Effect of contaminated expired and unexpired penicillin G injection on the haematological indices of juvenile wistar rats

*Takon, I. A. And Antai, S. P.

Department of Microbiology, Faculty of Science, University of Calabar, P.M.B 1115 CALABAR, NIGERIA

ABSTRACT

The effects of contaminated expired and unexpired penicillin G injection on the haematological indices of juvenile Wistar rats have been investigated. The microbial load of penicillin G injection revealed contamination with microorganism such as Bacillus subtilis 3.0 x 10^2 cfu/ml, Staphylococcus aureus 1.2 x 10^3 cfu/ml and Escherichia coli 4.0 x 10^3 cfu/ml in the unexpired form. This result was significant at P > 0.05. The values of the packed cell volumes (PCV), platelet count (PC), white blood cell counts (WBC) after daily dosage intake for a period of 5 days showed significant difference at P > 0.05 when compared with the control. The danger posed by contaminated unexpired and expired penicillin G injection on antimicrobial therapy in healthcare delivery system is of significance.

Keywords: Haematological indices, Toxicity, Penicillin G, Wistar rats.

INTRODUCTION

The formation of an elegant, efficacious medicine which is both stable and acceptable to the patient may necessitate the use of a wide variety of ingredients in a complex physical state [1]. The aspects of which have created conditions conducive to the survival and even extensive replication of contaminant microorganisms, that might enter the product during its manufacture or use by patients or medical staff and spoil the drug [2].

Pharmaceutical products are substances used in the prevention, treatment and diagnosis of disease [3]. These products are meant to be safe and potent during manufacture, storage and use. Microorganisms have posed serious threat on these pharmaceutical products [4, 5]. The metabolic versatility of microorganism is such that almost any formulation ingredient, from simple sugars to complex aromatic molecules, may undergo chemical modification by a suitable organism, thus leading to spoilage of the product [6]. This spoilage may lead deterioration of these products, which in turn may lead loss in potency of the drug or initiate infection in the user [7, 8]. These microorganisms ranged from true pathogens to motley collection of opportunistic pathogens such as Pseudomonas aeruginosa, Staphylococcus aureus, Serratia sp, Klebsiella sp etc.

According to Waterman [9], the physical and chemical status of a pharmaceutical formulation influences considerably the type and extent of microbial spoilage it is at risk. Toxic metabolites may persist even after removal of any microorganisms originally present or detectable physical and chemical changes have occurred in the product [10].
According to Andrew and Russell [11], the outcome of using a contaminated antimicrobial product may vary from patient to patient, depending on the type and degree of contamination and how the product is to be used. These effects include the use of contaminated injection products and eye drops, where generalized bacteraemia shock and in some cases death of patients have been reported [12, 21]. Medicament–borne infections could spread for sometimes before it is properly diagnosed. Treatment of patients with contaminated materials is bad in principle. The question is “how much harm is actually done?” This depends largely on the route of administration [13, 14]. The aim of this study was to determine the correlation between haematological parameters changes in Wistar rats and contaminated penicillin drug treatments.

Aneja [15] and Baby [16] observed that sterile and self-sterilizing products, which are most desirable, would sometimes have low levels of contamination without multiplication or with minimal multiplication during the intended shelf-life of the product. Deterioration had resulted in the loss of potency or initiation of an infection in the user [11, 20].

**MATERIALS AND METHODS**

**Materials:** The following materials were used:

Ten (10) drug samples (Penicillin G injection) were collected from both pharmacies and patent medicine stores in their expired and unexpired form in Calabar – Nigeria for analysis. Different media and reagents were used in the course of this work. These included general purpose and selective media, such as nutrient agar, MacConkey agar, cetrimide agar, Sabouraud dextrose agar, trypticase soy agar, Mueller-Hinton agar, DCA-Hynes medium and brain-heart infusion agar and peptone water mainly of Oxoid product (Oxoid Basingstoke, Uk) to support culturing growth, isolation and characterization of a wide spectrum of heterotrophic organisms encountered in this study. They were prepared according to the manufacturers specifications.

**Methods**

Different aseptic bacteriological techniques were employed in processing of the drug samples. Only representative portions (1ml) of the drug content were used for analysis. The powdered sample was dissolved in water for injection. Ethical condition was strictly observed.

