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RESEARCH

Quality Control Testing for Tracking Endotoxin-Producing Gram-Negative Bacteria during the Preparation of Polyvalent Snake Antivenom Immunoglobulin

NORHAN S. SHERABA,1 MOHAMED R. DIAB,1 AYMEN S. YASSIN,2,* MAGDY A. AMIN,2 and HAMDALLAH H. ZEDAN2

1Vacsera, The Holding Company for Biological Products and Vaccines, Giza, Egypt; 2Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, Cairo, Egypt ©PDA, Inc. 2015

ABSTRACT: Snake bites represent a serious public health problem, particularly in rural areas worldwide. Antitoxic sera preparations are antibodies from immunized animals and are considered to be the only treatment option. The purification of antivenom antibodies should aim at obtaining products of consistent quality, safety, efficacy, and adherence to good manufacturing practice principles. Endotoxins are an integral component of the outer cell surface of Gram-negative bacteria. They are common contaminants of the raw materials and processing equipment used in the manufacturing of antivenoms. In this work, and as a part of quality control testing, we establish and examine an environmental monitoring program for identification of potential sources of endotoxin-producing Gram-negative bacteria throughout the whole steps of antivenom preparation. In addition, we follow all the steps of preparation starting from crude plasma till finished product using a validated sterility and endotoxin testing.

Samples from air, surface, and personnel were collected and examined through various stages of manufacturing for the potential presence of Gram-negative bacteria. A validated sterility and endotoxin test was carried out in parallel at the different production steps. The results showed that air contributed to the majority of bacterial isolates detected (48.43%), followed by surfaces (37.5%) and then personnel (14%). The most common bacterial isolates detected were Achromobacter xylosoxidans, Ochrobactrum anthropi, and Pseudomonas aeruginosa, which together with Burkholderia cepacia were both also detected in cleaning water and certain equipment parts. A heavy bacterial growth with no fungal contamination was observed in all stages of antivenom manufacturing excluding the formulation stage. All samples were positive for endotoxin including the finished product.

Implementation and continued evaluation of quality assurance and quality improvement programs in aseptic preparation is essential in ensuring the safety and quality of these products.

KEYWORDS: Endotoxin, Pyrogen, Antivenom, Vaccine, Gram-negative.

LAY ABSTRACT: Antitoxic sera preparations are the only treatment option for snake bites worldwide. They are prepared by immunizing animals, usually horses, with snake venom and collecting horse plasma, which is then subjected to several purification steps in order to finally prepare the purified immunoglobulins. Components of the bacterial cell wall known as endotoxins can constitute a potential hazardous contamination known as pyrogen in antisera, which can lead to fever and many other adverse reactions to the person subjected to it.

In this work, we monitored the environment associated with the different steps of production and purification of snake antivenom prepared from immunized horses. We examined the air quality, surface, and personnel for possible sources of contamination, particularly the presence of Gram-negative bacteria, which is the major source of endotoxin presence. We also monitored all stages of preparation by sterility and endotoxin testing. Our results showed that air contributed to the majority of bacterial isolates. Sterility testing revealed the presence of bacterial contamination in all the intermediate steps, as only the final preparation after filtration was sterile. Endotoxin was present in all tested samples and the final product. Good manufacturing practice procedures are essential in any facility involved in antisera production.

*Corresponding author Aymen S.Yassin, PhD, Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, Kasr Eleini St., Cairo, 11562, Egypt; phone number: 2-02-25353100/200/300/400; mobile: 2-0100-9610341; e-mail: aymen.yassin@pharma.cu.edu.eg
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Introduction

Envenoming and subsequent death resulting from snakebites is a serious public health problem in rural and tropical areas of Africa, Asia, and Latin America (1). Intravenous administration of snake antivenom, taken at different doses on a case-to-case basis, is the principal therapy for the majority of medically significant poisoning cases (2). Snake antivenom sera are produced by immunizing horses with repeated nonlethal doses of snake venom. These sera contain the antibody [(Fab’)2 fragments] responsible for neutralizing the toxic effects of the venom (3). After immunization, the horse plasma is submitted to enzymatic treatment, fractional precipitation, and molecular cut-off ultrafiltration to purify a high level of specific immunoglobulins (4). Consequently, these preparations are rich in their protein content and are usually a mixture of several sera obtained after purifying plasma of animals immunized with different strains of snake venoms common to a certain geographic area where the final preparations are to be used.

