) samples were collected from camel’s feces (79 from Egdabya and 42 from El-mackeli) in Libya. Bacteriological examination of samples showed that the incidence of positive fecal samples were 16 (13.2%) according to their culture and morphological characteristics. Biochemical and serological identification revealed the following serotypes: six of *S. typhimurium* with incidence of (37.5%), four of *S. enteritids* (25%), three of *S. frintrop* (18.8%), in addition to three isolates were not identified. Antibi-otic sensitivity of isolated serotype showed that serovars were most sensitive to Ampicillin, Amoxicillin/Clavulanic acid (all isolates) and Chloramphenicol which could be used for treatment of salmonellosis in camels. The epidemiology and zoonotic significance of *salmonella* infection were discussed in the incidence of *Salmonella* species among apparently healthy camels in Libya.

**Material and Methods**

**Sample collection**

Fecal sample were collected from 79 camels from Egdabya and 42 camels from El-mackeli without clinical science of diarrhea. These camels are not treated with antibiotic. A single specimen was obtained from each animal.

**Isolation of Salmonella Species**

According to Harvey and Price (1974) each fecal specimen was homogenized by mixing with a tooth pick, then a pea sized amount added to 10 ml of selenite F broth in screw capped bottle and incubated at 37 °C for 24 hours, subcultures were then made into plates of MacConky agar (Merck, Germany), Xyloselysine Desoxycholate (XLD) agar (Oxoid), *Salmonella* agar (Merck), *Salmonella Shigella* (SS) agar (Oxoid) and incubated at 37 °C for another 24 hour. Pure colonies were sub cultured into nutrient agar and incubated for 24 hours.

**Identification of Salmonella Species**

The cultures on nutrient agar plates were subjected to gram staining, motility, urease production, hydrogen sulphide production and citrate utilization tests. All gram negative rod shaped motile, urease negative isolates that produced acid on triple sugar iron agar slants and able to utilize citrate as sole carbon source were identified as species of the genus *salmonella*. The...
plate cultures were examined for suspected colonies and confirmed by API-20E (Biomereux).

**Serological identification of Salmonella species**

Serologic identification of Salmonella species was determined in Animal Health Research Institute (Dokki, Egypt) and performed by slide agglutination test with (Andrews et al. 2005). An agglutination test was performed on a clear glass slide. The slide was divided into sections with a wax pencil and one small drop of physiological saline was placed in each test section on the slide. By using a sterile inoculating loop a portion of growth from the surface of TSI agar was removed and emulsified in each drop of physiological Saline on the slide.

It was then mixed thoroughly to create a moderately milky suspension. A bent inoculating loop was used to pick a small drop of antiserum and transferred to one of the suspensions; the second suspension served as the control (usually approximately equal volume of antiserum and growth suspension was mixed).

The suspension and antiserum was mixed very well and then the slide was rocked to observe for autoagglutination (agglutination is more visible if the slides are observed under a bright light and over a black background). If the reaction is positive, clumping will appear within 30 to 60 seconds. The saline suspension (control) was examined carefully to ensure that it was even and did not show clumping resulting from autoagglutination.

**Antimicrobial susceptibility**

All Salmonella strains were tested for susceptibility to a panel of 9 antimicrobial disks by the disc–agar diffusion method on Mueller -Hinton agar (oxoid), following the National Committee on Clinical Laboratory Standard. Each Salmonella serovar was cultured on Nutrient broth and incubated at 37°C for 12 hours. Adjusted turbidity sample was then spread over the surface of Mueller Hinton agar.

Antibiotic discs were disposed on the surface of inoculated agar media aseptically and incubated at 37°C for 18-20 hours. The inhibition zones of each disk were measured and the results were interpreted based on comparison to standards. Antibiotic discs used were: ampicillin (10), amoxicillin/clavulanic acid (30), ciprofloxacin (30), gentamycin (10), streptomycin (10), chloramphenicol (10), oxytetracycline (30), neomycin (30) and cephalaxin (30).