**Determination of Powder Properties**

The particle size distribution and shape of the powdered dosage form were determined by optical microscopy on approximately 300 particles per sample. The flow properties of the powders were evaluated by measuring the angle of repose. Exactly 5 g of each powder was poured into a cylindrical glass fixed to a flat base of diameter 28 mm. The cylinder was slowly pulled out vertically so as to form a cone of powder on the base. The height of the cone was measured and the angle of repose, θ, was calculated using the equation:

\[
\tan \theta = \frac{h}{r}
\]

Where:
- h = height of the conical powder heap
- r = radius of the circular base

Determinations were done in triplicates.

**Determination of solution properties**

The density of the solutions and suspensions were determined by measuring the weight of 1 ml of the sample. The density was taken as the mass per unit volume of the preparation.

**Microbial content/load determination**

**Solid samples:** Exactly 1 g of the powdered sample was weighed and suspended in 90 ml of sterile distilled water and 10-fold serial dilution was carried out. Viability was assessed using 0.02 ml volume of the final dilution for spread-plate method for bacteria count and the plates were incubated at 37°C for 24 h. Sabouraud dextrose agar was poured into plates and allowed to set and 1 ml aliquot of each sample spread on the surface and used for fungal count. Plates were incubated at 28°C for 72 h. Each sample was inoculated in duplicates. The plate was placed on a
colony counter and the number of colony forming units determined. The microbial content was taken as the mean of each sample calculated and expressed as mean colony forming units per milliliter (cfu/ml).

Isolation and identification of microbial contaminants
A loopful of the sample was streaked on centrimide agar, manitol salt agar, MacConkey agar, kligler iron agar, and Sabouraud dextrose agar. The viable aerobic bacterial count and viable count for molds (dry surface method) isolated were assessed and identified using well established methods by their morphological and biochemical characteristics.

Toxicity Test
Toxicity test according to [10] was carried out on drug sample (Penicillin G injection).

a) Animals: Six (6) adult wistar rats, weighing between 100 g and 250 g were bred locally in Microbiology Department of University of Calabar. The animals were kept two (2) per cage and divided into three (3) groups and fed with standard rat chow (Royal Livestock Feeds Plc, Nigeria) and 75cl tap water supplied to each cage daily, with 12 hour light – dark cycle exposure. Environmental temperature ranged from 20°C (night) to 34°C (daytime). The rats were handled according to standard protocols for the use of animals for toxicological experiments. The investigation was conducted in accordance with internationally accepted principles for laboratory animals use and care as found in EEC Directive of 1986; 86/609/EEC.

b) Preparation and Administration of Penicillin G Injection
Crystalline penicillin G injection were commercially purchased from Unical Pharmacy and Bez Pharmacy, which served as positive controls and also Essential Drug store of Cross River State in Calabar, Nigeria and water for injection served as negative control. The active ingredient was benzyl penicillin sodium BP 1,000,000 IU (600 mg). The penicillin powder was distributed in 2 ml water for injection (manufacture’s prescription) and administered at 360mg per kilogram rat body weight two times daily or a day, intramuscularly using a sterile and disposable syringe for 5 days. The dose effect was monitored at divided doses of 6 hours interval.

c) Assessment of Toxicities
Animal body weight and locomotor activity were monitored on days 1, 3 and 5 of daily administration. On each day, the animals were examined for general pharmacological and toxicological signs of lethargy, morbidity, mortality, food consumption rate, haematological and blood chemistry measurements. Locomotion was recorded for 30 minutes on each day by the use of sensitive electronic locomotor meter (40 fc, Motron products, Sweden), using the method described by [19]. After 2 days from the last day of administration, the animals were anaesthetized with Bouin’s fluid soaked cotton wool, in a dessicator container. It was sacrificed and dissected longitudinally. Five millimeters blood samples were collected from the carotid artery cannulations into blood sample bottles containing 0.5 ml of 3.8 % sodium citrate and gently mixed. Samples were cooled and assayed soon after collection. These samples were used for haematological analysis.