When highly stringent measures against microbial contamination are not taken, the horse hyperimmune snake antivenom sera might be contaminated by endotoxins (pyrogens) from accidental microbial contamination. Bacterial endotoxins are lipopolysaccharides present at the outer cell surface of Gram-negative bacteria, whether the organisms are pathogens or not, and they constitute one of the major problems in the formulation of pharmaceutical products (5). Endotoxins are released into the environment in the course of bacterial cell lysis or during active growth of the cell (6). Because Gram-negative bacteria are present all over the environment, endotoxin can be found in the soil, water, house dust, and humans (7).

Many selective culture media are used for the isolation of Gram-negative bacteria in clinical samples and for assessment of water quality. However, culture media has not been used for the detection of airborne bacteria in manufacturing environments. Previous work has shown that endotoxin levels in the air of waste water treatment plants can be estimated from bacterial counts by use of a selective medium for Gram-negative bacteria, eosin methylene blue medium (8).

According to World Health Organization (WHO) guidelines (9), the purification of immunoglobulins and immunoglobulin fragments for the production of antivenoms should aim at obtaining products of consistent quality, safety, and efficacy. This covers all stages leading to the finished antivenoms, including production of water, production of plasma, purification, and processing of the finished product. Of particular relevance is the control of microbiological risks and contamination with particulates and pyrogens. All processing areas should be monitored with special attention paid to air, surfaces, and personnel. An environmental monitoring program controls the viable microbial load and provides data about the air system efficiency in operational procedures. Alert and internal action at specific microbiological levels can be determined through frequent monitoring of the environment and operators. Consequently, deviations from normal conditions can be detected, allowing corrective procedures before product quality is affected (10).

The application of the sterility test aims to assure the quality of the sterilizing process and the sterile products, considering the probabilistic and contamination risks. Due to the statistical limitations of the sterility test, it is necessary to use validated assays that present sensibility, accuracy, reproducibility, and avoid any false results caused by external factors. All installations and parameters such as the bacteriostatic and fungistatic effects of the preservative system should be previously qualified (11). Similarly, the application of a validated endotoxin testing is essential to carefully detect the presence of endotoxins in a particular product.

The aim of the present work is to apply in-process quality testing on the different stages of antivenom preparation. In an attempt to track the sources that might contribute to the presence of endotoxins, an environmental monitoring program was applied, as well as a validated sterility test to detect viable microorganisms, in addition to a validated bacterial endotoxin test to detect endotoxins from both viable and non-viable Gram-negative bacteria throughout the whole process. Determination of the sources of contamination is a critical step towards the completion of a successful quality control testing in any facility.

Materials and Methods

Statement of Ethical Approval

All experiments, involving any samples taken from personnel in this study, were done in accordance and approval of the Ethical Committee at Cairo University, Cairo, Egypt. In addition, all personnel who contrib-
uted any samples did this according to their written informed consent.

**Study Location, and Design**

The study was conducted in the antisera production plant of Vascera (The Egyptian Company for the Production of Sera, Vaccines and Drugs and the main vaccine producing facility in Egypt), which has three production units; a fractionation unit (class C), a purification unit (class C), and a filtration and filling unit (Class B). The fractionation unit is where the digestion of horse plasma proteins by pepsin takes place, followed by ammonium sulphate precipitation of (Fab')$_2$ and desalting by tangential flow diafiltration. In the second unit, both the purification of (Fab')$_2$ by caprylic acid and precipitation of non (Fab')$_2$ proteins take place. The third unit, which is the sterile filtration and filling unit, involves the sterile filtration through a 0.45 μm filter, followed by a 0.22 μm filter, and dispensing into final containers. Operators were equipped with sterile lab uniforms, gloves, caps, and face masks. The floor and horizontal surfaces were cleaned and sanitized daily before working hours with Lysoformin 1% (Lysoform, Berlin, Germany) by trained workers and supervised by appropriate personnel. The work surfaces inside the laminar air flow (LAF) hoods were swab-disinfected with ethanol 70% before commencing work and after each shift. Temperature and relative humidity were monitored with a portable combined thermo-hygrometer.

Stream samples were taken at different stages of production and collected aseptically in sterile, endotoxin-free tissue culture flasks for detection of microbial contamination using validated sterility testing. Representative samples were taken from various locations within each unit for each method under operational conditions. Non-exposed plates were also incubated as negative control to confirm the sterility of media for each method used.

