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## of Medical Research: C

# Microbiology and Pathology



Prevalence, Isolation of Bacteria

Hemorrhagic Syndrome in Erysipelas

Highlights

Preliminary Survey of Norovirus

Bacteriological Profile and Antibiotic

Discovering Thoughts, Inventing Future

VOLUME 16 ISSUE 1 VERSION 1.0

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Microbiology and Pathology

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# Global Journal of Medical Research: C
# Microbiology and Pathology

Volume 16 Issue 1 (Ver. 1.0)

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# Disfibrinogenemiya and Hidden Hemolysis - Indicators of Hemorrhagic Syndrome in Erysipelas

By Elena. G. Fokina

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*Abstract-* *Purpose of the work:* The work was aimed at the study of the changes of the system of hemostasis and of blood rheology during the progression of infection in patients with lower-limb and facial erysipelas and to validate the advisability of replacement and/or antithrombotic therapy.

*Material and methods:* the author studied the indices of external (time of prothrombin (PT), international normalized ratio(INR)) and internal (activated partial thromboplastin time (aPTT)) coagulation pathways, the degree of disfibrino-genemiya (thrombin time (TT), functional fibrinogen activity and D-dimer level), the amount and functional activity of the platelets (aggregation with adenosine diphosphate (ADP) and the erythrocytes (aggregation with lanthanoid (LaCl3) and protamine sulfate( PS)) in 60 cases of erysipelas. The studied indices also included endothelial dysfunction – the decrease of athrombogenicity of vascular wall endothelium (antithrombin III (AT III) and protein C) and the increase of endothelial adhesive properties (von Willebrand factor(vWf)).

*Keywords:* *facial erysipelas, lower-limb erysipelas, hidden hemolysis, DIC-like syndrome, erythrocyte aggregation, platelet aggregation, fibrinogen, D-dimer, protein C, antithrombin - III, von Willebrand factor, haptoglobin, β – hemolytic streptococcus (β-HS).*

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# Disfibrinogenemiya and Hidden Hemolysis - Indicators of Hemorrhagic Syndrome in Erysipelas

Elena. G. Fokina

*Abstract- Purpose of the work:* The work was aimed at the study of the changes of the system of hemostasis and of blood rheology during the progression of infection in patients with lower-limb and facial erysipelas and to validate the advisability of replacement and/or antithrombotic therapy.

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The comparison groups included normal volunteers (n=32) and patients with inflammation localized on the face (n=24), and the legs (n=36) at various stages of the disease (day 1–3; 4-6; 7-10; and 11-15 from the onset of the disease). These patients underwent in-hospital treatment in 2nd Moscow Clinical Hospital for the Infectious Diseases.

*Results and discussion:* The thesis, according to which the rate of hemorrhagic complications in lower limb erysipelas (LLE) is by 3,9 times higher than in facial erysipelas (FE), was confirmed by laboratory findings. In particular, a significant decrease of protein C was noted in patients with LLE and concomitant chronic venous insufficiency (CVI). We have found increased D-dimer and decreased α2-macroglobulin ($\alpha_2$ MG) levels, suggesting potent activation of proteolytic enzymes (plasmin, matrix metalloproteinases and neutrophil elastase), which can be one of the causes of bullae, erosion and ulceration formation in the erysipelas focus on the lower limb.

The signs of intravascular (hidden) hemolysis - the decrease of haptoglobin concentration and the increase of indirect bilirubin and lactic dehydrogenase (LDH) blood levels; the changes of rheological properties of erythrocytes – the increase of deformability (aggregation with lanthanum chloride ($LaCl_3$)) and the decrease of elasticity (aggregation with protamine sulfate (PS)) – have been identified as one of the main factors for disseminated intravascular coagulation (DIC)-like syndrome in erysipelas.

*Keywords:* *facial erysipelas, lower-limb erysipelas, hidden hemolysis, DIC-like syndrome, erythrocyte aggregation, platelet aggregation, fibrinogen, D-dimer, protein C, antithrombin - III, von Willebrand factor, haptoglobin, β – hemolytic streptococcus (β-HS).*

## I. Introduction

To date, clinical research focuses on the study of the relation between the inflammation and the coagulation. The dysfunction of vascular endothelium, common for these two pathological processes, is an early pathophysiological sign and an independent predictor of unfavorable prognosis of most diseases. The prevention of endothelial dysfunction of the microcirculatory bed helps to prevent and to treat many diseases.

Erysipelas is a widely spread acute infectious disease. Its development does not depend on industrial development and social security in different countries. General and local predisposing factors form an important pathogenic aspect of this condition's development. Lower limb erysipelas (LLE) is often associated with obesity, type 2 diabetes mellitus, chronic venous insufficiency (CVI) and feet and nail mycoses [1, 2]. Facial erysipelas (FE) often develops in association with otomycosis and chronic ear, nose and throat (ENT) diseases [3].

Despite modern methods of treatment, up to 10% cases of erysipelas are complicated by skin necrosis at the sites of hemorrhages and vesicles formation, by venous incompetence (periphlebitis, phlebitis and thrombophlebitis). The frequency of hemorrhagic form of erysipelas increased [4, 5]. Hence, the study of the system of hemostasis and blood rheology during the developing infectious process in patients with lower limb and facial erysipelas is of thrilling importance.

### a) Purpose of the work

The purpose of this work was to study the changes of hemostasis and of blood rheology during infective process in patients with lower limb and facial erysipelas and to validate the advisability of replacement and/or antithrombotic therapy.

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## II. Material and Methods

*a) Patient profiles*

We have studied 60 patients aged 25 to 71 years. Thirty-six of them had 2nd degree lower limb erysipelas and 24 had 2nd degree facial erysipelas (24). 67% of patients had primary form of the disease. Erythematous form of erysipelas was found in 33% of cases (52% in facial erysipelas), erythematous-bullous in 15%, erythematous -hemorrhagic in 22%, and bullous-hemorrhagic in 30% of cases. Erythematous-hemorrhagic (n=11) and bullous-hemorrhagic (n=15) erysipelas affected the lower limb more frequently than the face (2 and 3 cases, respectively). The risk of hemorrhagic lesions was significantly higher in cases with local inflammatory process (78%) affecting the legs, than in face affection (20%); the odds ratio (OR) = 9,9 (2,8; 34,7).

Primary facial erysipelas diagnosed in 92% of cases was more common in women (16 women, 8 men). LLE was primary in 50% of cases, repeated in 31% and recurrent in 19% of cases. Facial erysipelas was primary in 92%, repeated in 4% and recurrent in 4% of cases. The risk of LLE recurrence was significantly higher than of the facial erysipelas (OR =5,55 (1; 51,2), p=0.009).

Gender ratio in LLE was almost equal (males-17, females - 19). Feet and nail mycosis onychomycosis were the most frequent (88%) concomitant diseases. Eleven patients had grade 2 to 4 obesity, 5 patients had subcompensated type 2 diabetes mellitus.

Skin diseases (retroaural dermatitis, streptoderma, psoriasis) were the underlying pathology in 37,5% of patients with facial erysipelas. 29% of these patients had chronic ENT diseases (otitis, tonsillitis and rhinitis). Four patients had type 2 diabetes mellitus.

The patients underwent in-hospital treatment in Erysipelas department of the 2nd Moscow Clinical Hospital for Infectious Diseases. Thirty-two patients received antibacterial monotherapy: intramuscularly (IM) Benzylpenicillin novocaine salt twice daily (1,2 mln ME per day) for 7-10 days. Another two patients received IM Cephazolin three times daily (3 g per day) for 5 days. Combined two-antibiotics therapy (IM Benzylpenicillin novocaine salt twice daily (1,2 mln ME per day) for 7-10 days and Ciprofloxacin twice daily per os (1 g per day) for 10 days) was used in 14 patients. Twelve patients got a three-antibiotics combination (IM Benzylpenicillin novocaine salt (1,2 mln ME per day) for 7 days + intravenously (IV) Ciprofloxacin (800 mg per day) for 3 days with subsequent passage to per os (1 g per day) for 10 days + IM Cephazolin three times daily (3 g per day) for 5 days). Additionally the patients received: antihistamine agents (Cetirizine dihydrochloride); topical physiotherapy (ultraviolet irradiation (UVI) therapy and low frequency current (LFC) for facial erysipelas; UVI therapy, LFC and magnetic therapy for lower limb erysipelas). The focus of LLE erysipelas was regularly treated with tanning solution of potassium permanganate. The studied patients did not receive medications capable to influence their hemostasis' state.

Mean duration of in-hospital stay was 11,9 + 4,1 days for the patients with LLE and 8,4 + 1,6 days for the patients with FE.

The hemostasis indices were studied in the beginning of the disease (days 1-3) - study point 1, during the course of the disease (days 4-6; 7-10) – points 2 and 3, and during the recovery period (days 11-15) – study point 4. Every third patient with LLE underwent the follow-up examination (5 months after the discharge). It helped to differentiate between erysipelas-induced changes and the underlying concomitant diseases.

The control group comprised 32 normal subjects aged 24 to 50 years, with equal gender distribution.

*b) Statistical analysis*

The results were processed on Statistica 10.0 for Windows 7.0 software. The statistically significant (reliable) differences between the groups was studied using: Mann-Whitney test for quantitative values, two independent groups; Kruskall-Wallis test for more than two independent groups; Wilcoxon test for quantitative values, related groups (pre-and post-therapy, in dynamics); chi-square test, and, if necessary, two-tailed Fisher's exact test for qualitative values. The differences evaluated with the use of parametric and non-parametric methods were considered statistically significant with confidence level >95% ($p<0.05$). In case of statistically significant inter-group differences, the odds ratio (OR) and 95 confidence interval (CI) were calculated OR (-95% CI; + 95 CI). The values in the Table are presented as Me (median) and SD – standard deviation.

## III. Methodology

Laboratory investigations of biochemical indices, the parameters of hemostasis, as well as the determination of blood serum protein fractions using electrophoresis were conducted together with the specialists from the express-laboratories of the 2nd Clinical Hospital for Infectious Diseases; the determination of protein C was conducted together with the specialists from the express-laboratories of Filatov' Pediatric City Clinical Hospital. The aggregation properties of the platelets, the elasticity and the deformability of the erythrocytes were studied together with senior researchers of the laboratories of hemostasis of the clinical department of Central Research Institute of Epidemiology of Federal Service on Customers' Rights Protection and Human Well-being Surveillance (Moscow). Aggregation capacity of the platelets (using Born' method) and the erythrocytes (using Sheremetiev'



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original technique [6]) was determined by photometric aggregometers SOLAR AP-2110 (Belarus) and BIOLA LA - 230 (RF). Platelet aggregation was induced using 2x10-5 M ADP (Reanal); erythrocyte aggregation – using 1% protamine sulfate (RF) solution and LaCl3 (RF) solution (at a dilution of 150 mg of reagent in 100 ml water.

The indices of the external (prothrombin time (PT), International Normalized Ratio (INR), Quick prothrombin index and the internal (activated partial thromboplastin time (aPTT)) coagulation pathways were turbodimetric determined on an automatic coagulometer ACL ELITE PRO (USA). The degree of disfibrinogenemiya was evaluated by the thrombin time (TT), functional fibrinogen activity (using Clauss method) and D-dimer dimension.

The endothelial dysfunction was determined by the degree of athrombogenicity of the vascular wall endothelium (antithrombin III (AT-III) and protein C levels) as well as by the adhesive properties of the endothelium (using von Willebrand factor). Blood plasma levels of AT-III was determined on ACL ELITE PRO coagulometer; protein C– on SYSMEX CA-500 coagulometer (Siemens Healthcare, USA). Von Willebrand factor (vWf) was determined by manual method (Renam reagents, RF).

Blood serum protein fractions were studied on agarose gel in HYDRASYS analyzer (Sebia, France).

The inflammatory level was estimated on the base of C-reactive protein (CRP) on HITACHI - 902 analyzer (Roche, Japan). The same analyzer was used to determine the content of haptoglobin and lactic dehydrogenase (LDH) in blood serum. According to the design of the study, we determined the substrates and the enzymes of human biochemical passport [1, 7, 8], performed clinical blood and urine analyses.

## IV. Results and Discussion

During acute infection, the disturbances of hemostasis can evolve to hidden or evident signs of disseminated intravascular coagulation with threatening venous thromboses. The mechanisms of venous thromboses can be triggered by tissue lesion (in this case – infectious inflammation caused by β-hemolytic streptococcus (β-HS), the edema of the affected limb, the presence of lymphostasis and chronic venous insufficiency, the excess and/or the hyperactivity of the plasma factors. All the above factors accentuate the already existing dysfunction of the vascular wall endothelium. For this reason, a special attention is given to the study of athrombogenic and adhesive properties of the vascular wall and to the control of native anticoagulants' level (protein C and antithrombin-III) [1, 9, 10, 11].

*a) Athrombogenic and adhesive properties of the vascular wall*

Baseline level of protein C during the first 3 days of the disease (at admission) in patients with lower limb erysipelas (81,9±4,9%) was significantly lower than in patients with facial erysipelas (94,1±6,0%), and reliably below the control values (100±5%, $p<0,05$) as shown in Table 1.

With the decrement of the erysipelas focus, the level of protein C recovered gradually in both groups.

Protein C in patients with LLE without CVI (n=28) was 99,8±4,7% during the acute stage of the disease, and increased to 140±4,5% during the recovery stage ($p<0,001$), (Figure 1). The low baseline level of protein C in lower-limb erysipelas with CVI (69,8±8,1%) did not significantly change during the recovery (79,15±4,0%, $p=0.21$) and remained refractory low during the treatment (Figure 1).

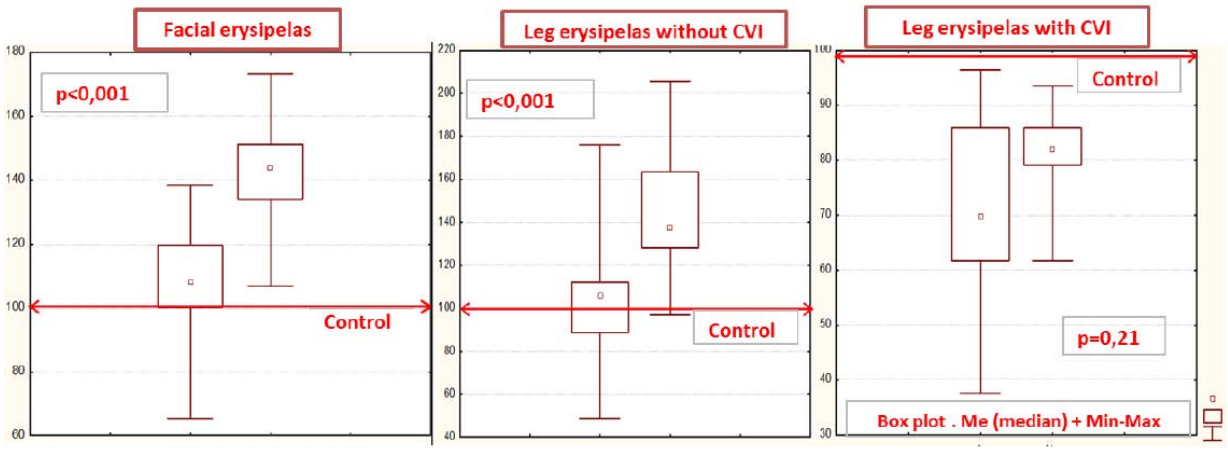



*Figure 1 :* Protein C in patients with facial erysipelas (1), lower-limb erysipelas without (2) and with CVI (3)
*Note:* Left columns - at admission (1st week of the disease), right columns –2nd-3rd weeks of the disease, recovery period (Wilcoxon test).