d) Assessment of Haematological Parameters of Test Animals
Blood samples were collected after the last drug treatment. Exactly 10 ml of blood was drawn using disposable syringe with needle gauge 22. Exactly 3 ml of blood sample collected aseptically from each animal was put into tri-potassium ethylenediamine tetra-acetic acid (K₃EDTA) anticoagulant bottle, for haematological analysis using an Automated System Behringer Mannhein Sysmex, Model NT800 at the haematological laboratory of the University of Calabar Teaching Hospital, Calabar. Ethical approval was obtained from Ethical Review Board of University of Calabar. Certain haematological parameters of test animals were assayed to detect possible drop in number and potency of these parameters due to adverse effect of drug and drug contaminants. These parameters were WBC, RBC, HGB, HCT, MCV, MCHC, MCH, RDW, PCV, RDV and PLT. Platelet counts (PLT) and white blood counts (WBC) were determined microscopically using the method of Dacie and Lewis (1991). Packed cell volume (PCV) was measured using microhaematocrit tubes with blood samples, centrifuging at 150g for 5 minutes and reading with a haematocrit reader. The stomach and intestine were washed with 10 % formaldehyde saline and examined for lesions using a magnifying glass attached to a dissecting fluorescent lamp (Thousand and one lamps, England). The controls were blood samples from healthy Wistar rats not treated with drugs. Results obtained were compared with known standard reference.
Antimicrobial studies:

Agar dilution method was used to determine the antimicrobial activity. The inoculum size for each organism (Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli and candida albicans) were prepared from broth cultures containing approximately $5 \times 10^{6}$ to $9 \times 10^{7}$ colony forming units per milliliter (cfu/ml). The diluted (1:20) inoculum was applied as a streak with a loop of 0.002 ml, containing $9 \times 10^{7}$ cfu. The plates were incubated for 24 h at 37°C. The penicillin powder was prepared at a concentration of 2 mg/ml, by dissolving in sterile distilled water, which also served as control. 1iu concentration of penicillin was used. Observations were performed in duplicates.

Statistical Analysis

The results were analyzed using a one-way analysis of variance (ANOVA) for the significant difference in the effects of expired, unexpired and control samples of penicillin G on haematological indices of Wistar rats. Data were presented as mean ± standard error of the mean (SEM) and for the animal weight as standard deviation (SD), where n represents number of rats used per group. Data were compared using student t-test (graph pad prism software, UK). P < 0.05 was regarded as statistically significant.

RESULTS

The result of the microbial content of Pencillin G is presented in Table 1. Pseudomonas aeruginosa had the highest viable count among expired Penicillin (6.0 x $10^{3}$ cfu/ml) and unexpired (4.0 x $10^{3}$ cfu/ml). The least count of 1.0 x $10^{3}$ cfu/ml was observed for Salmonella sp and Aspergillus niger respectively in the expired penicillin. Salmonella sp and Aspergillus niger were not present in unexpired Penicillin.

The effect of penicillin treatment on blood parameters of three groups of Wistar rats is presented in Figure 1. The results shows significant difference (P < 0.05) between means of red blood cells (RBC) of rats treated with contaminated expired penicillin and that of the control. There was no significant difference (P > 0.05) in the means of the blood platelets (PLT) and white blood cells (WBC) among the three treatment groups. Figure 2 shows significant difference (P < 0.05) in means of haemoglobin (HGB) and haematocrit (HCT). The mean corpuscular volume (MCH), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were highly significant (P < 0.05) across the three penicillin treatments when compared with control (Figure 3). The toxicity test result of the three penicillin treatment groups is presented in Table 2. The toxic effect of expired and unexpired penicillin on the three groups showed no significant difference (P > 0.05).

Table 1: Microbial content of the three treatment groups of penicillin

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Expired penicillin</th>
<th>Unexpired penicillin</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis</td>
<td>5.0 x $10^{3}$</td>
<td>3.0 x $10^{3}$</td>
<td>-</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>5.0 x $10^{3}$</td>
<td>2.2 x $10^{3}$</td>
<td>-</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>4.0 x $10^{3}$</td>
<td>3.0 x $10^{3}$</td>
<td>-</td>
</tr>
<tr>
<td>Salmonella sp</td>
<td>1.0 x $10^{3}$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>6.0 x $10^{3}$</td>
<td>4.0 x $10^{3}$</td>
<td>-</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>2.0 x $10^{3}$</td>
<td>1.0 x $10^{3}$</td>
<td>-</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>1.0 x $10^{3}$</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Cfu/ml = colony forming unit per millilitre, - = no growth.
Figure 1: Differences in blood cells levels across the 3 Penicillin treatment groups of wistar rats and control.