**Microbiological Monitoring of Air, Surfaces, and Personnel**

The microbial air contamination was measured by passive air sampling using MacConkey and Sabouraud dextrose agar (SDA) settling plates of 90 mm diameter exposed horizontally for 4 h in high-risk positions in the fractionation, purification, and sterile filtration and filling units (12). Surface samples were taken with contact plates (diameter 55 mm) at predetermined positions in all units at the end of a shift. The Replicate Organism Detection and Counting (RODAC) plates contained MacConkey agar medium with polysorbate 80 and lecithin to neutralize residues of disinfectants; the entire surface of each plate was put over the surface to be tested. After sampling, any residual medium was wiped off the surface with alcohol (13). Glove prints were taken from personnel by pressing the five fingertips of each gloved hand on one plate before changing gloves; the same type of RODAC MacConkey plates that were employed in surface sampling were used for personnel (14). All collected samples were incubated at 37 °C for 5 days for MacConkey medium and at 20–27 °C for 7 days for SDA (12). Colony counts and identification of the microbial isolates were carried out as soon as colony-forming units (CFU) were visible by established microbiological laboratory methods (15). Identification was carried using the API Staph identification software (BioMérieux, Marcy l’Etoile, France).

**Stoppers, Vials, and Rinsing and Manufacturing Water Samples**

Aseptically, 20 stoppers to be tested were placed into a sterile container containing 50 mL of Tryptic soya broth with 0.1% Tween solution for 1 h and shaken for approximately 30 minutes then incubated at 30–35 °C for no less than 72 h. For unprocessed vials, each vial was filled about halfway with Tryptic soya broth with 0.1% Tween 80 solution. After filling, depyrogenated parafilm was placed onto each vial and placed onto a mechanical shaker for approximately 30 min. All vials were pooled into sterile, endotoxin-free container and then incubated at 30–35°C for no less than 72 h. One milliter was pipetted from each of the previously mentioned containers and streaked on MacConkey agar plate for detection of Gram-negative bacteria followed by incubation at 30 to 35°C for no less than 72 h; then colonies were identified. In each collected water sample, bacteria counts were done in duplicate on MacConkey’s agar plates by the spread plate technique, and plates were incubated at 37 °C for 3 days and then identified.

**Sterility Testing and Controls**

Direct inoculation of culture media was applied in which 1.5 mL was aseptically transferred from each of 20 containers, then 1 mL from the pooled sample was used to inoculate each vessel of the culture media [fluid thioglycollate medium (FTM), soybean casein...
digest medium (SDM) using sterile syringe followed by gentle mixing. All the inoculated media were incubated for not less than 14 days at 30–35 °C for FTM and 20–25 °C for SDM. All the inoculated media were examined during the incubation period and at its conclusion for macroscopic evidence of visible microbial growth (16). The results are interpreted as shown in Table I.

Microbiological monitoring was performed for evaluating the environment in which sterility tests were conducted including settling plates containing trypticase soy agar medium placed on both sides of LAF, RODAC plates poured with trypticase soy agar medium and containing Polysorbate 80 & lecithin with convex surface to examine the LAF workbench and operators’ gloved hands. All plates were incubated at 30–35 °C for 72 h; non-exposed plates were also incubated as negative control to confirm the sterility of media (17). This is done to verify the efficacy of the LAF.

Growth promotion test (positive control) was performed to demonstrate that the medium will support the microbial growth as follows: 15 mL of both FTM and SDM were inoculated with 10–100 CFU of each of the microorganisms indicated in Table II. FTM was incubated at 30–35 °C for 3 days and SDM at 20–25 °C for 5 days. The abovementioned challenge inocula were verified by concurrent viable plate counts. The media were considered suitable for sterility testing if a clearly visible growth of the microorganisms occurs within the specified time period for each organism. A media sterility test (negative control) was performed to demonstrate the sterility of the media used; all FTM and SDM used were incubated at 30–35 °C and 20–25 °C, respectively, for 14 days. A negative product test was performed to check the reliability of the test procedure and the validity of test outcome; sterile diluting fluid was used periodically as a negative control in sterility testing (18).

A validation test (bacteriostasis and fungistasis) was employed to determine whether the therapeutic antisera contained any antimicrobial activity that may adversely affect the outcome of sterility testing as