In facial erysipelas, the level of protein C returned to the norm during the third week of the disease (108,2±5,1% at admission and 144±4,6% at discharge; p1-4 <0,001). In LLE without CVI it recovered during the fourth week (99,8±4,7% at admission and 140±4,4% at discharge; p1-4<0,001). In LLE with CVI,



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the level of protein C did not change: 69,8±8,1% at admission, 79,15±4,07% at discharge; p1-4=0.21), (Figure 1).

In the presence of endothelial dysfunction, the level of protein C in certain patients significantly increased during recovery - from study point 1 to study point 4 (from 49,7 to 112%, increase by 125%; from 48,9 to 110,7% , increase by 126%; from 65,5 to 119,7% increase by 83%). This is a good example of the adaptation mechanism (Figure 2).

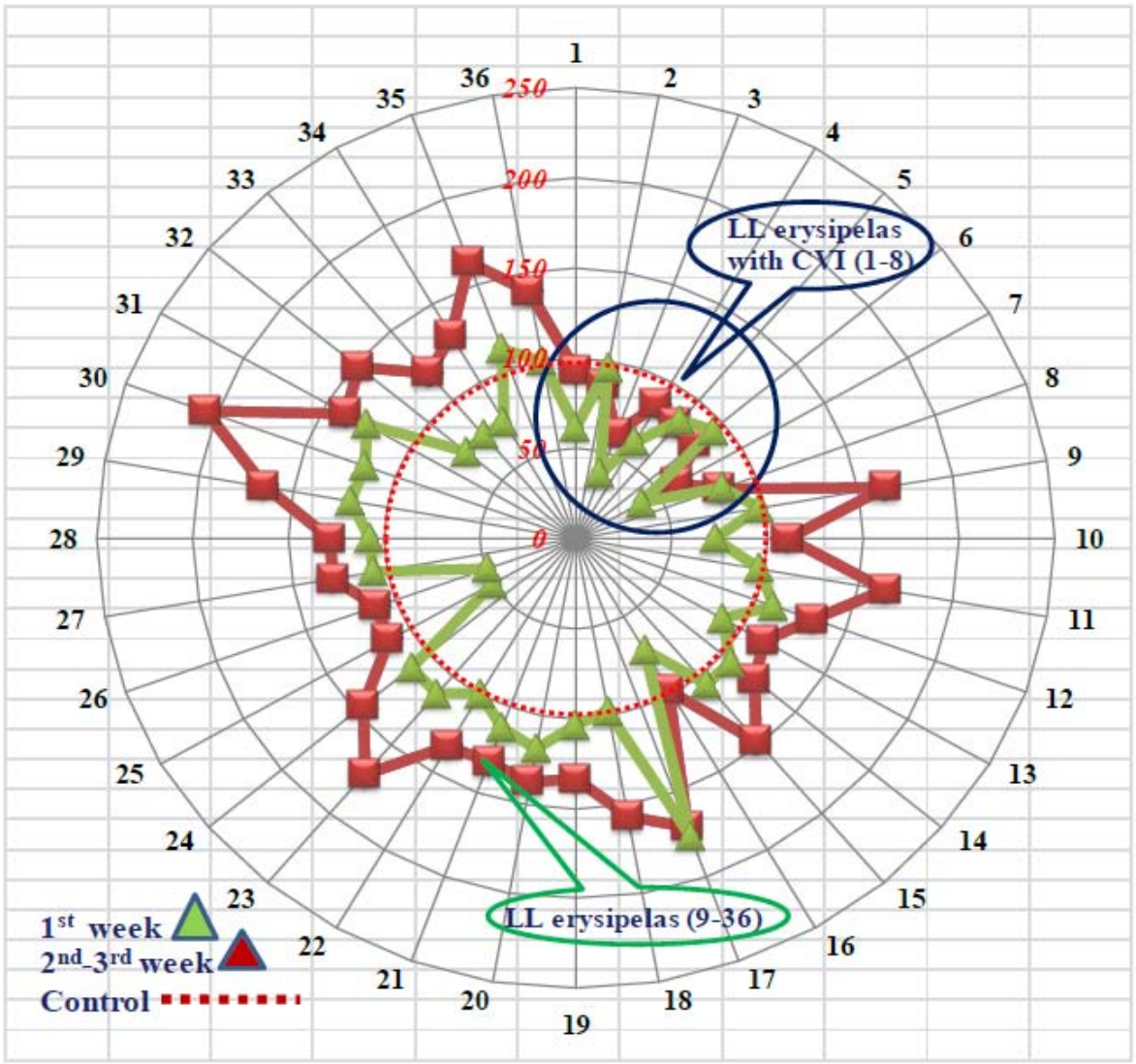



*Figure 2 :* Changes of protein C levels in patients with lower limb erysipelas

*Note:* 1-8 with CVI, 9-36 without CVI, LL - lower limb

The recovery in LLE without CVI (and with positive dynamics of protein C level) was more favorable than in LLE with concomitant CVI. According to some authors [1, 4, 9, 10, 12], CVI leads to the prolongation of the healing time of erysipelas focus and of the recovery. We have shown that in the presence of normal protein C (100±5%), the chances for the favorable course of LLE are significantly higher (OR=2,89 (0,15; 55)), than in erysipelas with low protein C and concomitant CVI.

We determined also the level of another, not less important natural anticoagulant – the antithrombin - III. According to the literature [13, 14], the thrombosis (strokes, infarctions) develop when AT-III level is 80-90%. AT-III deficit occurs in the presence of clinical signs of disseminated intravascular coagulation and the multiorgan failure syndrome.

AT-III deficit was more pronounced in LLE, than in facial erysipelas (Table №1). The baseline values of AT-III in patients with LLE (87,6±2,5%) were significantly lower than in facial erysipelas (91,8±2,5%), and lower than the control values (p<0,05). AT-III level did not recover up to the discharge (Table №1). Bigger AT-III deficit seen in patients with LLE can explain higher incidence of hemorrhagic forms of erysipelas in this group of patients in comparison with facial erysipelas (78% in LLE and 22% in FE). Besides, AT-III (as well as



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fibrinogen, CRP, α1-acid glycoprotein) is an acute-phase protein. Our previous studies had shown that the level of acute-phase proteins in patients with lower limb erysipelas was higher than in patients with facial erysipelas [8].

The concentration of α2-macroglobulin decreased by 25%. The minimal level of α2–MG (an inhibitor of various proteases, including plasmin) was seen at the end of the 1st week of the disease: 3,78±0,16% in LLE and 3,96±0,16% in FE.

β-hemolytic streptococcus produces pathogenicity factors as streptokinase, hyaluronidase, streptodornase, etc. These factors destroy the protective level of heparansulfate, lining the vascular endothelium. This is accompanied by an increase of prothrombogenic properties of the vascular wall, the release of von Willebrand factor (vWf) and the decrease of AT-III activity. We noted an increase of vWf (187% to 220%) during 1st week of the disease in all patients with erysipelas. With the extinction of the inflammatory focus, high values of vWf tended to decrease, however they never reached normal values (Table 1).

Thus, the results of the study of endothelial markers in erysipelas patients are suggestive of a compromised endothelium-related hemostasis regulation. It concerned not only antithrombotic (decreased levels of protein C and AT- III), but also adhesive characteristics (high level of vWf). The deficit of natural anticoagulants was more pronounced in LLE cases. The refractivity of protein C in patients with LLE and CVI is a diagnostic marker of CVI and indication for vascular replacement therapy [13, 14].

It is known, that the body uses natural anticoagulants for the isolation (delimitation) of infectious inflammation area. The decrease of anticoagulants' concentration «opens the gate» for the generalization of infectious inflammation [11, 15, 16, 17].

*b) Changes in plasma hemostasis and disfibrinogenemiya*

We have found the following shifts in the indices of the external (prothrombin time, prothrombin index, INR) and the internal (aPTT) coagulation pathways, in the degree of disfibrinogenemiya (thrombin time, functional platelets' activity and D-dimer level) in patients with erysipelas (Table 2):

- The activation of coagulation cascade during the acute stage of the disease (decreased TT in facial and lower-limb erysipelas during the days 1-3 of the disease in comparison with the control (p<0,05). It means the active processes of thrombin and fibrin formation in the blood flow;
- The activation of the external coagulation pathway (increased PT time in lower- limb erysipelas in comparison with the control (p=0,033), decreased prothrombin index and increased INR);
- The activation of the internal coagulation pathway (the increase of aPTT at admission and during recovery in LLE. In facial erysipelas, the baseline aPTT was below the control (p < 0,05) and increased by the end of the 1st week of the disease (p < 0,025);
- The disfibrinogenemiya (decreased TT with the activation of fibrin polymerization process and the appearance of a great amount of D-dimers in the patient's blood (Table № 3). The fibrinogen level in LLE was higher than in FE: 8,0 ±2,3 g/l vs 5,9±1,8 g/l (p=0,008) in 1- 6th days and 6,6 ±2,4 g/l vs. 4,3 ±1,0 g/l (p<0,0001) in 7-15th days. Fibrinogen is acute-phase protein, as well as: AT-III, CRP, presepsin, procalcitonin, α - tumor necrosis factor, interleukin - 6 [16, 14].

Hence, the disfibrinogenemiya and the activation of coagulation cascade were higher in lower limb erysipelas (see above). The peak changes occurred at 4-6th days (study point 2), (Table **3**).

The initial fibrinogen level in LLE (7,7±0,38 g/l) was by 18% higher than in FE (6,53± 0,49 g/l). D-dimer level in lower-limb erysipelas (399±46 ng/ml) was more than twofold higher in comparison with FE patients (160,2±41 mg/ml) and by 27 times (!) higher than in control group (14,5+3,18 ng/ml, p<0,001).

The documented differences in D-dimer level allowed us to conclude that the processes of intravascular coagulation in lower limb erysipelas are more intense than in facial erysipelas. The presence of the clots of polymerized fibrin is a necessary condition for the increase of D-dimer level, as the process of plasminogen transformation to plasmin takes place inside these clots [5, 11, 13].

The increase of D-dimers and the decrease (expenditure) of α2-macroglobulin also are suggestive of a potent local activation of proteolysis enzymes (plasmin, matrix metalloproteinases and neutrophil elastase). These enzymes destroy the extracellular matrix and induce the process of erosions, ulcers and necrosis formation in the area of infectious inflammation.

Starting from the 2nd week of disease, with the improvement of patients' condition, the levels of acute-phase proteins (fibrinogen, CRP, α1-antitripsin (α1-AT) etc.) decreased (Table **3**).

The presence of disfibrinogenemiya, the activation of external and internal coagulation pathways, as well as the increase of acute-phase proteins suggest a close relation between the systemic inflammatory response and the compromised hemostasis there.

The risk of severe (erythematous-bullous, erythematous-hemorrhagic, bullous-hemorrhagic) forms of LLE was significantly higher compared with FE (OR= 4,9 (1,5; 16)).



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*c) Platelets' aggregation activity and erythrocytes' rheological properties*

In normal settings, the circulating platelets do not interfere with the internal surface of the vessel, covered by a thin layer of heparansulfate, that confers athrombogenic and anti-adhesive properties to vascular endothelium. The vascular wall injury results in the exposition of subendothelium components, mainly collagen, into the blood flow. In case of the participation of vWf (interaction with the platelet GP1b receptors) and of fibrinogen (interaction with the platelet GPIIb/IIIa receptors), the processes of platelet adhesion and aggregation are significantly enhanced [**Ошибка! Источник ссылки не найден.**, 15].

In our study, the ADP-induced platelet aggregation was minimal at 1-3th days: 41,7 ±4% in facial erysipelas and 64,8 ±3,9 % in LLE ($p<0.05$), which is statistically lower in comparison with platelets' functional activity in normal subjects (76±3,1%, $p<0.05$). At 11-15th days of the disease, the platelets' functional activity tended to recover (71,7±3,4% in LLE and 59±3,2% in FE). The number of platelets increased in both groups: from 224±10x109/l to 408±21x109/l ($p=0,002$) in lower limb erysipelas and from 234±30 x109/l to 317±29x109/l ($p=0,04$) in facial erysipelas. Hence, the platelets' amount and their functional activity in erysipelas occurred on the 2nd week of disease.

Herewith, the initially normal amount of erythrocytes at admission (study point 1) decreased by the days 7-10 of the disease (study points 2 and 3). The number of cells decreased by 7% of the initial value (from 4,7 to 4,39 x1012/l) in facial erysipelas and by 5% (from 4,6 to 4,37 x1012/l) in lower limb erysipelas. Simultaneously, erythrocyte sedimentation rate (ESR) rose to the maximum: 53,7±8,5 mm/hour in lower limb erysipelas and 26,3±5,8 mm/hour in facial erysipelas ($p=0.006$).

Earlier, we have found an increase of indirect bilirubin and LDH blood levels in patients with erysipelas. Together with the decreased of haptoglobin found in this study (3,86±,26 g/l in FE and 3,79±0,3 g/l in LLE ($p<0,05$ with the control), it suggests the presence of hidden hemolysis [7]. Further changes of haptoglobin level confirm this conclusion. The level of this protein increased by 153 % of the initial values in facial erysipelas and by 61% — in LLE (Figure 3).

Hence, intravascular (hidden) hemolysis is one of the leading pathogenetic mechanism of DIC-like syndrome. This syndrome is often described as a clinically unapparent (local) disseminated intravascular coagulation. In our study, we have seen the transformation of DIC-like syndrome to the classic DIC in three cases - 5% of 60 studied patients [8].

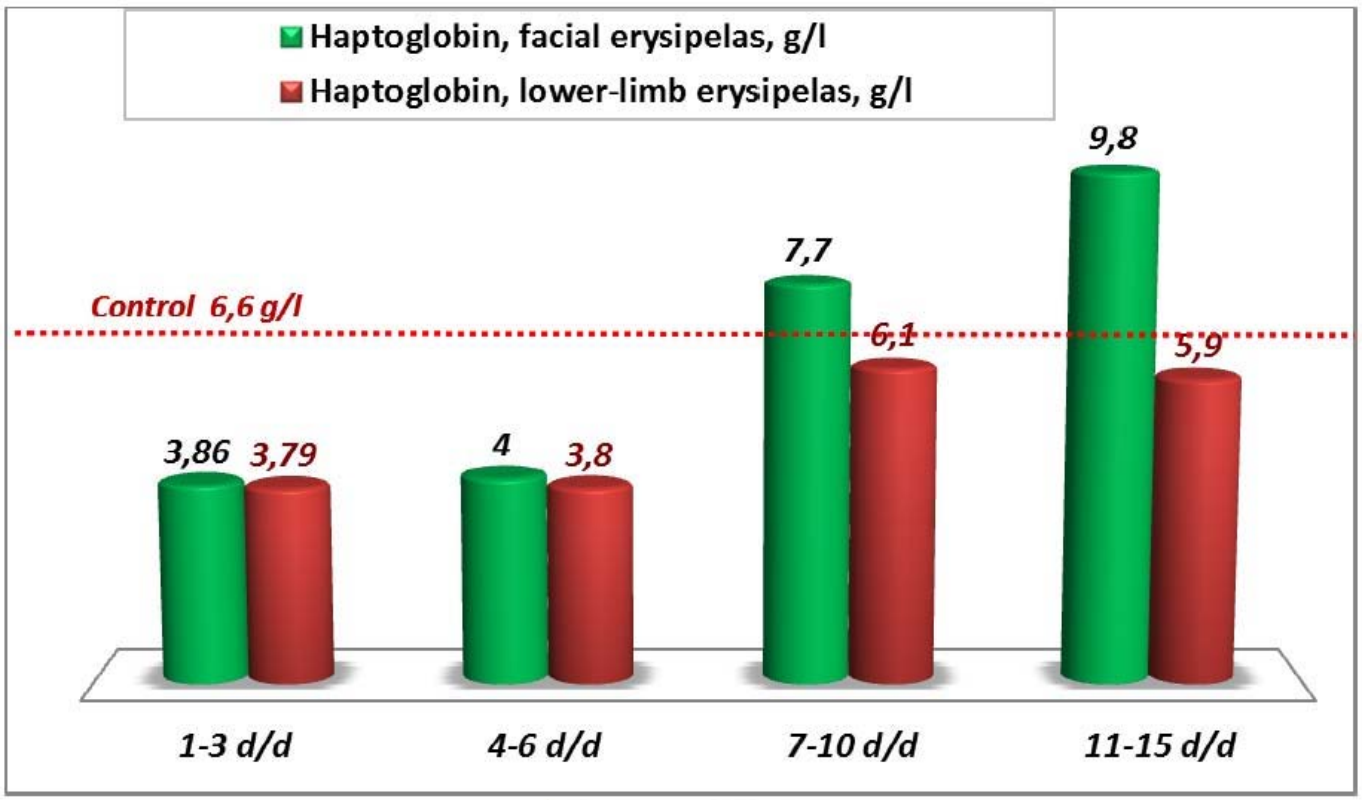



*Figure 3 :* Changes of haptoglobin level (g/l) in facial and lower limb erysipelas at 1st-2nd week of the disease

*Note:* d/d - days of the disease

We studied also the changes of rheological properties of erythrocytes – their elasticity (the aggregation with protamine sulfate) and deformability (the aggregation with lanthanum chloride) in erysipelas.