**Results and Discussion**

Out of 121 examined samples 16 *Salmonella* isolates (13.2%) were recovered from feces. The recorded infection rate of *Salmonella* spp. in camel feces was near to those reported by (Molla et al. 2004), whose result was 15.1% and (Al-Ruwaili et al., 2012) whose record a rate of 14.7%. On other hand, a higher infection rate with salmonellosis in camel (11/15, 73.3%) was reported by (Pegram and Tareke 1981). This study recorded the incidence of salmonellosis in camels (13.2%) which is higher than that found in UAE (4.3%) by (Wernery1992). The isolation of *Salmonella* spp. from camel fecal samples may probably due to fecal-oral contamination of feedstuffs, feeding surfaces, water troughs and equipment from carrier human or animals sources.

Results of serotyping of 16 isolated *Salmonella* spp. revealed that *S. typhimurium* represented the higher incidence, it was recovered from 6 samples (37.5%), followed by *S.enteritidis* 4 (25%), *S.frntrrop 3 (18.8%) in addition to three isolates could not be identified (Table 1). These results were similar to the finding of (Faye et al. 1997). They reported that the most important Salmonella serotypes in camels were *S.typhimurium, S.enteritidis, S.kentucky and S.saint-paul*. Also agreed with the results of (Wernery and Kaaden 2002). They identified 69 different *Salmonella* serotypes isolated from camels all over the countries they studied including *S.typhimurium, S.enteritidis, S.muenchen, S.bovisnorbificans* and *S.derby*.

From zoonotic point of view Matofari and colleagues (2007) mentioned that healthy camels can be carriers of *Salmonella* spp. which could be isolated from their feces and lymph nodes. Camels that are chronic carriers of Salmonella may present as human health hazard through consumption of camel’s products like milk (Matofari et al. 2007). Salmonellosis in camels was reported in Sudan (Curason 1998), United States of America (Bruner and Moran 1949) and from Somalia (Cheyna et al.1977).

The literature showed that *Salmonella* spp. could be present in camels of all ages (Wernery 1992; Salih et al.1998; Berada et al. 2000). Camels and their products could be a potential reservoir for *Salmonellas* pp. not only to the remaining camels but also to human and other animal species (Morpeth et al. 2009). Continuous surveillance studies for *Salmonella* in human and animals are important since new *Salmonella* serovars are emerging yearly and serotyping is very important to the epidemiological studies (Kim, 2010; Al-Ruwaili et al., 2012).

Concerning the susceptibility of isolated Salmonella serovars to antibiotics is presented in Table (2). The highest sensitivity rate was recorded to ampicillin (100%), amoxicillin / clavulanic acid (100%) and chloramphenicol (93.75%) and the highest resistance rate was recorded to oxytetracycline (100%), Neomycin (93.75%) and cloxacin (81.25%).

Some isolates were intermediate to gentamicin (75%), Streptomycin (68.75%) and Cephalaxin (87.5%). Although antibiotic therapy is important in treatment of salmonellosis in most endemic countries; antibiotics such as ampicillin, amoxicillin/clavulanic and chloramphenicol have been banned in Asia and Sub Saharan Africa (Amyes and Gupta 2002). Not all
antimicrobials at the concentration required to be effective are completely non toxic to human cells. However, the degree of susceptibility in determining the length of therapy and choice of cheaper antimicrobial agents with less side effects (Edward and Ewing 2003; Ngozi and Onyenekwe 2003) The high antibiotic resistance demonstrated by these isolates in our study is correlated with the high level of antimicrobial resistance in Enterobacteria, in fecal flora as well as in clinical isolates reported by (Velonakis et al. 2001; Aarestrup 2005).