### Table I
**Interpretation of the Sterility Test Data (N Represents the Total Number of Vials in the Lot)**

<table>
<thead>
<tr>
<th>Original Test Sampling = (0.4/N)</th>
<th>1° Re-Test Sampling = (0.4/N)</th>
<th>2° Re-Test Sampling = (0.8/N)</th>
<th>Final Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong> Negative growth</td>
<td>-</td>
<td>-</td>
<td>Satisfactory</td>
</tr>
<tr>
<td><strong>B</strong> Positive growth</td>
<td>Negative growth</td>
<td>-</td>
<td>Satisfactory</td>
</tr>
<tr>
<td><strong>C</strong> Positive growth</td>
<td>Positive growth</td>
<td>Negative growth</td>
<td>Unsatisfactory*</td>
</tr>
<tr>
<td><strong>D</strong> Positive growth</td>
<td>Positive growth</td>
<td>Positive growth</td>
<td>Satisfactory**</td>
</tr>
<tr>
<td><strong>E</strong> Positive growth</td>
<td>Positive growth</td>
<td>Positive growth</td>
<td>Unsatisfactory***</td>
</tr>
</tbody>
</table>

* Same microorganism detected in the original test and first re-test.
** Different microorganisms isolated in the original test, first re-test, and negative second re-test.
*** Contamination in the three tests, even by different microorganisms.

### Table II
**Standard Microorganisms Used in Growth Promotion and Validation Tests**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Types of Microorganisms</th>
<th>Strains of Microorganisms</th>
</tr>
</thead>
</table>
| FTM    | Aerobic                 | *Bacillus subtilis* ATCC 6633  
|        |                         | *Pseudomonas aeruginosa* ATCC 9027  
|        |                         | *Staphylococcus aureus* ATCC 6538  |
|        | Anaerobic               | *Clostridium sporogenes* ATCC 19404  |
| SDM    | Aerobic                 | *Bacillus subtilis* ATCC 6633  
|        |                         | *Pseudomonas aeruginosa* ATCC 9027  
|        |                         | *Staphylococcus aureus* ATCC 6538  |
|        | Fungi                   | *Aspergillus niger* ATCC 16404  
|        |                         | *Candida albicans* ATCC 10231  |

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follows: After aseptically transferring 1.5 mL from each container to be tested to the culture medium (FTM and SDM), 10–100 CFU of the challenge inocula mentioned in Table II were added to the medium. FTM was incubated at 30–35 °C for 3 days and SDM at 20–25 °C for 5 days. The abovementioned challenge inocula were verified by concurrent viable plate counts.

Endotoxin Testing

After test validation and including all the appropriate controls, a total of 100 μL of reconstituted Limulus amebocyte lysate (LAL) reagent (Endosafe, Charles River, Wilmington, MA) was added to each tube to be tested, and the pH of the product/LAL mixture at the compatible dilution/concentration was adjusted between 6.5 and 8.0 if necessary using 0.1 M Tris HCl solution or 0.25 M Tris Base solution not exceeding 10% of sample volume. Immediately, the contents for each tube were mixed thoroughly and all tubes were placed in a non-circulating water bath at 37 ± 1 °C for 60 ± 2 min. After the incubation period, each tube was carefully removed and inverted slowly by 180° (upside-down). Each tube was scored as (positive) for a firm gel, or (negative) for non-gel formation.

Results

Levels of Gram-Negative Bacteria in Different Locations

A total of 101 samples were collected from air sampling. The distribution of the colonies found on MacConkey agar medium was as follows: At the fractionation unit (designated as class C), the count was from 50 to 140 CFU/4 h (at the corners and under tanks). These numbers do not meet the requirements of class C, as the acceptance criteria is ≤50 CFU/4 h for air monitoring. At the purification unit (also designated as class C), the level was 25–35 CFU/4 h (within the limit of class C). At the filling unit (designated as class B), the level was from 0 to 5 CFU/4 h. The numbers at the filling meet class B requirements criteria of ≤5 CFU/4 h for air monitoring. No fungi were detected on SDA plates.

A total of 51 samples were collected from surfaces; the Gram-negative bacterial count varied from 20 to 65 CFU/25cm² in the fractionation unit (Class C) to 15–35 CFU/25cm² in the purification unit (Class C). Consequently, both sections did not meet grade C requirements (acceptance criteria ≤25 CFU/25cm² for surface monitoring). In the filling unit (Class B), the requirements were not fulfilled as well, as the levels were 0–10 CFU/25 cm² (acceptance criteria ≤5 CFU/25 cm² for surface monitoring).

On operators’ glove prints (38 samples), the range from for the fractionation (Class C) and purification (Class C) units exceeded the limits of grade C (≤5 CFU/fingerprint for personnel monitoring). For the operators working in the filling unit (Class B), the levels were below the limits of grade B (<1 CFU/fingerprint for personnel monitoring). All negative controls for each method used showed no bacterial growth. All microbial limit counts were evaluated according to the European Commission’s Volume 4. EU Guidelines for Good Manufacturing Practice for Medicinal Products for Human and Veterinary Use—Annex 14. Manufacture of Medicinal Products Derived from Human Blood or Plasma.