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The degree of blood cells' elasticity and deformability in normal subjects is almost equal: 62±4,9 % for PS and 66,4±4,2 % for LaCl3.

In erysipelas, the cells' elasticity decreased twofold (aggregation with PS), while the deformability (aggregation with LaCl3) increased by 37% (Table **4**). The aggregation with two types of inductors (LaCl3 and PS) was recorded simultaneously on aggregometer «BIOLA». The aggregates were 3,6 times bigger in size and 7,8 times higher in aggregate's degree on LaCl3 compared with PS.

After addition of LaCl3, the erythrocytes of erysipelas patients interacted faster (3-5 minutes) and quickly precipitated as large conglomerates [1, 8].

Some authors also described the changed conformation properties of the erythrocytes' membrane, the cells' form transformation from concave-discoid to spherical. Our experiments showed that with the addition of LaCl3 to the erythrocytes of erysipelas patients, cytoarchitectonics of cellular membranes is disturbed. It leads to fast adhesion between the cells and to the formation of cell conglomerates. The aggregation with LaCl3 helps to reproduce the picture of erysipelas-associated hidden hemolysis and increased erythrocyte "frailness" in vitro.

The mechanism of protamine sulfate action is different. It does not induce conformational rebuilding of the erythrocytes' membrane. With the loss of negative charge of the membrane (preventing cells adhesion to each other), the erythrocytes sediment and form the coin columns. The aggregates on the PS look smaller and softer, and the aggregation time is slower (≥10 minutes) [1, 8].

The found differences in erythrocytes' elasticity and deformability persisted during the recovery period. The high "frailness" of erythrocyte membrane persisted up to the end of the 2nd week, and its low "plasticity"- up to the discharge (Figure 4).

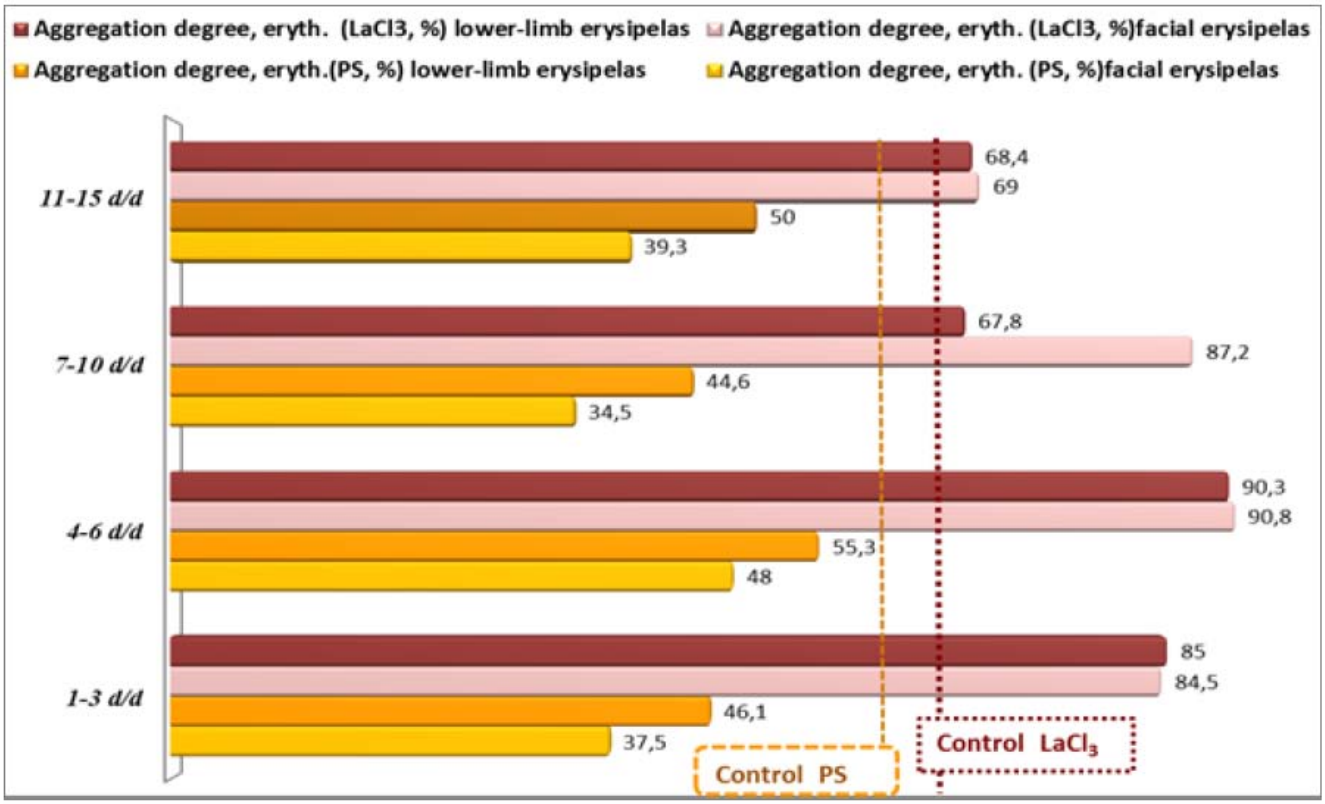



*Figure 4 :* Erythrocytes' deformability (LaCl3) and elasticity (PS) in aggregation tests in facial and lower limb erysipelas

*Note:* d/d — days of the disease

One has to note, that the erythrocytes play not only the role of oxygen transporter in the blood flow. They also serve as a potent buffer of the system of hemostasis and as a second, in order of importance (after albumin), detoxification barrier. The body uses the erythrocyte pool for supplementary refilling of protein deficit in the blood flow [7]. The discoid form allows the erythrocytes to perform their functions with maximal effectiveness. For this reason, the recovery of erythrocytes' elasticity is of clinical interest for certain therapeutic practices.

## V. Conclusion and Recommendations

1. Laboratory studies confirmed that clinical hemorrhagic complications are by 3,9 times more



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common in lower limb erysipelas than in facial erysipelas.

2. The results of determination of the markers of hemostatic endothelial function in erysipelas patients suggest the disturbances of endothelium-related regulation of hemostasis, as judged by antithrombotic (decreased levels of protein C and of AT- III), as well as by adhesive indices (high level of von Willebrand factor).
3. Increased D-dimer and decreased alpha-2-macroglobulin levels suggest potent activation of the system of proteolysis enzymes (plasmin, matrix metalloproteinases and neutrophil elastase). This is pathogenetically associated with the formation of bullae, erosions and ulcers in the erysipelas focus.
4. Low level of protein C, persisting during standard therapeutic procedures, is not only a laboratory indicator of chronic venous insufficiency in lower limb erysipelas, but also serves as a marker of the development of DIC-like syndrome.
5. Documented evidence of protein C deficit can be an indication for the consideration of antithrombotic therapy. Maximally early start of the replacement therapy (with protein C products) can contribute to the delimitation of the inflammation area and to the decrease of the severity of local proteolysis reactions.
6. We found the signs of intravascular (hidden) hemolysis and of disturbed rheological properties of erythrocytes – their increased deformability (aggregation with lanthanum chloride) and decreased elasticity (aggregation with protamine sulfate). The disturbed erythrocytes elasticity is an indication for the supplementation of standard erysipelas therapy with the agents contributing to the increase of erythrocytes' plasticity and to the improvement of blood rheology (Pentoxifylline).
7. Clinically important risk of hemorrhagic complications in lower limb erysipelas is higher, than in facial erysipelas, OR = 9,88 (2,81; 34,7).

## VI. Acknowledgement

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*Table 1 :* Changes in protein C, AT-III and vfW levels in facial and lower-limb erysipelas (Me ±SD)

| Changes by days/Index | Facial erysipelas (n=24) | | | Lower-limb erysipelas (n=36) | | |
|---|---|---|---|---|---|---|
| | Protein C, % | AT-III,% | vfW,% | Protein C, % | AT-III,% | vfW,% |
| Days 1-3 of the disease (point 1) | 94,1±6,0*,** | 91,8±2,5* | 187,0±4,2** | 81,9±4,9*,** | 87,6±2,5* | 190±2,0** |
| Days 4-6 of the disease (point 2) | 119,6±3,1*,** | 96,4±0,8** | 183,0±2,7** | 103,0±3,2*,** | 91,3±1,7*,** | 187,6±2,9* |
| Days 7-10 of the disease (point 3) | 129,0±6,4* | 91,4±2,2* | 175,6±6,2* | 134,5±4,7* | 89,6±1,5* | 180,0±2,8* |
| Days 11-15 of the disease (point 4) | 153,0±4,4* | 93,0±1,1* | 174,8±4,4* | 139,0±6,7* | 89,4±3,2* | 176,0±3,1* |
| Follow-up (in 5 months) | _____ | _____ | _____ | 106,4±10,2 | 93,0±1,2* | 167,0±3,7* |
| Normal subjects (n=32) | 100±5,0 | 97,3±0,38 | 150,4±3,9 | 100,0±5,0 | 97,3±0,38 | 150,4±3,9 |

*Note: *— significant difference with the control value, **— reliable differences between the groups (p<0,05)*



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*Table 2 :* Changes in plasma hemostasis in facial and lower-limb erysipelas (Me ±SD)

| Changes by days/Index | Facial erysipelas (n=24) | | | | | Lower-limb erysipelas (n=36) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | PT, sec | INR | Prothrombin, % | TT, sec | aPTT,sec | PT, sec | INR | Prothrombin, % | TT, sec | aPTT,sec |
| Days 1-3 of the disease (point 1) | 12,2±0,7*,** | 1,27±0,04* | 79,2±4,5*,** | 12,2±0,5*,* | 28,5±1,3*,* | 15,4±0,5*,* | 1,47±0,04*,** | 59,3±2,8*,** | 10,9±0,36*,** | 39,5±1,4*,** |
| Days 4-6 of the disease (point 2) | 14,9±0,6* | 1,42±0,05* | 68,7±4,3* | 12,7±0,7*,* | 45,1±2,7*,* | 15,1±0,4* | 1,47±0,04* | 61,3±3,8* | 11,9±0,5* | 38,5±1,7*,** |
| Days 7-10 of the disease (point 3) | 12,2±0,7*,** | 1,27±0,04* | 79,2±4,5*,** | 12,2±0,5*,* | 28,5±1,3*,* | 15,4±0,5*,* | 1,47±0,04*,** | 59,3±2,8*,** | 10,9±0,36*,** | 39,5±1,4*,** |
| Days 11-15 of the disease (point 4) | 12,2±0,7 | 1,16±0,03 | 79,4±4,8* | 12,2±0,3*,* | 37,3±3,0*,* | 13,3±0,25*,** | 1,31±0,03*,** | 73,3±2,8*,** | 12,1±0,26* | 37,4±1,3*,** |
| Follow-up (in 5 months) | – | – | – | – | – | 11,2±0,3 | 1,1±0,02 | 93,2±2,9 | 13,4±0,15 | 30,4±1,0 |
| Normal subjects ($n$=32) | 10,9±0,14 | 1,11±0,02 | 98,2±1,8 | 14,6±0,26 | 33,7±0,66 | 10,9±0,14 | 1,11±0,02 | 98,2±1,8 | 14,6±0,26 | 33,7±0,66 |

*Note: PT — Prothrombin time, INR — International Normalized Ratio, TT — Thrombin time, aPTT — activated Partial Thromboplastin time;*— significant difference with the control value, **— reliable differences between the groups (p<0,05).*

*Table 3 :* Signs of disfibrinogenemiya, (Me ±SD)

| Changes by days/Index | Facial erysipelas ($n$=24) | | | | Lower-limb erysipelas ($n$=36) | | | |
|---|---|---|---|---|---|---|---|---|
| | Fibrinogen, g/l | D-dimer, ng/ml | CRP, mg/l | $\alpha_1$-AT +$\alpha_1$-acid GP, % | Fibrinogen, g/l | D-dimer, ng/ml | CRP, mg/l | α1-AT +α1-acid GP, % |
| Days 1-3 of the disease (point 1) | 6,53±0,49*,** | 160,2±4*,** | 126±14* | 3,63±0,2* | 7,7±0,38* | 399±46*,** | 126±8,5* | 3,79±0,29* |
| Days 4-6 of the disease (point 2) | 5,62±0,47*,** | 164±2*,* | 44,5±7,1*,** | 3,34±0,08* | 6,75±0,42* | 371±88*,** | 117±13*,** | 4,2±0,3*,** |
| Days 7-10 of the disease (point 3) | 5,0±0,33* | 147,7±27*,** | 1,2±0,2* | 3,22±0,14 * | 5,3±0,17* | 381±43*,** | 45,7±15* | 3,45±0,2* |
| Days 11-15 of the disease (point 4) | 4,2±0,2*,** | 88,6±12,7*,** | 2,0±0,3*,** | 2,98±0,13 * | 5,6±0,4*,** | 467±46*,** | 9,4±3*,** | 3,2±0,24* |
| Follow-up (in 5 months) | ——— | ——— | ——— | ——— | 5,1±0,6* | 202,6±5* | 1,7±0,4* | 2,5±0,2 |
| Normal subjects ($n$=32) | 2,96±0,09 | 14,5±3,18 | 0,6±0,2 | 2,6±0,1 | 2,96±0,09 | 14,5±3,18 | 0,6±0,2 | 2,6±0,1 |

*Note: CRP — C reactive protein, α1-AT — α1-antitrypsin, α1-acid GP — α1-acid GP glycoprotein;*

**— significant difference with the control value, **— reliable differences between the groups (p<0,05).*



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*Table 4 :* Platelets' number and aggregation activity and erythrocytes' rheological properties in facial and lower-limb erysipelas, (Me ±SD)

| Changes by days/Index | Platelets, $x10^9/l$ | ADP-aggregation degree: platelets, % | Erythrocytes, $x10^{12}/l$ | ESR, mm/hour | $LaCl_3$-aggregation degree: erythrocytes, % | PS - aggregation degree: erythrocytes, % | Haptoglobin, g/l |
|---|---|---|---|---|---|---|---|
| **Facial erysipelas (n=24)** | | | | | | | |
| Days 1-3 of the disease (point 1) | 234±30,0 | 41,7±4*,** | 4,5±0,08* | 20,4±1,8*,** | 84,5±6,4*,** | 37,5±3,7*,** | 3,86±0,26*,** |
| Days 4-6 of the disease (point 2) | 249±9,4 | 67,2±5,1 | 4,7±0,08 | 18±2,8*,** | 90,8±5,3*,** | 48±3,1*,** | 4,0±0,4*,** |
| Days 7-10 of the disease (point 3) | 296±39 | 47±2,6*,** | 4,39±0,08 | 26,3±5,8*,** | 87,2±3,6*,** | 34,5±1,7*,** | 7,7±1,2*,** |
| Days 11-15 of the disease (point 4) | 317±29 | 59±3,2*,** | 4,3±0,2 | 16,5±2,3* | 69±7,3 | 39,3±3,9*,** | 9,8±,4*,** |
| **Lower-limb erysipelas (n=36)** | | | | | | | |
| Days 1-3 of the disease (point 1) | 224±10 | 64,8±3,9*,** | 4,7±0,06* | 33,5±3,9*,** | 85±4,5*,** | 46,1±2,7*,** | 3,79+0,3*,** |
| Days 4-6 of the disease (point 2) | 253±14 | 68,2±4,3 | 4,6±0,08 | 35,2±3,7*,** | 90,3±4,8*,** | 55,3±5,0 | 3,8+0,4*,** |
| Days 7-10 of the disease (point 3) | 314±12,8 | 53,7±3,0*,** | 4,37±0,14 | 53,7±8,5*,** | 67,8±5*,** | 44,6±3,4*,** | 6,1+0,8 |
| Days 11-15 of the disease (point 4) | 408±21 | 71,7±3,4* | 4,5±0,1 | 33,7±6,4*,** | 68,4±4,9 | 50,0±6,0*,** | 5,9+0,6 |
| Follow-up (in 5 months) | — | 52,7±5,5* | — | — | 63,0±6,4 | 36,7±3,4*,** | 5,4+0,8 |
| Normal subjects (n=32) | 250+5,2 | 76±3,1 | 4,5±0,13 | ≤ 10 | 66,4±4,2 | 62±4,9 | 6,6±0,5 |

*Note: ESR - erythrocyte sedimentation rate,*— significant difference with the control value, **— reliable differences between the groups (p<0,05)*

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# The Histological Changes of the Skin Lesion in Diabetic Foot

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*Abstract-* *Objective:* To investigate the histological changes in the skin tissue covering the area surrounding the ulceration of the diabetic foot.