Figure 2: Differences in HGB and HCT levels across the 3 Penicillin treatments groups of wistar rats and control.
DISCUSSION

Drugs are supposed to maintain their original status during storage and use. In this study it was discovered that it was not so, as certain microorganisms contaminated this drug (Penicillin G injection) and consequently spoilt the drug. Result obtained in this work is in agreement with the assertion by Ozolua [17] that the ability to exist on almost any material, characterizes microorganisms as primary agent of deterioration. Bos [18] reported that most of these organisms utilize the active ingredient as carbon and substrate. The following organisms were implicated (Table 1): *Pseudomonas aeruginosa* (6.0x10^cfu/ml), *Bacillus subtilis* (5.0x10^cfu/ml), *Staphylococcus aureus* (2.0x10^cfu/ml) and *Candida albicans* (2.0x10^cfu/ml) their presence in this drug is objectionable, since this drug is meant to be sterile even on expiration. These organisms have been reported by [8] as drug contaminants. Drug contamination could be prevented by proper selection of suitable preservatives for the formulations and proper storage facility. These organisms are indicator of poor hygiene practice, pathogenic potential for route of administration and survival profile of these microorganisms and recoverability in the products.

Statistical database of the parameters have shown that contaminated penicillin product brought about a change in the haemoglobin (HGB) counts of these organisms. The erythrocyte indices showed no significant differences (P > 0.05) between the three groups upon drug administration. This indicates that the haematological parameters of the animals were similar with slight variations in the packed cell volume (PCV), the mean corpuscular haemoglobin concentration (MCHC) (P > 0.05) and red cell distribution width (RDW) (P >0.05), when compared with standard range of control. In contrast, group C had lower haemoglobin concentration, with hypochromic and rouleaux band formed. There was no toxic effect of contaminated penicillin across three treatment groups. This result is in agreement with that obtained by [10], who stated that most contaminated drugs had little or no toxic effect on usage.
The packed cell volume (PCV) values were significantly different at P < 0.05, when compared with the control. This may be as a result of breakdown of viable red blood cells by drug contaminating agents which might lead to anaemic conditions. This result is of great concern because of the risk this effect might have on antibiotic therapy and vulnerable patients, such as children, the elderly, maternal and compromised hosts. This result agrees with that obtained by [19], where it was observed that, contaminated drug products were implicated in most anaemic conditions on children.

The microbial nature and content of penicillin as presented in Table 1, showed an increase in the microbial load above stipulated level. Penicillin samples were contaminated to varying degrees with bacteria and fungi. Of concern were the Gram negative bacteria (Escherichia coli, Pseudomonas aeruginosa) which are considered pathogenic. Pseudomonas aeruginosa, a soil bacterium and a pathogen was found in both expired and unexpired drug form. Similarly, Escherichia coli, an intestinal bacterium and an indicator organism for faecal contamination was also present in the expired and unexpired penicillin. Salmonella sp and Staphylococcus aureus were also present in both expired and unexpired penicillin samples. Also Bacillus subtilis, an aerobic organism were also isolated from these drug samples. Candida albicans and Aspergillus niger were also detected in these products, which is not expected in a sterile product. The microbiological quality of these drug samples may be influenced by improper drying, storage and handling conditions by personnel. The presence of microbial contaminant in sterile pharmaceutical products can reduce or even inactivate the therapeutic activity or potentials of the products and has the potential to adversely affect patients taking the medicine [8].

Some infections outbreak has been traced to the use of heavily contaminated raw materials [2]. The microbial quality of pharmaceutical products is influenced by the environment and quality of raw materials used during formulation. Thus, manufactures should ensure the lowest possible level of microorganism in the raw material, finished dosage forms and packaging components to maintain appropriate quality, safety and efficacy of the products.

Both expired and unexpired penicillin did not show acceptable pharmaceutical and microbial qualities. There is need for constant monitoring and stringent quality control of sterile pharmaceutical products manufactured, sold and used by the general public or consumer.

**SUMMARY AND CONCLUSION**

Recent increase in the number of medicament – borne disease had necessitated this research. Medicines are an essential part of human life and the safety of medicine is of utmost importance in providing pharmaceutical health needs of patients. Ethical, economic and competitive reasons, as well as those of safety and efficacy support the need to monitor contamination of drug products. Some of these isolates were indicator organisms and pathogens, which is of great health concern. The problem of drug contamination had been unfavorable to both the government and the patients, who may have to pay first for drugs and later for the consequences. Due to the health risk posed by injection of contaminated drug, knowledge of the manufacture, appropriate storage environmental conditions, packaging and use will be helpful in the development of best formulations.

Despite extensive development and rigorous laboratory analysis, it is not possible to totally ascertain that a drug product will be sufficiently robust to withstand all the rigors of real – life usage. The task of implementing this approach inevitably rest with appropriate government legislations and public enlightenment on proper products. This task, though daunting, is achievable.

**REFERENCES**