Table 2. Antimicrobial susceptibility of *Salmonella* spp. (n = 16) by agar disc diffusion method.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Disc Concentration</th>
<th>Susceptible No</th>
<th>Susceptible %</th>
<th>Intermediate No</th>
<th>Intermediate %</th>
<th>Resistant No</th>
<th>Resistant %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicilline</td>
<td>10 mg</td>
<td>16</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Amoxicilllin/Clavulanic acid</td>
<td>AMC 30 mg</td>
<td>16</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>S 10 mg</td>
<td>5</td>
<td>31.25</td>
<td>11</td>
<td>68.75</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Neomycin</td>
<td>N 30 mg</td>
<td>1</td>
<td>6.25</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>93.75</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>CX 30 mg</td>
<td>2</td>
<td>12.5</td>
<td>1</td>
<td>6.25</td>
<td>13</td>
<td>81.25</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>G 10 mg</td>
<td>2</td>
<td>12.5</td>
<td>12</td>
<td>75</td>
<td>2</td>
<td>12.5</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>C 10 mg</td>
<td>15</td>
<td>93.75</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>6.25</td>
</tr>
<tr>
<td>Oxitetracycline</td>
<td>30 mg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>16</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Cephalexin</td>
<td>CL 30 mg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>87.5</td>
<td>2</td>
</tr>
</tbody>
</table>

Results are expressed as a percentage of (n = 16) (*Salmonella* spp. isolates susceptible, intermediate/moderately susceptible and resistant, respectively, for each antimicrobial.

References:


The effect of Indoxacarb on blood parameters and liver tissues in Mice

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Abstract
This study was conducted to evaluate the effect of Indoxacarb (Avant), an oxidiazine compound on hematological, some biochemical parameters of liver functions and histological changes in the liver. Sixteen albino adult mice were divided into two groups (n=8). Group1 (control group). Group 2 injected intraperitoneally with 90 mg/kg indoxacarb for a period of two weeks. Hematological investigations revealed a significant decrease in red blood cells count (RBC), haematocrit, mean corpuscular volume (MCV) and hemoglobin content (Hb) compared to the control group, whereas, white blood cells (WBC) and platelets counts a highly significant increase. Biochemical analysis illustrated that indoxacarb caused a marked elevation of alanine amino transferase (ALT) and aspartate amino transferase (AST) enzymes are increased in the blood of buffalo calves after exposure to indoxacarb for 90 days (Goyal and Sandhu, 2009). Previous studies on indoxacarb acute toxicity following suicidal ingestion have led to clinical effects such as cyanosis, dyspnea, lethargy, coma and acute kidney injure (Chhabra et al., 2010; Wu et al., 2010; Park et al., 2011; Jin 2012; Rathnayaka et al., 2016) and Rhabdomyolysis (Jin 2012; Rathnayaka et al., 2016). However, indoxacarb is a relatively new pesticide, so there are few studies on its side effects on mammals. Blood parameters are considered a potential biomarker of exposure to chemical agents, as they can increase and decrease parameters (Van der Oost et al., 2003). Therefore, the present investigation was performed to evaluate the effects of indoxacarb on blood parameters and histoarchitectural of liver of albino mice.

Keywords: Indoxacarb, Hematology, Histopathology, Liver, Mice

Introduction

Large numbers of pesticides with novel modes of action have been developed to substitute several old pesticides that had been used. Recent pesticides have been particularly planned for fast assassination of pests and relative safety to non-target organisms and environment. The efficiency and safety of new pesticides are a consequence of using special physiologic differences between mammals and specific pests (McCann et al., 2001; Poppengal and Oehme 2010).

Indoxacarb an oxadiazine compound is a new synthetic insecticide produced by DuPont (EPA, 2000; Dinter and Wiles, 2000; Arahashi, 2001), and marketed in the U.S. as Steward and Avant (EPA, 2000). Indoxacarb has a broad spectrum insecticide that is considered a safe substitute for organophosphate insecticides (Rathnayaka et al., 2016) effective against the insects of the Lepidoptera, Coleoptera, Hemiptera, Diptera and Hymenoptera (Nassar, 2016). Indoxacarb (DPX-MP062) is a 75:25 mixture of active and inactive enantiomers displays potent insecticidal activity and low toxicity on non-target organisms (Dinter and Wiles, 2000; Arahashi, 2001; Litchfield et al., 2015, Nassar, 2016). Moreover, it was reported that indoxacarb metabolized inside the insects by esterases and amidases to an insecticidal metabolite, Decarbomethoxylated JW062 (DCJW) (Tsurubuchi and Kono, 2003, von Stein et al., 2012, Nassar, 2016). Also, it is bioactivated in mammals, but decarbomethoxylation to DCJW is less efficient than in insects and detoxication is accomplished through different biochemical pathways (Silver et al., 2010; von Stein et al., 2012).