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*Keywords:* *diabetic foot, microangiopathy, neuropathy.*

*GJMR-C Classification :* *NLMC Code: QW 4*




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# The Histological Changes of the Skin Lesion in Diabetic Foot

Muna Z. Al-Hamdany [α], Professor Dr. Abduljabbar Y. Al-Hubaity [σ] & Modhar S. Al-Omary [ρ]

الخلاصة- **هدف البحث** : هدف هذا البحث الى التعرف على التغيرات النسيجية في نسيج الجلد المحيط بقدم السكري. **المرضى و طريقة العمل**: اجريت هذه الدراسة على ثلاثين شخص تم تقسيمهم الى ثلاث مجاميع عشرة في كل مجموعة.المجموعة الاولى هي مجموعة السيطرة تضم المرضى الاصحاء والمجموعة الثانية يعانون من مرض السكري النوع الاول والمجموعة الثالثة يعانون من مرض السكري من النوع الثاني . جميع مرضى السكري في هذه الدراسة لديهم درجات مختلفة من تقرح الجلد المحيط بالقدم.اخذت النماذج من نسيج الجلد المحيط بتقرح قدم السكري وتم تجميعها من مستشفى الجمهوري التعليمي بين امراير الفحص النسيجي في قسم التشريح عقب الانسجة الطبية في كلية طب الموصل في الفترة الممتدة بين نوفمبر ٢٠١٥ الى حزيران ٢٠١٦ تم تحضير النماذج لغرض الفحص النسيجي باستخدام صبغة الهيماتوكسلين و **النتائج** : اظهرت النتائج وجود صبغة Van-Gieson. تقرن شديد في طبقة البشرة مع تسرب شديد في الخلايا الالتهابية في طبقة البشرة، انعدام او انحلال في الغدد العرقية مع تسرب الخلايا الملفاويه حول الاوعية الدموية ووجود منطقة بؤرية نخرية للنسيج في طبقة الادمة .اما الشرايين الكبيرة والشرايين العضلية متوسطة الحجم فقد اظهرت تليف واضح في الطبقة الثانية من جدارها. اما الاعصاب المحيطية فقد اظهرت انحلال واضح في خلايا شوان. **الاستنتاجات:** التغيرات الوعائية في قدم السكري تظهر اغلبا في الاوعية الدموية الشعرية ثم تنتقل الى الشرايين الكبيرة والمتوسطة الحجم وتكون تغيرات تخرية في الاعصاب المحيطية. اما النسيج البشرة الجلدي المحيط بتقرح قدم السكري وهو يشمل طبقتي البشرة الوعائية والادمة عام. كما ان اكتشاف التغيرات النسيجية والوعائية في قدم السكري يتيح فرصة اكثر للتسبب والخلفية للتقرح. **الكلمات المفتاحية**: قدم السكري، مرض الاوعية الدموية،مرض الاعصاب

*Abstract- Objective:* To investigate the histological changes in the skin tissue covering the area surrounding the ulceration of the diabetic foot.

*Patients & Methods:* The study was performed on 30 patients who were classified into 3 groups 10 patient in each. Group I is the control group have no diabetes, group II with diabetes mellitus type I, and Group III patients with diabetes mellitus type II. All the diabetic patients showed various degrees of skin lesions in the foot. Specimens of skin tissue were obtained from the area surrounding the diabetic ulcer from Al-Jumhuri Teaching Hospital and the histological analysis was performed in the Department of Anatomy, College of Medicine, University of Mosul from November 2015 to June 2016. The specimens were processed for the standard histological examination using Hematoxylin and Eosin, and Orcein-VanGieson stains.

*Results:* The skin sections were dominated by the presence of hyperkeratosis in the epidermis with regular acanthosis in addition to dense chronic inflammatory cells infiltration in the dermis, absence or degeneration of the sweat glands, perivascular lymphocytic infiltration, focal areas of necrosis and melanin pigmentation in the dermis. The large arterioles and arteries of muscular type revealed fibrous tissue deposition at the level of media while the peripheral nerves showed an obvious degeneration of Schwann cells.

*Conclusions:* The vascular changes in the diabetic foot appears in the microcirculation level (capillaries) then involves arterioles and arteries of muscular type and were accompanied by morphological changes of the peripheral nerves. The morphological changes in the skin surrounding the diabetic foot ulcer involves both the epidermis and dermis. The identification of histological and vascular alterations in the diabetic foot allows more knowledge related to the pathogenesis and pathological background of this condition.

*Keywords: diabetic foot, microangiopathy, neuropathy.*

## I. Introduction

Diabetic foot is the most common complication of diabetes. The ulceration is frequently associated with peripheral neuropathy which is a well-known cause of morbidity and even mortality in the diabetic patients[1]. The incidence is rising as a result ageing and increased risk factors for atherosclerosis such as smoking and obesity commonly associated with diabetes[2]. The diabetic foot lesions involves a wide range of structural changes affecting the nerves in the form of autonomic and motor neuropathy, blood vessels as diabetic macro and microangiopathy, joint and bone lesions of the sole, and skin and nail lesions[3]. The exact mechanism underlying the pathogenesis of diabetic ulceration is not well known, many mechanisms have been proposed even there may be a genetic influence which increase the susceptibility to such complications[4]. However, peripheral neuropathy is the major cause combined with arterial insufficiency caused by atherosclerotic occlusion of the tibioperoneal arteries[5]. The first pathological change in the development of diabetic foot ulcer is vasoconstriction associated with vascular abnormalities, such as thickening of the basement membrane of the capillaries and hyperplasia of their endothelial cells lining with subsequent diminished oxygen tension and hypoxia, as the disease progresses, neuronal dysfunction occurs[1]. Microvascular changes occurs early in diabetes, parallel to the progression of neuronal ischemia which is a characteristic feature in diabetic neuropathy thus both vascular and neural abnormalities determine the severity of structural, functional, and clinical dysfunction [6].

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Diabetic neuropathy is the major problem in diabetes mellitus, which may be prevented by the control blood glucose level and maintenance of normoglycaemia[7]. The term "diabetic foot" involves multiple changes on the level of small and large blood vessels, nerves, bone and soft tissues besides abnormalities of the microcirculation which results in capillary insufficiency, all lead to alterations in the foot biomechanics which promotes tissue destructions and severe infections even sometimes, resulting in amputations[8]. The microangiopathy in diabetes can affect different organs to a different degree, like diabetic nephropathy or retinopathy) (9). Recently it is well-established that there is a close connection between the abnormalities of the microcirculation and the diabetic neuropathy [10] proving the fact that microvascular changes are closely linked to the diminished nervous conduction and the potential of muscular action[11]. The abnormal neural function contributes to the development of microangiopathy in diabetic foot ulcer manifested as thickening of the basal membrane of the capillaries and proliferation of the endothelial cells lining of both arteries and arterioles, resulting in ischemic changes and ulceration[12]. A great improvement in the field of management of diabetic foot has been made by increasing range of the antibiotic therapy and by exploring invasive and noninvasive angiographic techniques [13].

Our present study aims to investigate the histological and vascular changes accompanied to the diabetic foot ulceration and to evaluate the severity and pattern of progress of the condition in relation to the type of diabetes and the control of blood glucose level.

## II. Patients, Materials and Methods

In this study, 30 patients were classified into 3 groups 10 patients in each. Group I is the control group have no history of diabetes, group II suffering from diabetes mellitus type I, and Group III patients with diabetes type II. The specimens of skin fragments were collected from the area surrounding the diabetic foot ulcer from diabetic patients who were admitted for surgical management. Detailed history, thorough physical examination and biochemical testing including serum glucose, renal function and liver function tests and x-ray of the affected foot were carried out, the history includes their age, sex, occupation, duration of diabetes, drug history and family history to exclude genetic predisposition of the condition. All the diabetic patients were affected by peripheral neuropathy and showed various degrees of skin lesions in the foot. Specimens of skin tissue were obtained from Al-Jumhuri Teaching Hospital and the histological analysis was performed in the Department of Anatomy, College of Medicine, University of Mosul from November 2015 to June 2016.

Following the debridement of the foot ulceration and removal of the skin tissue fragments from inside and around the ulcer, the specimens were put in a fixative solution (10% neutral formalin) for 24 hour then each specimen was cut into 1 cm thick slices and dehydrated in graded alcohol solutions (70% alcohol for overnight, two changes in 90% alcohol one hour for each and two changes in 100% alcohol for two hours) then the specimens were immersed in xylene using three changes with one-hour interval for each. Complete removal of the clearing solution was made by immersing the tissue specimens into three successive paraffin bathes in oven, one hour for each. Finally paraffin blocks were prepared by embedding the tissue specimens using paraffin wax (melting point is 55-60Co) and these paraffin blocks were now ready for sections using Reichert Rotary Microtome, serial paraffin sections of 4 micrometers in thickness were cut from each block, the sections were collected and mounted on glass slides then the sections were stained with Haematoxylin and Eosin for histological analysis.

## III. Results and Observations

This study was performed on 30 persons, 20 of them were diabetic and 10 of them were not, 8 (40%) male and 12 (60 %) female, 14(70%) smoker and 6(30%) were not, 16 (80%) hypertensive and 4(20%) were not, and ١٨(90%) suffering from kidney disease and ٢(10%) were not.

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*Table 1 :* Statistical significance of the demographic variables in patients with diabetes mellitus (N=20)

| Variables | | Number (N=30) | Percentage %(N=30) | P-values |
|---|---|---|---|---|
| Sex | Female | ١٢ | 60% | 0.01(S) |
| | Male | ٨ | 40% | 0.1(NS) |
| Smoking | Yes | 14 | 70% | 0.02(S) |
| | No | 6 | 30% | 0.2(NS) |
| Hypertension | Yes | 16 | 80% | 0.02(S) |
| | No | 4 | 20% | 0.2(NS) |
| Renal insufficiency | Yes | 18 | 90% | 0.03(S) |
| | No | 2 | 10% | 0.3(NS) |

*S=Significant (P≤0.05); NS=Non-significant (P>0.05)*

The table showed that female affected with diabetes more than male, and statistical significant increase in the incidence of diabetes in smokers (70%) and in those suffering from hypertension (80%) and renal insufficiency (90%) thus P≤0.05.

The histopathological examination of skin fragments from tissue around revealed specific microscopic changes which reflect the pathogenic background of the lesions of diabetic foot.

## IV. Histological Findings

*a) In the control group*

- Skin tissue from the control group showed normal epidermis as stratified squamous epitheliumand dermis which a connective tissue layer (Figure 1).

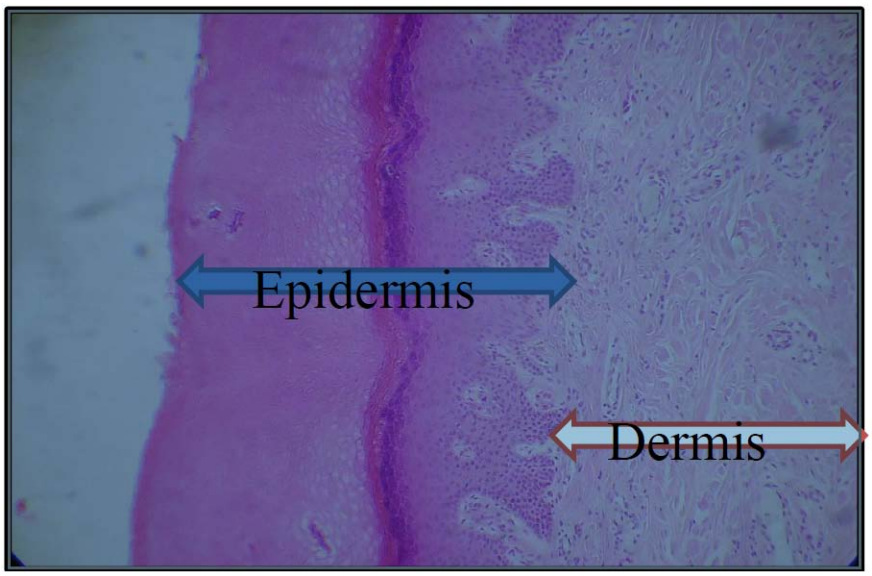



*Figure 1 :* Photomicrograph of skin tissue from control groupshowed normal epidermis and dermis(H&E X100).



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b) *Ininsulin dependent diabetes (IDD)*

- Mild hyperkeratosis in the epidermis (increase thickness of the keratin layer of the epidermis) with regular acanthosis due to hyperplasia of the stratum spinosum in addition to dense chronic inflammatory cells infiltration mainly lymphocytes and eosinophils and at some time associated with polymorphonuclear neutrophils (Figure 2).

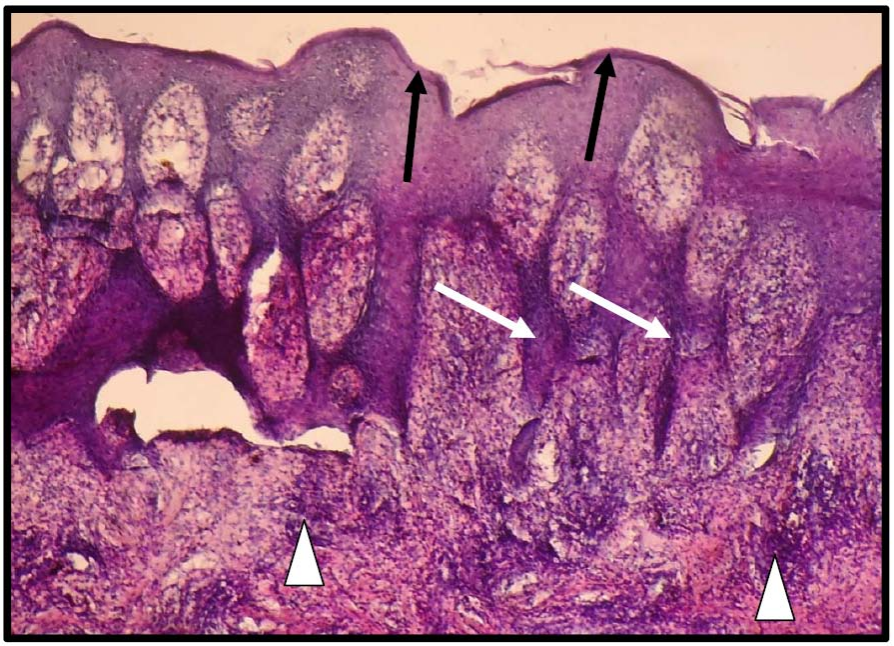


*Figure 2 :* Photomicrograph of skin tissue from Group II showedmild hyperkeratosis in the epidermis(black arrows) with regular acanthosis(white arrows) anddense chronic inflammatory cells infiltration (arrow heads)(H&E X150).