Identification of the Recovered Microorganisms

A total of 64 microbial isolates were recovered from the environmental monitoring program. Out of the total 64 isolates, 56 isolates (87.5%) were Gram-negative bacilli, oxidase-positive (Non-Enterobacteriaceae) as identified by Analytical Profile Index 20E (API 20E). A total of seven isolates (10.9%) were Gram-negative bacilli, oxidase-negative (Enterobacteriaceae), as they formed pink colonies on MacConkey’s agar medium, and one isolate (1.6%) was yeast. The 64 isolates were distributed as follows: 31 isolates from air sampling, 24 isolates from surface monitoring, and nine isolates from personnel. Among the total 31 isolates detected from air sampling, 30 isolates were non-Enterobacteriaceae and one isolate (1.6%) was yeast. Among the 24 isolates detected from surface monitoring, two isolates were Enterobacteriaceae and the rest were non-Enterobacteriaceae. Among the nine isolates detected from personnel, five isolates belonged to Enterobacteriaceae and four isolates were non-Enterobacteriaceae. Tables III to V list the names, numbers, and percentages of isolates detected in the three units (fractionation, purification, and filling) out of each section (air, surface, and personnel). From the environmental point of view, more than half of Gram-negative bacterial isolates that cause microbiological contamination originated from air, 48.43% (31 out of 64); from surface, 37.5% (24 out of 64); and less commonly isolated from humans, 14% (9 out of 64). Achromobacter xylosoxidans, Pseu-
domonas aeruginosa, and Ochrobactrum anthropi were the most common bacteria isolated from the air, surface, and personnel, respectively.

Monitoring of Bioburden on Stoppers, Unprocessed Vials, and Water Samples

Among the total of 20 stoppers examined, five isolates were recovered on MacConkey’s agar plates and identified as Burkholderia cepacia. However, no bacterial growth was observed in case of unprocessed vials. Throughout this study, 37 isolates were recovered from 12 water samples collected from the final rinse resulting from cleaning of tanks used in antisera formulation. Prevalent isolates were P. aeruginosa, 19 isolates (51.3%); Burkholderia cepacia, 13 isolates (35.1%); Pseudomonas fluorescens, three isolates (8.1%); Pseudomonas luteola, one isolate (2.7%); and O. anthropi, one isolate (2.7%). Water samples used in the preparation procedure themselves were free from any microorganism.

Sterility Testing

All sampled plates used for evaluating the working conditions of the sterility testing in the facility showed no microbial growth with non-exposed plates as a control for the sterility of media. Sterility testing was performed on all stages of the manufacturing process of antisera starting from the raw material (pooled plasma) acquisition, till the filling of the antivenom using direct inoculation, to assure the quality of the sterilizing process and of the sterile products considering the probabilistic and contamination risks.

TABLE III
List of Numbers and Percentage of the Recovered Microbial Isolates from Air

<table>
<thead>
<tr>
<th>Types of Isolated Microorganism</th>
<th>Fractionation Section N = 13</th>
<th>Purification Section N = 17</th>
<th>Filling Section N = 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burkholderia cepacia</td>
<td>2 (15.38%)</td>
<td>4 (23.5%)</td>
<td></td>
</tr>
<tr>
<td>Achromobacter xylosoxidans</td>
<td>2 (15.38%)</td>
<td>2 (11.76%)</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>1 (7.69%)</td>
<td>1 (5.9%)</td>
<td></td>
</tr>
<tr>
<td>Ochrobactrum anthropi</td>
<td>1 (7.69%)</td>
<td>9 (52.64%)</td>
<td></td>
</tr>
<tr>
<td>Stenotrophomonas maltophilia</td>
<td>1 (7.69%)</td>
<td>1 (5.9%)</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>2 (15.38%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast</td>
<td>1 (7.69%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Comamonas testosteroni</td>
<td>1 (7.69%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aeromonas hydrophilia/caviae</td>
<td>2 (15.38%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE IV
List of Numbers and Percentage of the Recovered Microbial Isolates from Surfaces