Indoxacarb blocks the flow of sodium ions through the inhibition of its voltage channels (Tsurubuchi et al., 2001; Chemical fact sheet, 2012, Litchfield et al., 2015, Rathnayaka et al., 2016; Sandeep et al., 2016). This causes an impaired nerve function, cessation of feeding, paralysis and death in insects (Dinter and Wiles, 2000; Laped et al., 2001; Nassar, 2016; Sandeep et al., 2016).

Earlier study reported that levels of alanine amino transferase (ALT) and aspartate amino transferase (AST) enzymes are increased in the blood of buffalo calves after exposure to indoxacarb for 90 days (Goyal and Sandhu, 2009). Previous studies on indoxacarb acute toxicity following suicidal ingestion have led to clinical effects such as cyanosis, dyspnea, lethargy, coma and acute kidney injure (Chhabra et al., 2010; Wu et al., 2010; Park et al., 2011; Jin 2012; Rathnayaka et al., 2016) and Rhabdomyolysis (Jin 2012; Rathnayaka et al., 2016). However, indoxacarb is a relatively new pesticide, so there are few studies on its side effects on mammals. Blood parameters are considered a potential biomarker of exposure to chemical agents, as they can increase and decrease parameters (Van der Oost et al., 2003). Therefore, the present investigation was performed to evaluate the effects of indoxacarb on blood parameters and histoarchitectural of liver of albino mice.

Materials and Methods

Indoxacarb 200 g/L (75:25) was purchased from a local pesticide store, supplied by Libyan Agrochemicals & Agriculture Supplies Specialized Co. it was...
suspension concentrate diluted in water before use and administrated to animal’s intraperitoneally (i.p) at a dose of 90 mg/kg.

Sixteen laboratory adult male mice had been used. These mice bred and housed in the animal house of the Zoology department / Faculty of Science / Tripoli University. Animals were housed in plastic cages containing wooden flakes under husbandry, and maintained at room temperature 22 ± 3° C under natural light/dark photoperiod. The mice were fed with a standard commercial pelleted and drinking water ad-libitum through the study. The experiments were performed on 10-12 weeks old albino mice with weight of 25 to 30 gram (g). The initial and final body weights of mice were recorded.

The mice were divided into two groups of eight animals each. Group 1 served as a control and injected with distilled water in the intraperitoneal. Group 2 was treated with 90 mg/kg body weight of indoxacarb for two weeks. At the end of the experimental period the blood from the control and treated mice were collected for the following parameters; Hb content, hematocrit, MCV, RBC, WBC and platelets counts. The second part of blood was used for serum preparation and assay some biochemical parameters of liver functions such as ALT, total protein and albumin.

Histopathology

Liver tissues were fixed in a 10% neutral buffered formalin solution. The tissues were passed through increasing gradient of ethanol then through xylene, embedded in paraffin and cut a 5-μm thick using rotatory microtome. The sections were stained with Hematoxylin and Eosin (H and E), microscopically examined and photographed. The histopathological examination was conducted in Faculty of Science, Tripoli University.

Statistical analysis

The results obtained in this study are represented as means ± standard deviation (SD) (n=8). Statistical analysis was performed using one-way analysis of variance (ANOVA) to evaluate significant difference between control and treated group for body weight and blood parameters. P-values less than 0.05 were considered statistically significant. All statistical analysis were performed using SPSS statistical version 16 software package.

Results

Morbidity and mortality

Male mice intraperitoneally injected with indoxacarb (90 mg/kg bw/day) for two weeks have shown signs of toxicity such as salivation, diarrhea, tremor, vigorous rolling, head tilt. No death was recorded throughout the experimental period.