- Disorganization and degenerative changes of the sweat gland which are surrounded by prominent deposition of lymphocytes (Figure 3).

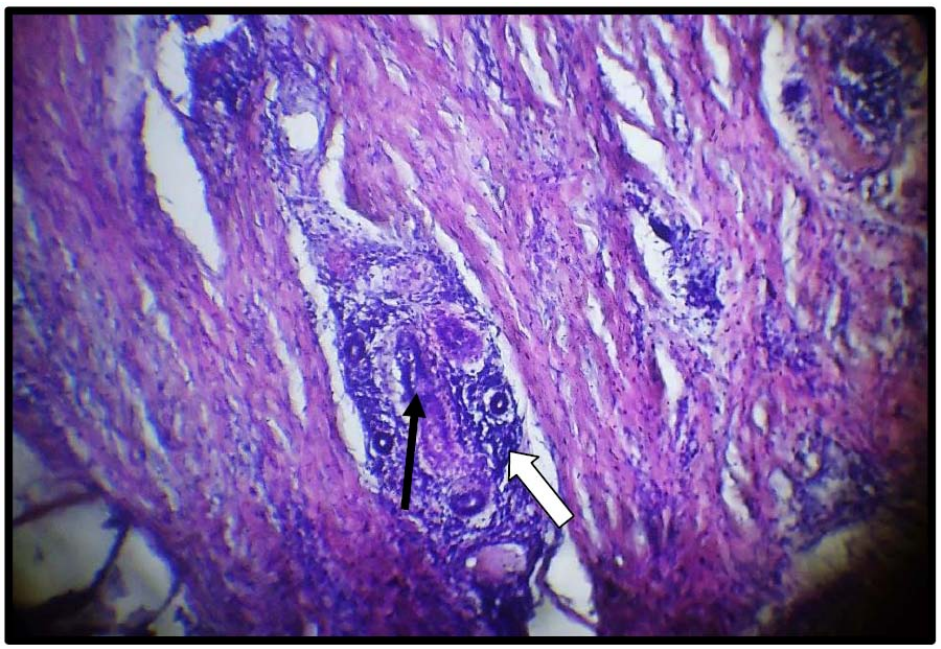


*Figure 3 :* Photomicrograph of skin tissue from Group II showeddegenerative changes of the sweat gland (black arrow) surrounded by prominent deposition of lymphocytes (white arrow)(H&E X150).

- Congestion of the blood vessels in the dermis with obvious perivascular lymphocytic infiltration arranged in concentric layers “muffs” around them, sometimes penetrating to the media layer (Figure 4).



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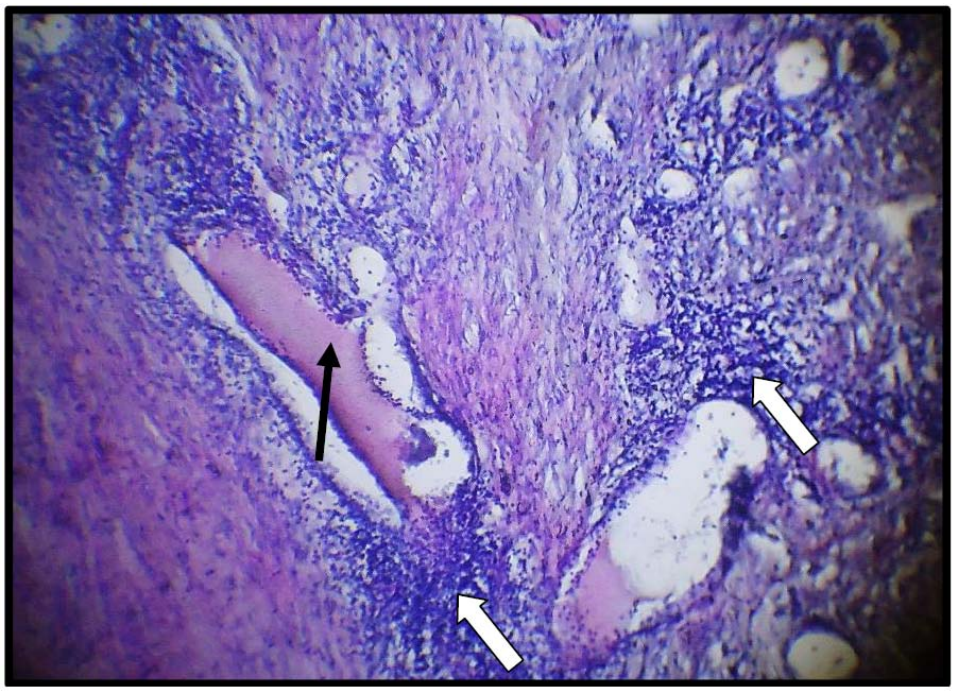


*Figure 4 :* Photomicrograph of skin tissue from Group II showed congestion of the blood vessels in the dermis(black arrow) with obvious perivascular lymphocytic infiltration (white arrows)(H&E X100).

- Disturbance of the histological architecture with focal areas of necrosis involving the destruction of the vascular structures **(Figure 5).** Focal melanin pigmentation in the dermis **(Figure 6).**

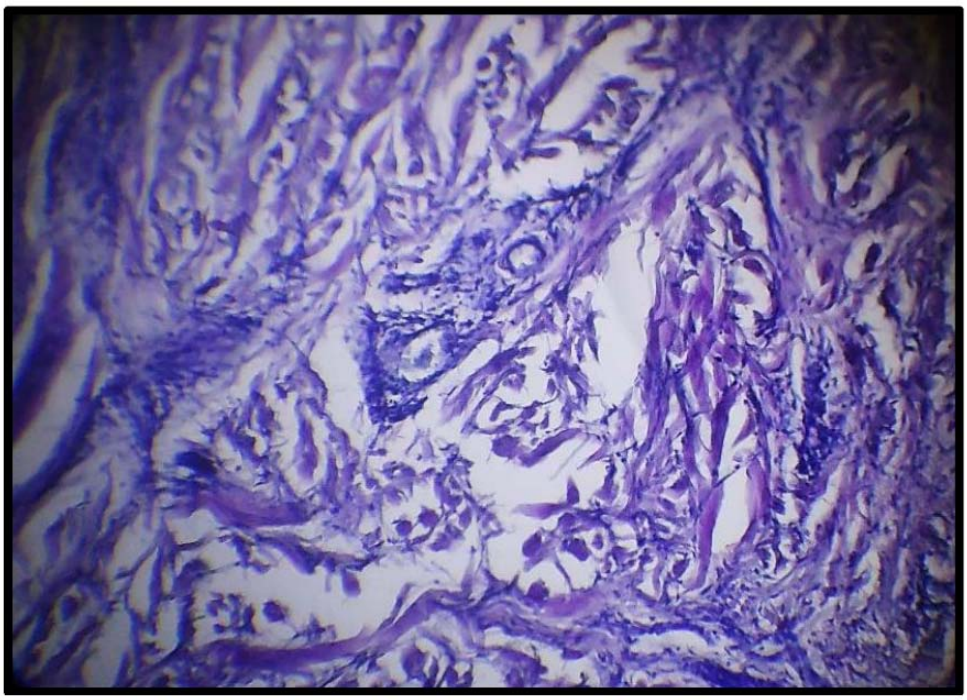


*Figure 5 :* Photomicrograph of skin tissue from Group II showeddisturbance of the histological architecture with focal areas of necrosis (black arrow)(H&E X100).



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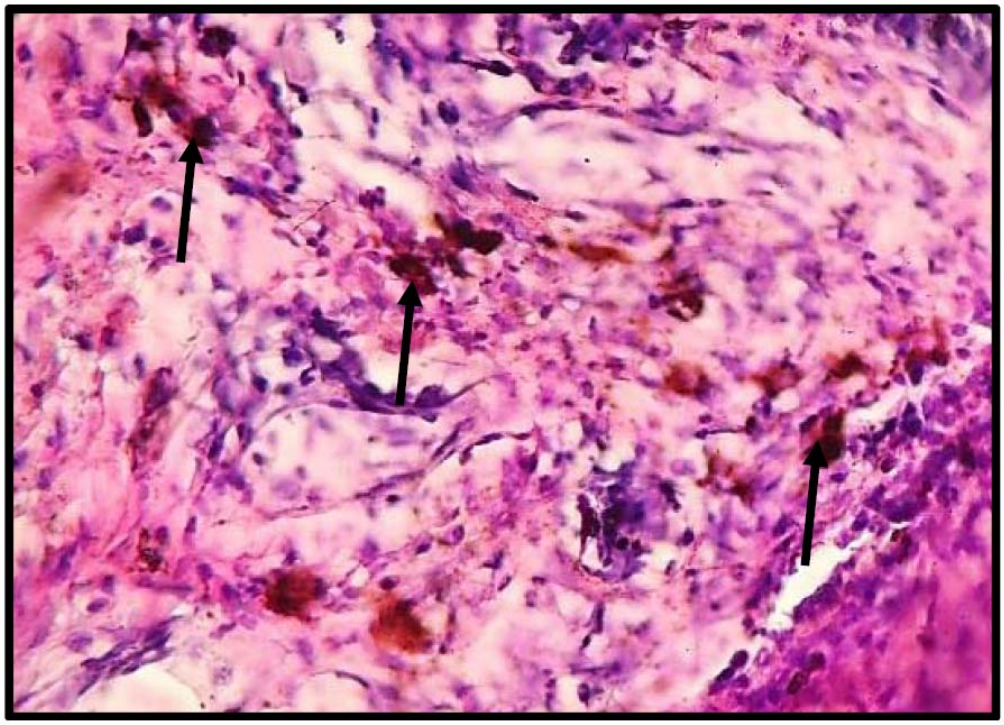


*Figure 6 :* Photomicrograph of skin tissue from Group II showedfocal melanin pigmentation in the dermis(black arrows)(H&E X150).

- The large arterioles and arteries of muscular type showed swollen endothelial cells lining, excessive proliferation of the subendothelial connective tissue layer and **fibrous tissuedeposition** at the level of media **(Figure 7).**

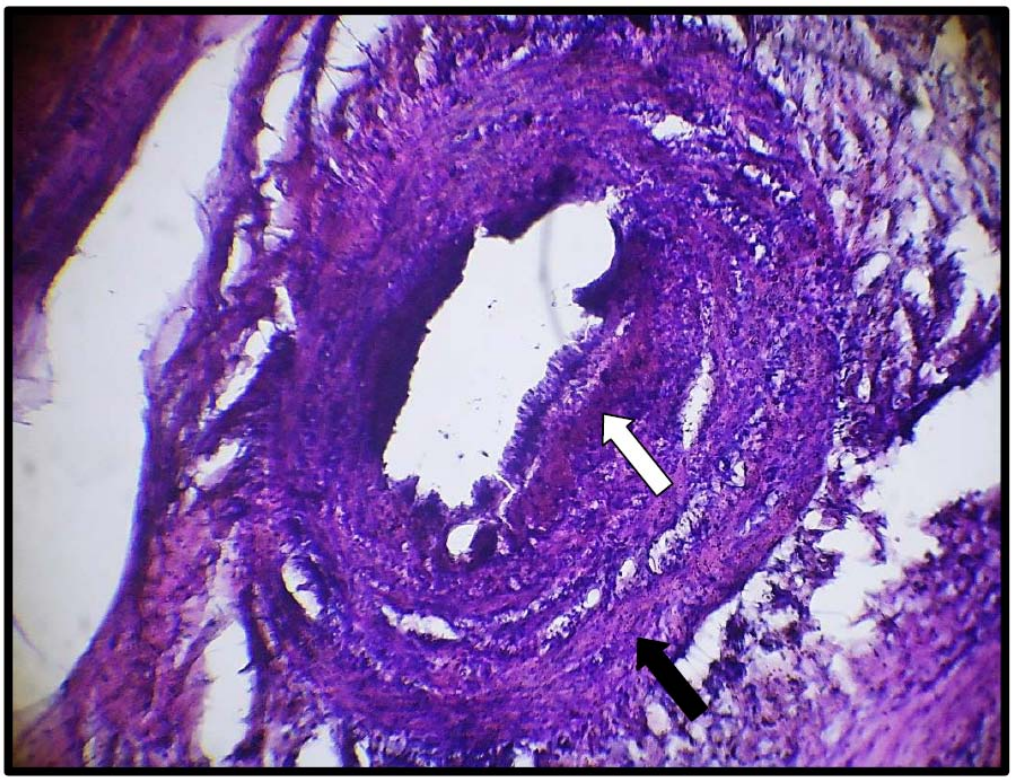


*Figure 7 :* Photomicrograph of skin tissue from Group II showed the artery with excessive proliferation of the subendothelial connective tissue layer (white arrow)and fibrous tissue deposition in the media (black arrow) (H&E X150).