<table>
<thead>
<tr>
<th>Types of Isolated Microorganism</th>
<th>Fractionation Section N = 15</th>
<th>Purification Section N = 8</th>
<th>Filling Section N = 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>5 (33.3%)</td>
<td>3 (37.5%)</td>
<td></td>
</tr>
<tr>
<td>Ochrobactrum anthropi</td>
<td></td>
<td>1 (12.5%)</td>
<td></td>
</tr>
<tr>
<td>Aeromonas hydrophilia/caviae</td>
<td>1 (6.66%)</td>
<td>3 (37.5%)</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>1 (6.66%)</td>
<td>3 (37.5%)</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas luteola</td>
<td>2 (13.33%)</td>
<td>1 (12.5%)</td>
<td></td>
</tr>
<tr>
<td>Achromobacter xylosoxidans</td>
<td>2 (13.33%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Comamonas testosteroni/Pseudomonas alcaligenes</td>
<td>2 (13.33%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacter sakazakii</td>
<td>1 (6.66%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>1 (6.66%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
After incubation for at least 14 days at 30–35 °C for all fluid thioglycollate media and at 20–25 °C for all soybean casein digest media, all fluid thioglycollate media showed a heavy bacterial growth in all stages of manufacturing of antivenom starting from pooling of crude plasma till mixing of different species of antisera. From these tubes, a subculture was made into trypticase soya agar followed by incubation at 30–35°C for 3 consecutive days. The isolated bacteria were microscopically visualized as Gram-positive bacilli and spore-forming, while no bacterial growth was detected in the bulk after the first and second clarification by filtration through 0.45 \( /H9262 \) and 0.22 \( /H9262 \) membranes filters, respectively. In all soybean casein digest medium, no fungal growth was detected in any stages of manufacturing of antivenom including the filling of the final product. A retest was performed as indicated in Table I to assure that the contamination was from the product and not from the test manipulation. The results showed that all samples failed the second sterility test as failed the initial one.

**Endotoxin Testing**

Endotoxin was detected in all tested samples starting from crude plasma to the finished product using a validated endotoxin testing (LAL test). No endotoxin was detected on stoppers and unprocessed vials used in the study. All water samples collected from the final rinse resulting from cleaning of tanks, and also all those from water sampling points used during the antisera manufacturing stages, contained endotoxins.

**Discussion**

The antivenom sera used for treatment of the envenomation caused by snake venom are immunological products for intravenous, subcutaneous, or sub-mucous injection. Among several requirements, these products must be microorganism and endotoxin-free, which may be obtained through a rigid microbiological control during the whole fabrication process. In addition, it is essential to implement an environmental monitoring program to identify out-of-trend conditions that can indicate any breach in the aseptic practices/effectiveness of a cleaning and sanitization program. Endotoxin contamination is a serious threat to the safety of parenteral drugs. Many severe biological reactions are induced by endotoxins which enter blood circulation, so it is necessary to determine and control the contaminated endotoxin levels in injections as a quality control measure (19).

This study addresses some of the methods for characterizing the types of microorganisms found in the monitored sections by investigating the potential origins of Gram-negative bacteria to indicate the potential sources of contamination and to characterize the bacterial flora in the air, on surfaces, and on glove prints of operators working in the fractionation, purification, and filling sections. Selective and differential culture medium (MacConkey) was the principle culture medium used in our study, as it is recommended for the isolation of Gram-negative bacteria, which is the main endotoxin contributor. SDA plates were also employed to rule out the presence of fungi, which might interfere with the LAL test due to presence of beta-D-glucan leading to a false-positive result (20). Our results showed that air contributed to the majority of bacterial isolates detected (48.43%), followed by surfaces (37.5%), and then personnel (14%). The most common bacterial isolates identified were *A. xylosoxidans*, *P. aeruginosa*, and *O. anthropi*. Sterility testing indicated a heavy bacterial growth contamination observed in all of the antivenom manufacturing stages excluding the final formulation (filtration and filling) one.
In air, settling plates enabled a direct assessment of the most likely microbial contamination of containers by measuring the number of microbe-carrying particles that fall onto a surface. The high counts of viable (culturable) particulate found in the fractionation and filling sections could be attributed to the poor air quality resulting from the lack of continuous high-efficiency particulate arresting (HEPA) filtration of the air in this monitored area. While the relatively low number of viable particulates found under LAF hoods could be attributed to the fact that the LAF hoods improve the air quality by continuously HEPA-filtering the air of the controlled area. This demonstrates the importance of a regular change of the prefilters at least once a month (21). This finding corroborates with the work done by Utescher et al. (10), who suggested a lower efficiency in the air system of a large institution dedicated to immunological production in Brazil. Microorganisms would increase to unacceptable levels in the absence of an adequate infection control regimen, less efficient ventilation systems, and contaminated air conditioning systems that recirculate microorganisms in the air or have old water distribution pipes which may be colonized with microorganisms, providing a niche for endotoxin growth (22). Airborne microorganisms are normally injured due to lack of nutrients and dehydration. Gram-negative bacteria are very sensitive to these conditions, and thus more difficult to recover. Even when present, stressed Gram-negative bacteria may fail to recover due to the lack of definite detection methods. The use of selective agar alone greatly underestimates contamination with Gram-negative bacteria (23).