Body weight

The body weight of the control group was increased by 1.5 g, while the treated group was decreased by 4.7 g when compared with the initial body weight (Table 1) these results showed high significant decrease in the body weight (P<0.001) compared with the control group.

Hematological and biochemical parameters

The effect of indoxacarb on blood parameters in the mice are presented in (Table 2). There were significant changes observed in treated group compared to the control group. RBC count, hematocrit and MCV significantly reduced (P<0.001). Hb content also decreased considerably (P<0.01). On the other hand, platelets and WBC counts significantly increased (P<0.001) in treated group compared to the control group. The effect of indoxacarb on some biochemical parameters of liver functions are presented in (Table 3). The findings of the present study revealed that treatment with 90 mg/kg indoxacarb showed significant increase in the level of ALT (P<0.05) as compared with that of the control group. However, a marked decrease in total protein (P<0.05) and albumin (P<0.01) was recorded.

Table 1. Effect of indoxacarb treatment on body weight (g) of mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial weight (g)</th>
<th>Final weight (g)</th>
<th>Weight change (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28 ± 0.894</td>
<td>30.5 ± 1.643</td>
<td>1.5</td>
</tr>
<tr>
<td>Treatment (90mg/kg)</td>
<td>27.5 ± 2.422</td>
<td>22.83 ± 1.941***</td>
<td>-4.7</td>
</tr>
</tbody>
</table>

Values are presented as means ± SD. the mean difference is significant at the P<0.05 level. *** Indicates highly significant difference P < 0.001 compared with the control group.
Table 2. Effect of indoxacarb on Hematological parameters in mice

<table>
<thead>
<tr>
<th>Hematological parameters</th>
<th>Control</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (x10^6/µL)</td>
<td>10.54 ± 0.114</td>
<td>9.68 ± 0.228 ***</td>
</tr>
<tr>
<td>WBC (x10^3/µL)</td>
<td>6.84 ± 0.586</td>
<td>15.2 ± 2.775 ***</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>14.46 ± 0.114</td>
<td>12.74 ± 0.865 **</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>53.62 ± 0.303</td>
<td>42.02 ± 2.855 ***</td>
</tr>
<tr>
<td>Plt (x10^3/µL)</td>
<td>442.4 ± 8.562</td>
<td>1534 ± 277.189 *</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>50 ± 0.791</td>
<td>43.3 ± 2.661 ***</td>
</tr>
</tbody>
</table>

RBC, red blood cells; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet; and WBC, white blood cells. Values are presented as means ± SD. ** Indicates very significant difference $P < 0.01$ compared with the control group. *** Indicates highly significant difference $P < 0.001$ compared with the control group.

Table 3. Effect of high dose of indoxacarb on some biochemical parameters of liver functions in mice

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Control</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein (mg/dl)</td>
<td>7.5 ± 0.678</td>
<td>5.78 ± 1.205*</td>
</tr>
<tr>
<td>Albumin (mg/dl)</td>
<td>3.1 ± 0.122</td>
<td>2.1 ± 0.381**</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>34.6 ± 8.532</td>
<td>95 ± 26.457*</td>
</tr>
</tbody>
</table>

ALT, alanine aminotransferase. Values are presented as means ± SD. * Indicates very significant difference $P < 0.05$ compared with the control group. ** Indicates significant difference $P < 0.01$ compared with the control group.

Histopathological

Light microscopy of the liver sections that stained with H and E, from albino mice of the control group (Figure 1. A and B) revealed normal histological architecture of the hepatic tissues with intact cells, that have normal nuclei and the lobules contain central and portal triad space with normal bill duct. However, in the treated mice, histological observations revealed some moderate or mild changes in the architecture of the hepatic tissues (Figure 2). Biliary inflammation within the triads of the liver, intense mononuclear inflammatory infiltrates in portal tracts with loss of bile ductless (A and B). Additionally, focal aggregations of lymphocytes and heterophils were seen in the hepatic tissues of the indoxacarb group (C and D). Moreover, (E and F) showed the cytoplasm was disintegrated, area of necrosis and hepatic architecture with mild hydropic degeneration of hepatocytes. Besides, atrophy, hemorrhage, apoptosis, pyknosis, karyolysis and hyperplasia are seen and hepatic sinusoidal dilatation with blood was observed as well (G). Also, presence of hemosedrin could be seen (H).