- Heavy collagen fiber deposition around the blood vessels on using Orcien-Van Gieson stain **(Figure 8).**



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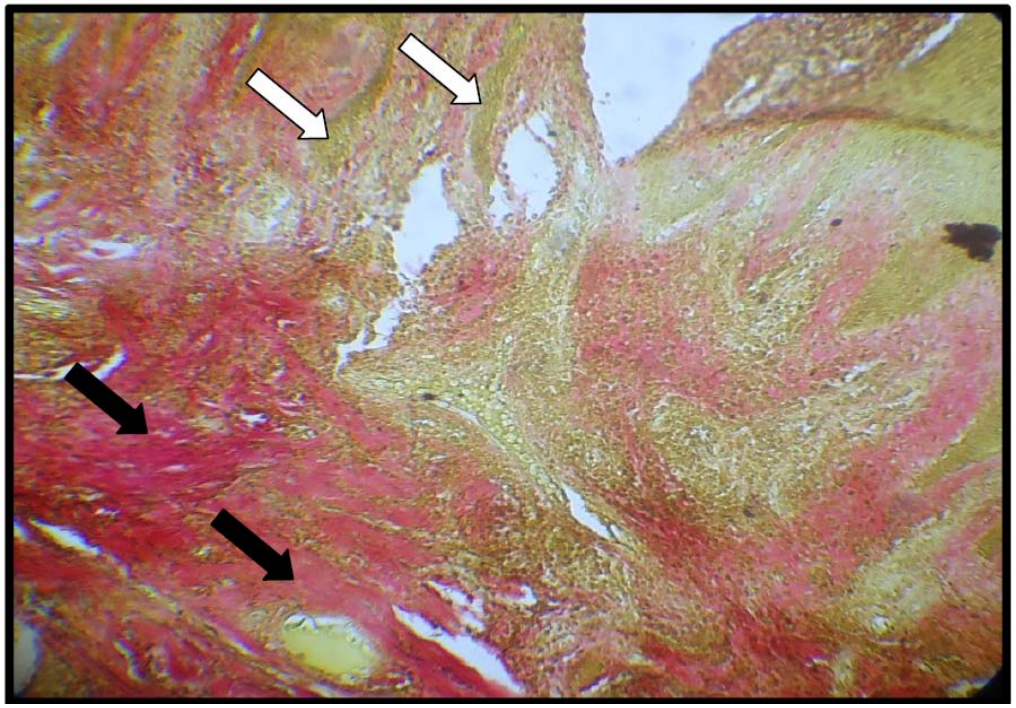


*Figure 8 :* Photomicrograph of skin tissue from Group Ishowedhyperacanthosis of epidermis (white arrows) with heavy collagen fiber deposition around the blood vessels (black arrows)(Orcien-Van Gieson X400).

c) *In Non-insulin dependent diabetes (NIDD)*

- Marked hyperkeratosis more than insulin dependent diabetes appeared as thickened keratin covering the epidermis with obvious sever acanthosis **(Figure 9).**

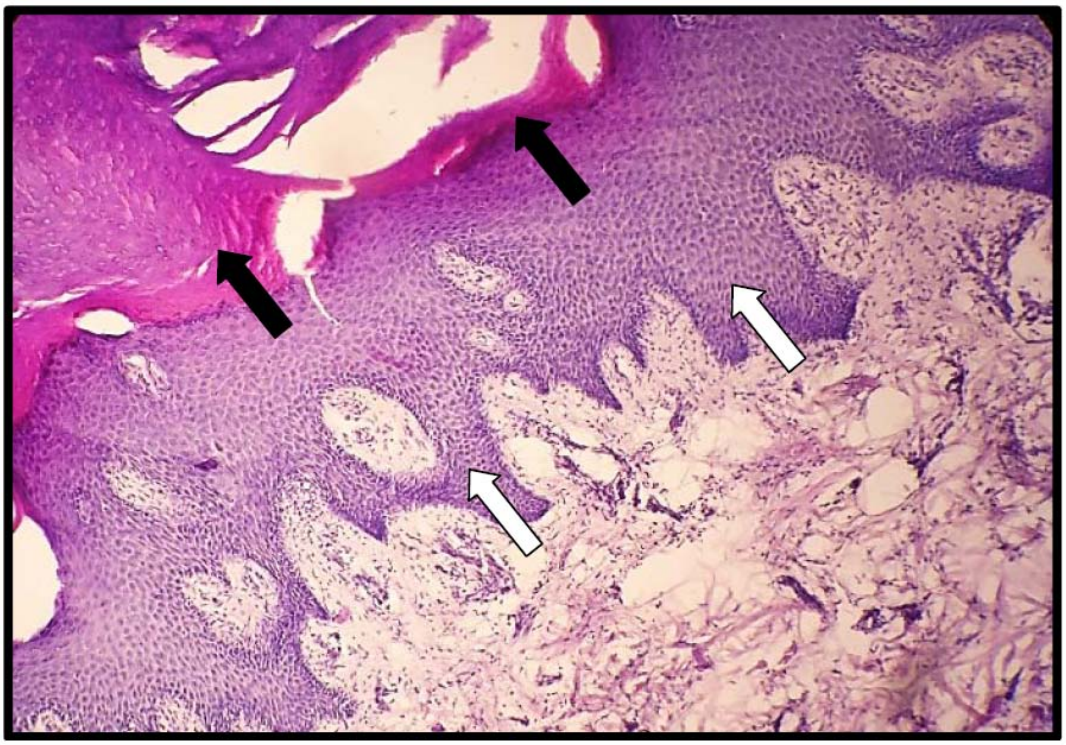


*Figure 9 :* Photomicrograph of skin tissue from Group III showedmarked hyperkeratosis as thickened keratin covering the epidermis (black arrow)with sever acanthosis (white arrows)(H&E X100).

- Mild inflammatory cells deposition including lymphocytes and plasma cells **(Figure 10).**



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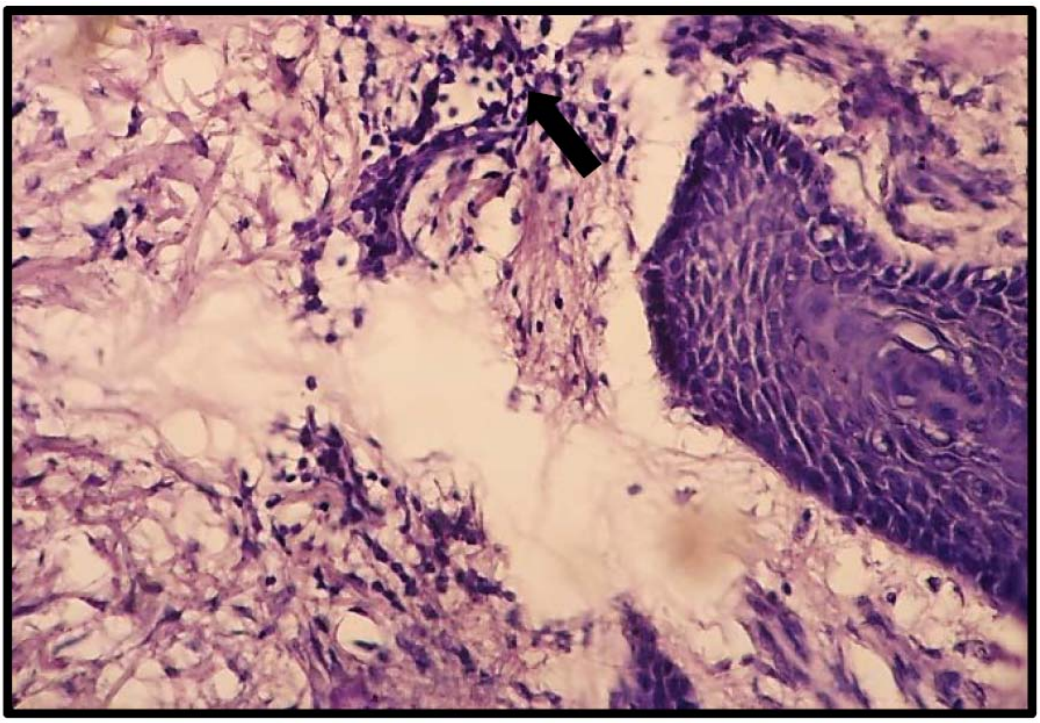


*Figure 10 :* Photomicrograph of skin tissue from Group III showedmild inflammatory cells deposition in thedermis (black arrow)(H&E X150).

- Dilated acini of the sweat glands with degenerative changes of their lining epithelium and dilatation of their ducts (Figure 11).

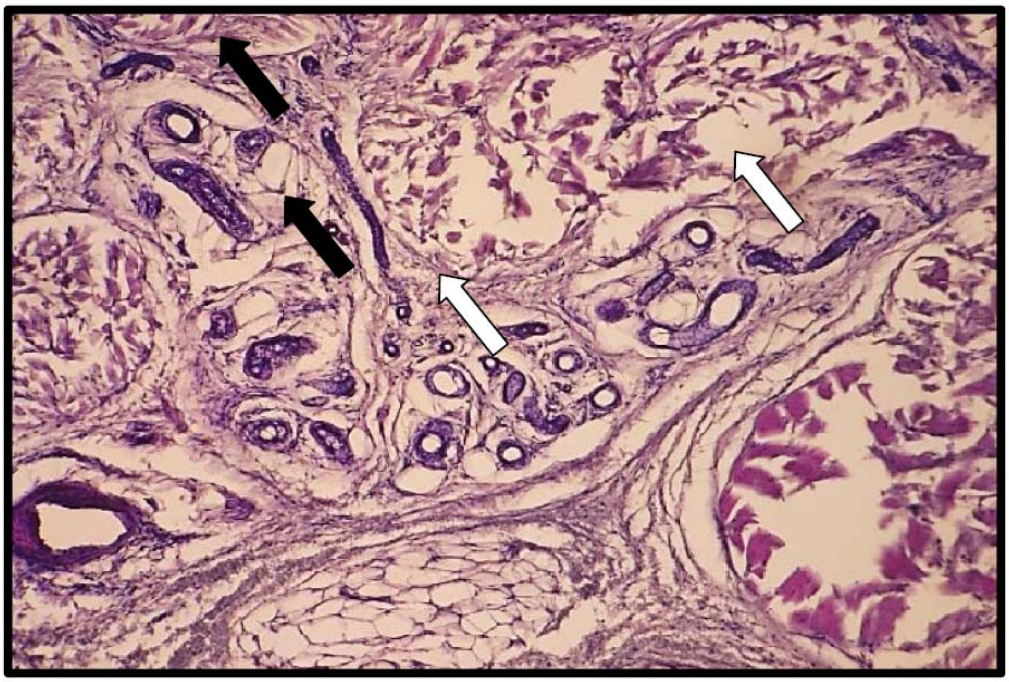


*Figure 11 :* Photomicrograph of skin tissue from Group III showeddilated acini of the sweat glands with degenerative changes of their lining epithelium (black arrows) and dilatation of their ducts (white arrow)(H&E X150).

- The peripheral nerves showed an obvious vacuolar degeneration of Schwann cells that eventually have resulted in the disappearance of the nerve fibers (Figure 12).



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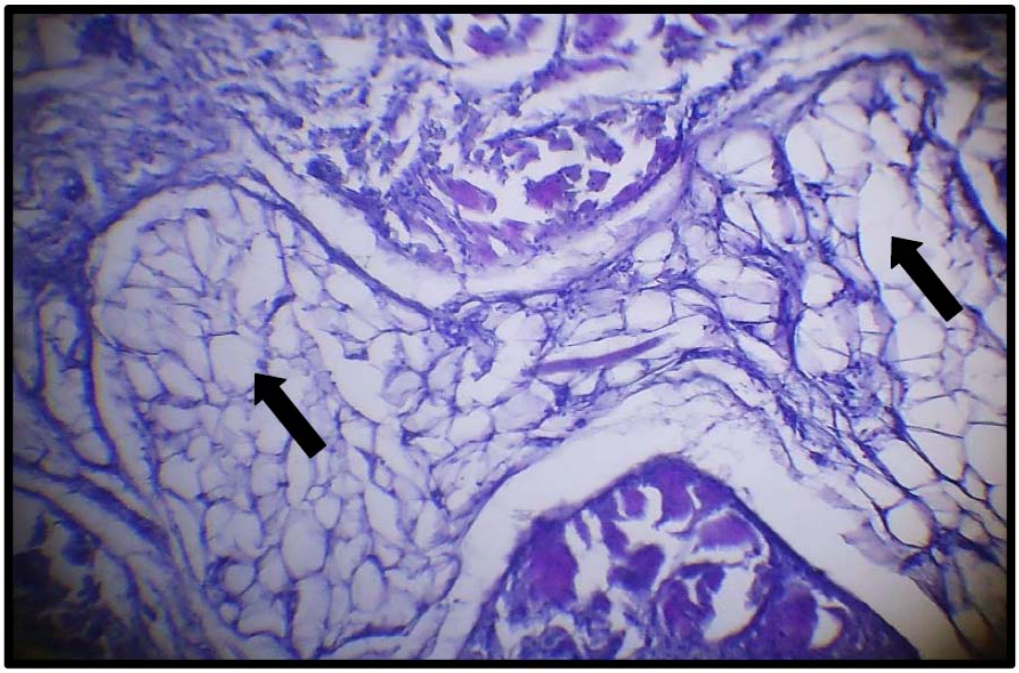


*Figure 12 :* Photomicrograph of skin tissue from Group III showedvacuolar degeneration of Schwann cells of the peripheral nerve(black arrows)(H&E X400).

## V. Discussion

The duration of diabetes, hypertension, smoking, and raised serum cholesterol are important risk factors for the development of diabetic foot ulcers in patients with diabetes. However, various pathogenic mechanisms of vascular and haemodynamic dysfunction have been proposed[14]. Platelet dysfunction, immunological mechanisms, presence of adhesion molecules have been described in relation to diabetic foot[1].

Aguiar et.al, 2007[15] stated that microcirculation is involved in the pathogenesis of diabetic foot which is sometime termed as "small vessel disease".

Endothelial dysfunction precedes the appearance of the microvascular lesions and it is proven by vasoconstriction, marked increase in the microvascular blood flow and vascular permeability and alterations of anti-thrombotic properties of the endothelium[16].

In this study, hyperkeratosis with regular acanthosis in the epidermis and dense chronic inflammatory cells infiltration might be due to release of proinflammatory cytokines like prostaglandins, leukotrienes, and interleukins which cause inflammatory response. This finding previously reported by[17]. Furthermore, vascular congestion in the dermis could be due to the release of vasodilator substances then the stagnant blood in the dilated vessels will cause tissue hypoxia followed by degenerative changes of the sweat gland and focal areas of necrosis. The finding agrees with that observed by[١٨]. deposition of collagen fibers occurs due to chronic inflammatory reaction, thus more fibroblasts might reach the area leading to more collagen fibers deposition[١٩]. Moreover, it has been suggested that alveolar macrophages may release fibroblast chemotactic factors leading to more fibroblast proliferation and **fibrous tissuedeposition** at the level of media[٢٠].

The focal necrotic areas could be provoked by mitochondrial changes mediated by oxidative stress. Ibrahim, (2013)[21] stated that oxidative mitochondrial swelling may lead to rupture of the outer mitochondrial membrane and release of cytochrome C to activate the proapoptoticBax protein which triggers cellular apoptosis followed by necrosis depending on the level of ATP.

Abnormalities in the vascular structure and function are caused by loss of the sympathetic tone, especially on the level of capillaries and small and medium arterioles leading to local ischemia, increase of the arteriolar resistance and consequently a decrease of the blood flow and nutritive circulation of the tissues [22]. The alterations of the microcirculation can also explain the late healing diabetic foot ulcer and even a raised suspicion of infections [23]. The frequency and severity of wound infection may be related to high glucose levels and the contribution of occlusive microvascular disease [24]. During the progression of the diabetic foot ulceration, the nervous involvement is sustained by the morphological changes on the level of the peripheral nerve [25].

Our present study makes a difference by identifying the vascular and nervous changes on the level of cutaneous areas, along with the presence of the



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inflammatory infiltrate on the dermis and structural modifications of the sweat glands.

## VI. Conclusions

- The identification of vascular and nervous morphological structures in the complicated diabetic foot allows the extension of the knowledge related to the pathological background of this condition.
- With the progression of diabetes mellitus, the vascular lesions, which appeared on the microcirculation level are aggravating consequently involving arterioles and arteries of muscular type and are being accompanied by nervous lesions shown through morphological changes of the peripheral nerves and these changes were accompanied with lesions involving the epidermis, dermis, and muscles.

## VII. Recommendations

- The microvascular changes in relation to the severity and progress of the diabetic foot ulceration and the role played by the vascular mediators such as serotonin, 5-hydroxt tryptamine in diabetes require further studies and recordings using both light and electron microscopy.
- Angiographic studies of medium and small arteries to investigate occlusive changes in diabetic foot ulceration were recommended.
- Epidemiological studies to evaluate the influence of diabetes and its outcome on the quality of life particularly in patients with chronic ischemia.