On surfaces, the high CFU counts detected in both fractionation and purification sections were obviously a result of inadequate housekeeping, which gave reason to the assessment of the actual cleaning and sanitization plan and behavior of personnel; our results agree with the previous work of several groups (10, 24). In a previous study, sufficient immersion or spraying of a disinfectant supply prevented the ingress of contamination into the critical areas and led to low CFU counts in the processing area of centralized intravenous admixture services (12).

Glove prints are the most critical samples because of the high risk of direct product contamination by touch. Handling disinfected but not sterile vials, containers, and labels causes microbiological contamination of sterile gloves. In this work, the CFU counts were high with the exception of the glove prints from personnel within the filling unit, which were within the limits (25). Considering that glove prints were taken immediately before the gloves were changed, these counts can be regarded as the maximum counts. This demonstrates the necessity of a regular disinfection of the gloved fingertips during processing, especially after touching vials or containers and after labelling the finished preparation. These findings are consistent with those obtained by Singh et al. (26), who highlighted the need for adequate measures such as personnel protective equipment and training on good hygiene practices to prevent endotoxin exposure and to improve healthcare worker behavior.

During the present study, it was revealed that there exists a negatively strong correlation between the presence of Gram-negative bacteria and humidity and a positive correlation with temperature. Similar findings were obtained by Grisoli et al. (27). A microclimatic factor such as indoor temperature was shown to be a good predictor of airborne endotoxin levels, whereas air velocity can be a key element in a reduction strategy to control endotoxin levels. These findings suggest that increasing ventilation would assist in reducing the endotoxin load in similar settings that require a clean environment according to the recommendations of the U.S. Centers for Disease Control and Prevention (22).

Bacterial isolates were identified with macroscopic and biochemical methods, as recommended (28). The majority of overall isolates were oxidase-positive Gram-negative bacilli (Non-Enterobacteriaceae) and less common were oxidase-negative Gram-negative bacilli (Enterobacteriaceae) with percentages of 87.5% and 10.9%, respectively. Although there are a few reports of contamination in clean rooms for pharmaceutical production, the most frequently isolated microorganisms in controlled areas used for aseptic processing are bacteria from the human skin (e.g., Staphylococcus spp., Micrococcus spp., Corynebacterium spp.), airborne bacterial spores (e.g., Bacillus spp.), occasionally airborne fungal spores (e.g., Aspergillus niger, Penicillium spp.), and most infrequently Gram-negative bacteria (e.g., Enterobacter cloacae, Burkholderia cepacia) (29, 30).

Burkholderia cepacia isolates were recovered from the stoppers and identified on the basis of colony morphology on MacConkey’s agar medium and biochemical characteristics. Bacterial species determined within the filling unit, which were within the limits (25). Considering that glove prints were taken immediately before the gloves were changed, these counts can be regarded as the maximum counts. This demonstrates the necessity of a regular disinfection of the gloved fingertips during processing, especially after touching vials or containers and after labelling the finished preparation. These findings are consistent with those obtained by Singh et al. (26), who highlighted the need for adequate measures such as personnel protective equipment and training on good hygiene practices to prevent endotoxin exposure and to improve healthcare worker behavior.

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Burkholderia cepacia isolates were recovered from the stoppers and identified on the basis of colony morphology on MacConkey’s agar medium and biochemical characteristics. Bacterial species determined in the final rinse of collected water samples from
cleaning of tanks were mainly *P. aeruginosa* (51.3%) and *B. cepacia* (35.1%). *B. cepacia* is a life-threatening human pathogen usually associated with respiratory infections of cystic fibrosis patients. Unlike *Pseudomonas*, *Burkholderia cepacia* can be easily spread from patient to patient, and it is resistant to all amino-glycoside drugs, which are typically used to treat *P. aeruginosa* infections. Monitoring and controlling contamination of facilities, clean utilities, and products for the presence of *Burkholderia cepacia* have become priorities for certain manufacturers of drugs that are at risk of being contaminated with this known pathogen (31). This work emphasized that the methods currently in use for cleaning tanks used for antisera formulation weren’t efficient in the removal of Gram-negative bacteria.