Figure 1: Light microscopy of liver sections (Stain: H and E) from albino mice of the control group 1 (A and B), showing normal histological architecture of the hepatic tissues with intact cells, nuclei and central and portal triad space with normal bill duct (Bar=100 µm).
Figure 2: examined sections from Group 2 which was exposed to accumulated dose of indoxacarb of 90 mg/kg/day for two weeks showed some moderate and mild changes in the histological architecture of the hepatic tissues. Mild inflammation within the triads of the liver, intense mononuclear inflammatory infiltrates in portal tracts with loss of bile ductules (A and B) marked by arrows. The inflammatory infiltrate can have granulomatous features as well (C) marked by arrows and (D) by circle. (E) and (F) showed the disintegrated cytoplasm, area of necroses as showed by the (stars) and the head arrows showed the hydropic hepatocytes. Atrophy, hemorrhage, apoptosis, pyknosis, karyolysis and hyperplasia are seen and sinosodial dilatation were observed (G). The presence of hemosedrin could be seen (H).

Discussion

Human may be exposed to indoxacarb from residues on vegetables and fruits or during exposure in the occupational environment. There is no report on the effects of indoxacarb on the histopathology of the mammals organs and limited data about its effect on the blood parameters in mammals (EPA, 2004; California Environmental Protection Agency, 2004; EPA, 2007). Thus, the effect of indoxacarb on the hematological, biochemical parameters and histology of liver was performed.

Investigated study illustrates that administered 90 mg/kg/day of indoxacarb i.p to mice for two weeks has produced significant toxic effects. There was highly significant decrease in the body weight of treated mice. The decrease in body weight is in agreement with the previous studies (Arfat et al., 2014; Badgujar et al., 2013) who reported that high dose of the insecticide imidacloprid resulted in reduction of the body weight of mice. The animals were less susceptible to food intake
due to the toxic effect of pesticides that led to body weight reduction (Li et al., 2007).

The results obtained on the hematological parameters of the treated mice showed a significant variation from that of control group. Hematological index are sensitive to various chemicals and environmental factors (Vosyliene, 1999) and reflect the health condition of an organism (Ojutiku et al., 2013). This study illustrated that indoxacarb reduced the RBCs count, hematocrit, MCV and Hb in mice. This effect is likely due to the adverse effect of indoxacarb on bone marrow or enhanced destruction of RBC (Shit et al., 2008). The reduction of the level of the Hb, RBCs count and hematocrit was consistent with in vitro studies conducted on rat and dog exposed to indoxacarb (EPA, 2004; California Environmental Protection Agency, 2004; EPA, 2007). In addition, these results are in agreement with the previous report by Shit et al., (2008) who reported that RBCs in mice were decreased after exposure to a single dose of 500 mg/kg indoxacarb. Similar reduction of RBC and Hb were reported by Eissa and Zidan (2009) in albino rats exposed to the pesticide abamectin. Furthermore, other investigators following treatment with different pesticides reported reductions in Hb, RBC counts and hematocrit (Adedeji et al., 2009; Kim et al., 2009; Magar and Dube, 2012). It was suggested that reduction in RBC and Hb due to disruption of hematopoiesis (Ali, 1990; Anambama et al., 2001; El-Deeb et al., 2007; Adedeji et al., 2009; Magar and Dube, 2012; Selmanoglu et al., 2001; Choudhari and Deshmukh, 2007).