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# Bacteriological Profile and Antibiotic Susceptibility Pattern of Blood Culture Isolates from Patients Visiting Tertiary Care Hospital in Kathmandu, Nepal

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*Keywords: blood stream infections, multi-drug resistant, salmonella paratyphi A, janamaitri hospital.*

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# Bacteriological Profile and Antibiotic Susceptibility Pattern of Blood Culture Isolates from Patients Visiting Tertiary Care Hospital in Kathmandu, Nepal

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*Keywords: blood stream infections, multi-drug resistant, salmonella paratyphi A, janamaitri hospital.*

## I. Introduction

Bacteremia is the presence of viable bacteria in the circulating blood. The detection of bacteria in blood is always abnormal. A minor injury occurring during tooth brushing, tooth extraction, abscesses, infected wound or boils, insertion of intravenous of bladder catheter, surgery and existing infections like lung infection, Urinary tract infection (UTI), gastrointestinal tract (GTI), burns or bedsores or from areas of localized disease as in pneumococcal pneumonia, meningitis, pyelonephritis, osteomyelitis, cholangitis, peritonitis, enterocolitis and puerperal sepsis are the sources of bacteremia and blood culture is required for the detection of it [1]. Bloodstream infection (BSI) is one of the most important causes of morbidity and mortality globally [2]. Detection of bacteremia by rapid and reliable method is by culturing blood. The blood should be collected aseptically before the administration of antibiotics [3]. Septicaemia is a clinical term used to describe severe life-threatening bacteremia in which multiplying bacteria release toxins into the blood stream and trigger the production of cytokines, causing fever, chills, toxicity, tissue anoxia, reduced blood pressure and collapse. Septic shock is usually a complication of septicemia with Gram-negative bacilli, and less frequently, Gram-positive organisms and prompt treatment is essential [4]. Continuous septicemia occurs primarily in patients with intravascular infections like endocarditis, septic thrombophlebitis, infections associated with intravascular catheter, septic shock whereas intermittent septicemia occurs in patients with localized infections like lung, urinary tract, soft tissues infections [5].

Bloodstream infections are potentially life-threatening and require rapid identification and antibiotic susceptibility testing of the causative pathogen. Both Gram positive and Gram negative bacteria causes bacteremia and septicemia. Gram negative septicemia, also known as endotoxic shock, which is more severe than Gram positive septicemia [6].

If the infection is caused by multidrug resistant (MDR) bacteria morbidity and mortality will increase which leads to great economic loss encompassing use of more expensive antibiotics to treat infection as well as threat of resistance to them. The infections caused by MDR organisms are more likely to prolong the hospital stay, increase the risk of death and require treatment with more expensive antibiotics [7].

In almost all cases, antimicrobial therapy is initiated empirically before the results of blood culture are available by keeping in mind that high mortality and

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morbidity are associated with septicemia and right choice of empiric therapy is of importance [6]. The increasing frequency of antimicrobial resistance among microbial pathogens causing nosocomial and community acquired infections is making numerous classes of antimicrobial agents less effective resulting in emergence of antimicrobial resistance [7, 22]. For the treatment, the isolation of bacterium from blood is valuable, but there is also urgent need of antimicrobial therapy, so sample is taken and treatment is started and after blood culture result, patient is treated as redirected by in vitro antibiotic sensitivity test [8, 9]. Therefore, we conducted this study to determine the common bacterial agents associated with bacteremia and their antimicrobial susceptibility patterns in febrile patients visiting in Janamaitri Hospital, Balaju, Kathmandu, Nepal.

## II. Materials and Methods

The study was conducted in Microbiology Laboratory of Janamaitri Hospital, Kathmandu, Nepal from March 2014 to April 2015. Written informed consents were obtained from patients prior to their inclusion in the study. A total of 838 blood samples were processed during the research period. Five ml blood sample was collected from each adult, 2-5ml from each child and 0.5-2ml from infant's aseptically using 70% alcohol and 2% tincture of iodine and inoculated immediately into 50ml Brain Heart Infusion (BHI) Broth with 0.025% of sodium polyanethol sulphonate as anticoagulant. In pediatrics cases, 1-2ml of blood was inoculated in 5-10ml of BHI broth. Negative result was followed by examining the broth daily for the sign of bacterial growth (turbidity, haemolysis, clot formation) and by doing final subculture at the end of seventh day. Bottles that showed sign of growth were further processed by Gram stain, followed by subculture on Blood agar, Mac Conkey agar, Manitol salt agar and examined after 18-24 hrs of incubation. Bacterial isolates were identified by colony morphology, Gram staining, catalase test, coagulase test, oxidase test, methyl red/voges-proskauer test (MR-VP), Triple sugar iron agar test, citrate utilization test, Urease test and Sulfur Indole Motility (SIM) test using standard procedure for bacterial identification [10].

*a) Antibiotic susceptibility test*

Antimicrobial susceptibility testing was performed by using Kirby Bauer disc diffusion method following guidelines of Clinical and Laboratory Standard Institute 2012 [11]. The inoculums used for susceptibility testing was prepared in nutrient broth by touching 5/6 colony and matched to 0.5 McFarland standard (1.5 X $10^8$ CFU/ml). Within 15 minutes, a sterile cotton swab was dipped into the inoculums suspension and pressed inside the wall of tube above the fluid level and inoculated at 60°C over the dried surface of Muller-Hilton agar (MHA) plate. After 3-5 minutes of inoculation, the antibiotic discs were applied and gently pressed down to ensure complete contact with agar. Organisms which showed resistance to at least one antibiotic among three or more antimicrobial categories were considered as multidrug resistant (MDR) bacteria [12, 13, 14].

*b) Quality control*

Reference strains *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were used as a control reference strains for identifications and drug susceptibility testing [14, 15].

*c) Data analysis*

The data obtained from the research were analyzed by using statistical tools in SPSS-21 version. The chi-square test was used for statistical analysis of data. A 'P' value less than 0.05 was considered as statistically significant.

## III. Results

A total of 838 clinical blood samples were collected from the patients attending Janamaitri Hospital, Kathmandu to study the prevalence of bacteraemia and septicemia from 9th August, 2014 to 8th November, 2015.

*a) Ward wise distribution of positive samples*

There were all together 838 blood samples, out of which, 61(7.28%) samples showed growth and rest 777 (92.72%) showed no growth. Again higher percentage of growth was obtained from OPD (Outpatient Department) followed by emergency and wards (Table 1).

*Table 1 :* Ward wise distribution of positive samples

| Types of patients | Growth | | Total samples | |
|---|---|---|---|---|
| | Number | % | Number | % |
| OPD | 41 | 67.21 | 476 | 56.80 |
| Emergency | 14 | 22.95 | 261 | 31.15 |
| Wards | 6 | 9.84 | 101 | 12.05 |
| Total | 61 | 100 | 838 | 100 |

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*b) Pattern of bacteria isolated from blood culture*

Out of 61 bacterial isolates, 48 (78.69%) were Gram negative and 13 (21.31%) were Gram positive bacteria. Among Gram negative bacterial isolates *Salmonella* Paratyphi A 26/48 (54.17%) was found to be most predominant among all bacterial isolates followed by *Salmonella* Typhi (33.33%), *Escherichia coli* (6.25%), *Salmonella* Paratyphi B (4.17%), *Morganella morganii* (2.08%). In Gram positive isolates *Staphylococcus aureus* 5/13(38.46%) was most predominant followed by coagulase-negative staphylococci (CNS) 4/13 (30.77%), *Streptococcus pyogenes* 2/13 (15.38%) and *Enterococci* 2/13 (15.38%) (Table2).

*Table 2 :* Pattern of bacteria isolated from blood culture gender wise

| Bacteria | Number | Percentage | Male | | Female | |
|---|---|---|---|---|---|---|
| | | | Number | % | Number | % |
| Gram negative | | | | | | |
| *Escherichia coli* | 3 | 6.25 | 1 | 2.08 | 2 | 4.17 |
| *Morgenella morganii* | 1 | 2.08 | 1 | 2.08 | 0 | 0 |
| *Salmonella Paratyphi A* | 26 | 54.17 | 18 | 37.50 | 8 | 16.67 |
| *Salmonella Paratyphi B* | 2 | 4.17 | 2 | 4.17 | 0 | 0 |
| *Salmonella Typhi* | 16 | 33.33 | 9 | 18.75 | 7 | 14.58 |
| ***Total*** | 48 | 78.69 | 31 | 64.58 | 17 | 35.42 |
| Gram positive | | | | | | |
| *Staphylococcus aureus* | 5 | 38.46 | 3 | 23.08 | 2 | 15.38 |
| *CNS* | 4 | 30.77 | 2 | 15.38 | 2 | 15.38 |
| *S. pyogenes* | 2 | 15.38 | 2 | 15.38 | 0 | 0 |
| *Enterococci* | 2 | 15.38 | 0 | 0 | 2 | 15.38 |
| ***Total*** | 13 | 21.31 | 7 | 54.85 | 6 | 46.15 |

*c) Age wise distribution of the isolates*

Out of 61 bacterial isolates, highest number of bacteria was isolated from age group 16-30 years 33(54.10%) followed by the age group 31-45 years 13(21.31%) and age group 76-90 years was found to be least affected 1(1.64%). Age group 16-30 years was most affected by wide range of bacteria (Table3).

*Table 3 :* Age wise and Ward wise distribution of the isolates

| *Bacteria* | Age group (year) | | | | | | OPD | | Emergency | | Wards | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1-15 | 16-30 | 31-45 | 46-60 | 61-75 | 76-90 | Number | (%) | Number | (%) | Number | (%) |
| ***Gram Negative Bacteria*** | | | | | | | | | | | | |
| *Escherichia coli* | 0 | 3 | 0 | 0 | 0 | 0 | 1 | 2.08 | 2 | 4.17 | 0 | 0 |
| *Morgenella morganii* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2.08 |
| *Salmonella Paratyphi A* | 0 | 16 | 6 | 3 | 1 | 0 | 19 | 39.58 | 6 | 12.50 | 1 | 2.08 |
| *Salmonella Paratyphi B* | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 2.08 | 1 | 2.08 | 0 | 0 |
| *Salmonella Typhi* | 2 | 7 | 4 | 2 | 0 | 1 | 12 | 25.00 | 3 | 6.25 | 1 | 2.08 |
| ***Total*** | 3 | 28 | 10 | 5 | 1 | 1 | 33 | 68.75 | 12 | 25.00 | 3 | 6.25 |
| ***Gram Positive Negative*** | | | | | | | | | | | | |
| *Staphylococcus aureus* | 1 | 2 | 1 | 0 | 1 | | 3 | 23.08 | 1 | 7.69 | 1 | 7.69 |
| *CNS* | 0 | 1 | 2 | 0 | 1 | 0 | 2 | 15.38 | 1 | 7.69 | 1 | 7.69 |
| *Streptococcus pyogenes* | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 7.69 | 0 | 0 | 1 | 7.69 |
| *Enterococci* | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 15.38 | 0 | 0 | 0 | 0 |
| ***Total*** | 2 | 5 | 3 | 1 | 2 | 0 | 8 | 61.54 | 2 | 15.38 | 3 | 23.08 |

*d) Seasonal variation on the prevalence of bacterial growth*

Out of 48 Gram negative organisms isolated, 20(41.67%) showed growth in summer season and followed by autumn season 20 (41.67%). Growth in winter was 4/48 (8.33) and in spring was 4/48 (8.33%). Out of 13 Gram positive bacterial isolates, 6/13(46.15%) showed growth in summer and followed by spring season 4/13(30.77%). Growth in winter was 2/13(15.38%) and in autumn was 1/13(7.69%). Sample showed highest growth in summer and autumn season (Table 4).



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*Table 4 :* Seasonal variation on the prevalence of bacterial growth

| | Season | | | |
|---|---|---|---|---|
| | Summer | Autumn | Winter | Spring |
| **Gram Negative Bacteria** | | | | |
| *Escherichia coli* | 1 | 0 | 1 | 1 |
| *Morgenella morganii* | 0 | 0 | 0 | 1 |
| *Salmonella Paratyphi A* | 9 | 14 | 2 | 1 |
| *Salmonella Paratyphi B* | 0 | 1 | 1 | 0 |
| *Salmonella Typhi* | 10 | 5 | 0 | 1 |
| ***Total*** | 20 (41.67%) | 20(41.67%) | 4 (8.33%) | 4 (8.33%) |
| **Gram Positive Bacteria** | | | | |
| *Staphylococcus aureus* | 3 | 1 | 0 | 1 |
| *CNS* | 1 | 0 | 1 | 2 |
| *S. pyogenes* | 1 | 0 | 0 | 1 |
| *Enterococci* | 1 | 0 | 1 | 0 |
| ***Total*** | 6 (46.15%) | 1 (7.69%) | 2 (15.38%) | 4 (30.77) |

e) *Antibiotic resistance pattern of Gram negative bacteria*

Out of 838 blood samples, 48 different Gram negative bacteria were isolated. Among the isolates, *Salmonella* Paratyphi A was most predominant followed by *Salmonella* Typhi. Five different bacteria were isolated during study period. Antibiotic susceptibility was performed on them in which imipenem was found most effective followed by amikacin (Table 5).

*Table 5 :* Antibiotic susceptibility pattern of Gram-negative isolates

| Gram Negative Bacteria | Antibiotic susceptibility pattern (%) / number | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AMX | COT | CN | OF | CIP | C | CFM | NA | CTX | GEN | AK | IPM |
| *Escherichia coli (3)* | (33.3) 1 | (66.67) 2 | (33.33) 1 | (66.66) 2 | (33.33) 1 | (33.3) 1 | (33.3) 1 | - | (100) 3 | (100) 3 | (100) 3 | (100) 3 |
| *Morgenella morganii (1)* | - | - | - | - | - | 100 1 | 100 1 | - | 100 1 | 100 1 | 100 1 | (100) 1 |
| *Salmonella Paratyphi A (26)* | (96.15) 25 | (50) 13 | (88.46) 23 | (73.08) 19 | (73.08) 19 | (84.62) 22 | (100) 26 | - | (100) 26 | (96.15) 25 | (96.15) 25 | (100) 26 |
| *Salmonella Paratyphi B (2)* | (100) 2 | (100) 2 | (100) 2 | (100) 2 | (100) 2 | (100) 2 | (100) 2 | - | (100) 2 | (100) 2 | (100) 2 | (100) 2 |
| *Salmonella Typhi (16)* | (87.50) 14 | (62.50) 10 | (81.25) 13 | (75.00) 12 | (75.00) 12 | (75.00) 12 | (81.25) 13 | - | (93.75) 15 | (87.50) 14 | (68.75) 11 | (100) 16 |

AMX=Amoxycillin, COT=Cotrimoxazole, CN=Cefalexin, OF=Ofloxacin, CIP=Ciprofloxacin, C=Chloramphenicol, CFM=Cefixime, NA=Nalidixic acid, CTX=Cefotaxime, GEN=Gentamicin, AK=Amikacin, IPM=Imipenem

f) *Antibiotic resistance pattern of Gram positive bacteria*

Out of 838 blood samples, only 13 samples showed growth of Gram positive bacteria. Isolated bacteria were subjected to antibiotic susceptibility test (Table 6).

*Table 6 :* Antibiotic susceptibility pattern of Gram-positive isolates

| Gram positive bacteria | Antibiotic susceptibility pattern (%) / number | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | P | AMP | C | CIP | GEN | MET | E | VAN | PIP | IPM | AK | CLO |
| *Staphylococcus aureus (5)* | 0 | (20) 1 | (40) 2 | (40) 2 | 0 | (60) 3 | (60) 3 | (100) 5 | (100) 5 | (100) 5 | (80) 4 | (20) 1 |
| *CNS (4)* | 0 | (50) 2 | (25) 1 | (50) 2 | (75) 3 | (50) 2 | (75) 3 | (100) 4 | 0 | (50) 2 | (75) 3 | (50) 2 |
| *Streptococcus pyogenes (2)* | 0 | (50) 1 | (50) 1 | 0 | (50) 1 | (100) 2 | (100) 2 | (100) 2 | (50) 1 | (100) 2 | (100) 2 | (50) 1 |
| *Enterococci (2)* | 0 | 0 | (50) 1 | 0 | (50) 1 | (100) 2 | (100) 2 | (100) 2 | 0 | (100) 2 | (100) 2 | 0 |

P= Penicillin, AMP= Ampicillin, C=Chloramphenicol, CIP= Ciprofloxacin, GEN= Gentamicin, MET=Methicillin, E=Erythromycin, VAN=Vancomycin, PIP=Piperacillin, IPM=Imipenem, AK=Amikacin, CLO=Cloxacillin



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*g) Multi drug resistance of bacterial isolates*

Out of 48 Gram neative isolates 15 strains (31.25%) were resistant to ≥3 antibiotics and were considered as multidrug resistant. It was found that 16 (33.33%) isolates were resistant to 1 antibiotic and 17(35.42%) isolates were resistant to 2 antibiotics. Among the MDR strains, 43.75% (7 out of 16) of *Salmonella* Typhi were found to be MDR. Similarly 15.38% (4 out of 26) of *Salmonella* Paratyphi A were found to be MDR. All the isolates (100%) of *Escherichia coli, Morganella morganii*, and *Staphylococcus aureus* were found to be MDR (Table 7).