No endotoxin was detected in the case of stoppers in spite of the fact that bacterial growth was observed. Because endotoxins are a part of the cell wall of the bacteria, it was thought that their detection by specific endotoxin assays could be used instead of by a bacterial count. However, several studies reported disappointing results, showing the lack of correlation between the degree of bacterial contamination (CFU) and the endotoxin content (Endotoxin Units EU) measured by LAL (32). Neither detectable bacterial growth nor endotoxin was observed in the unprocessed vials. This situation may be due to effective washing and depyrogenation process.

Both bacterial growth and endotoxin presence were present in rinsing water; however, endotoxin content with no bacterial growth was observed in the water itself used during the antisera manufacturing stages. Two explanations for this observation may be offered. First, endotoxins are released into the environment after lysis of the bacterial cells, leaving the endotoxin molecule in the water and no viable cells. Second, limitations of culture methods may underestimate bacterial growth, as water bacteria have slow rates of growth (an adaptation to low nutrient concentrations).

Endotoxins have been detected in the air, dust, and water; however, typical exposures occur through air. Endotoxin exposure is highest when the water containing the endotoxin is aerosolized and creates droplets that can be inhaled by workers (33). Gram-negative bacteria can be found almost everywhere in nature. Endotoxins have been linked to water systems and have been found in buildings with humidifiers and those with sewage or gray water incursions. The types of Gram-negative bacteria that contain endotoxins include but are not limited to *Escherichia coli*, *Salmonella*, *Pseudomonas*, and *Haemophilus* (34). However, in this study, no Gram-negative bacteria were recovered in the filling section except one case of *P. fluorescens* from an air sample due to the effective use of cleaning and sanitization procedures.

The composition of Gram-negative flora recovered from air samples was similar to that recovered from surface samples and characterized itself by the prevalence of non-enterobacterial strains and the presence of potentially pathogenic species. Presence of enterobacterial species *Citrobacter* prevailed, forming 55.5% of the overall isolates of operators’ glove prints, possessing strong endotoxic and/or allergenic properties. In our study, the lack of Gram-negative bacterial isolates in the filling section should be noted.

From the environmental point of view, more than three-quarters of Gram-negative bacterial isolates that cause microbiological contamination originated from air (48.43%) and surfaces (37.5%), and less common from humans (14%). These results contradict those of Utescher et al. (10) and Cobo and Concha (14), who considered that humans are the main source of contamination in classified areas due to constant endogenous emissions. This study clearly demonstrates that fractionation and purification areas housed a more dense and diverse resident microbial profile. These data reinforce the impression that the positive control measures used in these sections were not efficacious in reducing the environmental microbial burden.

In the present study, the sterility test revealed that a bacterial contamination with Gram-positive spore-forming bacilli was detected in thioglycollate media during plasma processing as presented in almost all stages, excluding the formulation and filling (after filtration) stages of the final product, with no detection of fungal contamination for all stages of antisera production. The determination of the contaminating flora in the manufacturing of the immunological products is of great importance for preventing severe contamination problems. Bacteria spores detected in contaminated thioglycollate media are generally considered as the most resistant form to sterilization processes and less sensitive to inactivation than fungi spores. The presence of endotoxin in the samples collected from all manufacturing stages clearly demonstrates that sterility testing is not a guarantee to the absence of endotoxins.
Strict in-process quality control measures were taken in the facility following these results to minimize the probability of occurrence of contamination. All personnel were re-subjected to advanced training. All areas and equipment were subjected to additional cleaning, and appropriate disinfection and air filters were checked. Plans for a new facility are currently being finalized; when constructed the facility will house all the production lines of biological products.

Conclusions

We were able to identify the sources of contamination by monitoring air, surfaces, and personnel by carefully monitoring the various steps of production of snake antivenom in a vaccine production facility. Bacterial counts were above the limits in many samples and Gram-negative bacteria were detected, which explains the sources of endotoxin contamination.

As a final recommendation, an environmental monitoring program should be performed using selective medium to detect endotoxin-containing bacteria. Snake venom antisera plant need to follow good manufacturing practices (GMPs) and hazard analysis and critical control points (HACCPs) to minimize the contamination for improving the microbiological safety of the product in a cost-effective manner. The sterility and endotoxin testing should be always regarded as a part of the microbiological control of the antiphidian sera production. These two tests are important assays for the institutions involved in producing antisera preparations and are always a target for continued study and improvement of antisera preparation efficacy.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest. All authors read and approved the final manuscript.

References


30. Sheraba, N. S.; Yassin, A. S.; Amin, M. A. High-throughput molecular identification of Staphylo-
coccus spp. isolated from a clean room facility in an environmental monitoring program. BMC Research Notes 2010, 3 (1), 278.


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