The current study also showed a significant increase in platelets and WBC counts. Increased WBC count can assist in the removal of cellular debris of necrotic tissue rapidly and aids in survival and recovery of animal exposed to insecticide (Magar and Dube, 2012). These results are quite similar with those found by others who showed that the pesticide cypermethrin and malathion increased WBC in fishes (Magar and Dube, 2012; Ojutiku et al., 2013). Similar results was reported that ivermectin increase WBC in donkeys (Ismail et al., 2013). Considerable increase in platelets count in the treated group recorded in this study in the accordance with the study of Adedeji et al., (2009) who reported that platelets enhanced following treatment of African catfish with diazinon. Platelets play critical function in blood clotting, which prevent blood loss from hemorrhage and therefore high number of platelets reduces clotting time (Adedeji et al., 2009). However, this result is contradictory with other results suggested that abamectin (Eissa and Zidan, 2009) and chlorpyrifos (El-Deep et al., 2007) caused reduction in platelets and WBC in albino rats The difference in the results could be due to many factors such as species, pesticides, duration of exposure and route of administration.

Liver play significant role in the detoxification of various chemicals and drugs, therefore, it is adversely affected by different chemicals that enter into animal body regardless the way of administration (Guyton and Hall, 2006). Significant increase in ALT (P<0.05) was observed in the current study. Elevation ALT in the blood may be associated with degeneration and necrosis of hepatocytes which confirmed in this study through microscopic examination of the liver of treated mice. These findings are consistent with those reported by (Arfat et al., 2014) who pointed out that ALT and AST were increased following imidacloprid exposure in rats. Other studies illustrated that a considerable increase in the level of ALT and AST in pesticide exposed agricultural workers. (Ibrahim et al., 2011; Awad et al., 2014). Rise of transaminases in blood may be related to tissue damage which increased synthesis of these enzymes as an adaptive mechanism to the chemical stress (Rahman and Siddiqui, 2003). In addition, it was hypothesized that increase ALT in blood may be due to alterations in permeability of the cell membrane of hepatocytes after hepatotoxic damage (Choudhary et al., 2003; Gaskill et al., 2005; Mansour and Mossa, 2010). These results are in accordance with other studies (Eissa and Zidan, 2010; Ambali et al., 2011) who revealed a marked decrease of total protein in rats exposed to insecticides. Similarly, diminished total protein has also been reported by other researchers (Awad et al., 2014) in agricultural workers and (Adedeje, 2010) in African cat fish. It might attribute to increase catabolism of the biomolecules to meet energy demand under stress or as a result of impaired liver function (Ivanova-Chemishanska 1982).

Regarding to histopathological examination, various pathological changes observed in the liver of mice following exposed to indoxacarb included focal inflammation, karyolysis, pyknosis, monocellular infiltration and sinusoid dilatation. In this study the histological changes in the liver were similar to the result of Selmanoglu et al., (2001) who confirmed that carbendazim caused mononuclear cell infiltration, dilution of sinusoids and increase number of kupper cells in the liver of rats. In addition, Abd-Elhadry and Abu-Elghar (2013) reported that carbendazim caused degenerative and atrophy of hepatocytes and congestion of blood vessels of rats. Furthermore, these findings are in accordance with other studies who reported that mild focal necrosis of the liver and hepatocellular damage following imidacloprid exposure in mice (Arfat et al.,2014) and chickens (Kammon et al., 2010). Moreover, it was reported that treatment of rats with endosulfan (Choudhury et al., 2003) and mice with carbosulfan (Ksheerasagar and Kaliwal 2006) caused liver damage such as dilatation of sinusoids, hypertrophy of hepatocytes and lymphocytic infiltration in central vein. Additionally, histopathologic study of rats’ liver treated with DDT and permethrin individually revealed hepatocytes with pyknotic, and cell with nuclear destruction stimulated by permethrin, while DDT caused cytoplasmic vaculization and hepatocyte necrosis (Kostka et al., 2000).

Conclusion

According to our findings in this study, indoxacarb caused significant changes in hematological and
biochemical indices and mild to moderate damage in the liver in mice, therefore use of the pesticides extensively without regulation could lead to dangerous effects in non-target animals and human.

References