*Table 7 :* MDR pattern of the bloodstream isolates

| Organisms | Number | Number of bacteria resistance to | | | MDR | |
|---|---|---|---|---|---|---|
| Gram Negative Bacteria | | 1 antibiotic | 2 antibiotic | ≥3 antibiotics | Number | % |
| *Escherichia coli* | 3 | 0 | 0 | 3 | 3 | 100 |
| *Morgenella morganii* | 1 | 0 | 0 | 1 | 1 | 100 |
| *Salmonella Paratyphi A* | 26 | 9 | 13 | 4 | 4 | 15.38 |
| *Salmonella Paratyphi B* | 2 | 2 | 0 | 0 | 0 | 0 |
| *Salmonella Typhi* | 16 | 5 | 4 | 7 | 7 | 43.75 |
| Gram positive | | | | | | |
| *Staphylococcus aureus* | 5 | 0 | 0 | 5 | 5 | 100 |
| *CNS* | 4 | 0 | 0 | 4 | 4 | 100 |
| *Streptococcus pyogenes* | 2 | 0 | 0 | 2 | 2 | 100 |
| *Enterococci* | 2 | 0 | 1 | 1 | 2 | 50.00 |

## IV. Discussion

Blood culture is a well-established procedure of the standard diagnostic workup for many infectious diseases. In the countries like Bangladesh, where all kinds of drugs including the antibiotics, are sold over the counter, misuse of antibiotics has been found to be responsible for developing pool of resistant bacteria as well as negative results of blood culture [16]. Blood culture is employed for the detection of bacteremia and septicemia in blood. Blood stream infection is one of the main agent causing morbidity and mortality worldwide. Urgent and effective treatment is required to manage blood infections [17].

In the countries like Nepal, the overuse of antibiotics, random use of antibiotics as hit and trial method by clinicians without proper sensitivity test, unawareness of people about emergence of antibiotics resistance, random use of antibiotics without advice of physicians, prolonged intensive care unit (ICU) stay, nursing home residency, severe illness, use of instrumentation or catheterization etc. are the major causes of drug resistance in our country. The less growth percentage may due to previous exposure of patients to used antibiotics that hindered their growth or dominance of organism's growth [18].

In the present study the isolation rate was (61/838) 7.28% which was comparable to those study conducted by Karki et al where 4.2% were culture positive [19]. Gram negative bacteria were common organisms isolated during this study accounting 48/61 (78.69%). Among Gram negative isolates the most common was *Salmonella* Paratyphi A 26/48 (54.17%) followed by *Salmonella* Typhi 16/48 (33.33%). Similar finding was seen from the studies in Kathmandu Model hospital Nepal, where 71% of total isolates from blood were *Salmonella* Typhi and 16% of the total isolates were *Salmonella* Paratyphi A [20]. In this study the isolation rate was highest in age group between 16-30 years 33/61 (54.10%) followed by age group 31-45 years 13/61 (21.31%). Similar study conducted in showed that the isolation rate was highest in age group between 21-40 (28%) followed by 41-60 (24%) [21]. In this study, among Gram positive bacteria the most common isolated bacteria was *Staphylococcus aureus* 5/13 (38.46%) followed by CNS 4/13 (30.77%).

The most effective antibiotic in Gram negative bacteria was imipenem followed by amikacin. The others effective drugs were cefotaxime and gentamycin. Similar study conducted in Nepal in KIST Medical College by Surya et al showed that imipenem and amikacin followed by gentamycin were most effective antibiotics for the treatment of *Escherichia coli* and *Klebsiella pneumoniae* [22]. Those organisms which showed resistance to at least one agent in three or more antimicrobial categories were considered as multidrug resistant (MDR) bacteria [23].

In the present study among Gram negative bacteria, 100% of *E. coli* and *Morgenella morganii* isolates were MDR, followed by *Salmonella* Typhi (43.75%) and *Salmonella* Paratyphi A (15.38%). None of the isolates of *Salmonella* Paratyphi B was found to be MDR. Similar study conducted in Nepal showed that 96.10% of the *E. coli* isolates were MDR whereas 44.8% of the *Salmonella* isolates were MDR in Ghana [22, 24].

In this study, among Gram positive bacteria, *S. aureus*, CNS and *Streptococcus pyogenes* were found 100% MDR whereas 50% of *Enterococci* isolates were MDR. Similar study conducted in Ethiopia showed that 100% of the *S. aureus* and CNS were MDR whereas none of the *S. pyogenes* isolates were MDR [2]. In the present study vancomycin is the antibiotic of choice for the treatment of Gram positive bacterial isolates followed by imipenem and amikacin. Higher rate of infection by *Salmonella* species may be due to



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unhygienic practices, contaminated food and drinking water. The prevalence of typhoid was higher in summer and autumn season which may be due to contaminated water and food [25, 26].

In this study, Imipenem and Amikacin were found most effective for Gram negative isolates whereas vancomycin was found most effective for Gram positive bacteria followed by imipenem and amikacin. These three antibiotics should not be used indiscriminately and kept as reserve drug because if resistance is developed then treatment will be complicated.

## V. Conclusion

*Salmonella* Paratyphi A and *Salmonella* Typhi was the major Gram negative organisms causing blood stream infections whereas *Staphylococcus aureus* and CNS was the major Gram positive organisms. The antibiotic resistance pattern varies according to geographical location, country to country and even institute to institute in the same country and continuously changes over time so determination of antibiotic sensitivity pattern in periodic intervals is mandatory for choosing appropriate antibiotics for the treatment. The antibiotic susceptibility pattern of this study suggested that vancomycin is the drug of choice for Gram positive bacteria while imipenem and amikacin for Gram negative bacteria but the later drugs showed wide coverage for Gram positive organisms too.

## VI. Acknowledgements

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# A Preliminary Survey of Norovirus and Astrovirus Antigen in Diarrheic Stools of Children in Borno State, Nigeria

By Oyinloye, S.O., Jatau, E.D., Aminu, M. & Ella, E.E.

*University of Maiduguri, Nigeria*

*Abstract-* *Background:* Noroviruses and astroviruses are important agents of acute gastroenteritis among children. Acute gastroenteritis (AGE) is a major cause of morbidity and mortality in pediatric populations world-wide. Globally, an estimated 800 000 infants and young children die from diarrhea every year.

*Aims:* This was a cross-sectional study that included acute diarrheic children presenting in Specialist Hospital, Nursing Home Health Centre and Ngamdu pediatric clinic in Borno state with the aim of detecting norovirus and astrovirus antigen. Two hundred children whose parent/ guardian consented were enrolled in the study.

*Methodology:* Two hundred acute diarrheic and forty one nondiarrheic fecal samples were collected from children aged 5 or less between June 2013 – May 2014. Samples were screened for norovirus and astrovirus antigen using 3rd generation RIDASCREEN ELISA test kit. Demographic data of the children were obtained.

*Results:* All non-diarrheic stools were negative for both antigens whileof the two hundred diarrheic stools screened, a prevalence of 8% and 5% were obtained for norovirus and astrovirus respectively. The proportion of males (6/130) positive for norovirus antigen relative to female (10/70) was found to be significant ($p < 0.016199$). No significant difference was observed between male and female positive for Astrovirus ($p = 0.307574$). A significant prevalence of norovirus ($p = 0.00001$) and astrovirus ($p = 0.013321$) based on age group were obtained. Clinical sign and symptoms of infection with norovirus and Astrovirus between male and female showed no significant difference ($p=0.42018$).

*Keywords:* *norovirus, astrovirus, diarrhea, borno state, nigeria.*

*GJMR-C Classification :* *NLMC Code: QW 51*




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*Conclusion:* Norovirus and Astrovirus are a significant aetiology of diarrhea in the study area. Measures to mitigate their sequelae are required to circumvent potential public health crises in future.

*Keywords: norovirus, astrovirus, diarrhea, borno state, nigeria.*

## I. Introduction

Viral intestinal infections are the most common cause of acute infectious diarrhea in the pediatric group and accounted for approximately 70% of episodes of acute infectious diarrhea in children (1). Rotavirus, norovirus, adenovirus, and astrovirus are the recognized viral causes of pediatric gastroenteritis (2) and the World Health Organization (WHO) data showed that each child practically has viral diarrhea irrespective of race and socioeconomic status within the first 5 years of life and this has great economic burden for the system of public health services and all society (3)

Epidemiological studies on norovirus gastroenteritis have been conducted in countries such as the United States of America (4, 5), Finland (6), Australia (7), Italy (8), some developing countries such as Brasil (9), Iraq (10). In sub-Saharan Africa, molecular epidemiology studies of norovirus have also been performed in countries such as Malawi (41% prevalence), Ghana (16.4% prevalence), South Africa, Botswana, Cameroon and Burkina Faso (12% prevalence) (11-16). In a study in Lagos Nigeria, norovirus prevalence of 37.3% was obtained among children with acute gastroenteritis (17) while in Ife, Nigeria, norovirus single infections were found in 64.3% (9/14) of the norovirus positive diarrhoea samples (18). Also in Owo, Ondo state Nigeria, norovirus was found in 4/50 (8%) of the diarrheic children examined (19)

The first astrovirus infecting humans was described in 1975 (20). Since then, a total of 8 serotypes closely related to this original astrovirus ("classic human astroviruses" (HAstVs)) have been identified, all of which are believed to cause diarrhea. The prevalence of HAstV infection has been reported to be 2%–16% among children hospitalized with diarrhea and 5%–17% in community studies that used either Enzyme Immunosorbent Assay (EIA) or Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) analysis (21-23). Seroprevalence studies indicate that most children are infected during the first 2 years of life (21, 24, 25). Previous studies in Nigeria show different prevalence of astrovirus. In a study in northwest Nigeria, 5% astrovirus positivity was reported (26) while others (17) and (27) reported 16% prevalence in Lagos and Nasarawa states respectively.

Diarrhea of viral aetiology is under reported in north east region of Nigeria. The aim of this study was to conduct a preliminary survey of norovirus and astrovirus antigen in acute diarrheic stool due to increasing epidemiological significance of viral gastroenteritis in north eastern region of Nigeria and dearth of published data on the existence or otherwise of diarrhea of norovirus and Astrovirus aetiology. It is hoped that the

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information generated in this study will serve as baseline data to inform the relevant authority in Nigeria of the health burden posed by these viruses.

## II. Materials and Methods

*a) Study Population*

Samples were collected at random from children aged below 5years presenting with acute diarrhea at the In and Out Patient Departments and the Pediatric Wards of Specialist Hospital, Nursing Home Health Centre and Ngamdu pediatric clinic in Borno State, Nigeria.

*b) Exclusion Criteria*

Children above age of 5 years and those below 5 years, whose parents/guardians declined consent, were excluded from the study.

*c) Inclusion Criteria*

Diarrheic children aged below 5 years whose parents/guardians consented to participate in the research were included in the study. Diarrhea was defined as passage of three or more watery stool within the last 24-hour period.

*d) Study Design*

In this research, a cross sectional design was employed in order to allow for stool sample collection from every other child presenting at any of the selected hospital in the study area.

*e) Ethical Approval*

Ethical approval was sought and obtained from the Ethical Committees of the respective hospitals involved in the study.

*f) Analyses of stool sample*

Stool samples collected were assayed to detect norovirus, and astrovirus antigen using RIDASCREEN® ELISA Test Kit.

*g) Detection of Norovirus, and Astrovirus by Enzyme Linked Immunosorbent Assay (ELISA)*

i. *Sample preparation*

Each stool sample was prepared for analysis according to manufacturer's instruction:

One milliliter (1ml) RIDASCREEN® sample was placed in dilution buffer in a labelled test tube. Liquid stool was sucked up into a disposable pipette until it rose to just above the second mark (approx. 100 µl) and was suspended in the buffer which was placed in the tube beforehand. The stool suspension was homogenised either by suction and ejection from a disposable pipette or, alternatively, by mixing in a vortex mixer. The specimen was centrifuged at 5000 rpm (approx. 2300 – 2500 G) for 5 minutes and the resulting supernatant of the stool suspension was used.

ii. *ELISA Procedure*

One hundred microliter (100µl) of positive control, the negative control (specimen-dilution buffer diluent) and the stool supernatant were dispensed in the wells. One hundred microliter (100µl) of the biotin-conjugated antibody was added to the wells and incubated at room temperature (20 – 25 °C) for 60 minutes after mixing thoroughly (by lightly tapping on the edge of the plate), after this, the plates were washed 5 times using 300µl wash buffer each time using an automated machine. (The wells were emptied completely by knocking them out after each wash on a part of the absorbent paper which is dry and unused). One hundred microliter (100µl) of the streptavidin-peroxidase conjugate was added to the wells and incubated at room temperature (20 – 25 °C) for 30 minutes and washed as described above. One hundred microliter (100µl) of substrate was added to each well. Then the plate was incubated at room temperature (20°C - 25°C) for 15 minutes in the dark. The reaction was stopped by adding 50µl of stop reagent to each well. After mixing carefully (by lightly tapping the side of the plate) the extinction was measured at 450nm using a reference wavelength ≥ 600 nm (optional).

*h) Evaluation and interpretation*

*Calculating the cut-off*

In order to establish the cut-off, 0.15 extinction units are added to the measured extinction for the negative control.

Cut-off = Extinction for the negative control + 0.15

*Test result*

Samples are considered **positive** if their extinction is more than 10 % above the calculated cut-off.

Samples are considered **equivocal** and must be repeated if their extinction is within ± 10 % of the cut-off. If repeating the test with a fresh stool sample again yields a value in the grey range, the sample must be considered negative.

Samples with extinctions more than 10 % below the calculated cut-off must be considered **negative**.

## III. Results

Of the two hundred diarrheic stool screened, a prevalence of 8% (16/200) and 5% (10/200) were obtained for norovirus and astrovirus respectively (Table 1). The proportion of males (6/130; 4.62%) positive for norovirus antigen relative to female (10/70; 14.29%) was found to be significant ($P < 0.016199$; Table 1) but not so for Astrovirus ($p = 0.307574$; Table 1). A significant prevalence of norovirus ($P = 0.00001$) and astrovirus ($P = 0.013321$) based on age group were obtained in this study (Table 1). Clinical sign and symptoms of infection with norovirus and Astrovirus between male and female showed no significant difference ($P = 0.42018$; Table 2).



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*Table 1 :* Norovirus and Astrovirus distribution among children in Borno State according to Age and Sex

| Variables | No. of Sample | Number Positive | | p-value | |
|---|---|---|---|---|---|
| Age group (month) | | Norovirus (%) | Astrovirus (%) | Norovirus | Astrovirus |
| 1-6 | 13 | 0(0) | 1(7.69) | | |
| 7-12 | 44 | 3(6.80) | 1(2.27) | | |
| 13-24 | 49 | 6(12.20) | 5(10.20) | 0.00001 | 0.013321 |
| 25-36 | 35 | 4(11.40) | 2(5.71) | | |
| 37-48 | 36 | 2(5.60) | 1(2.78) | | |
| 49-60 | 23 | 1(4.30) | 0(0.00) | | |
| **Total** | **200** | **16(8.0)** | **10(5.0)** | | |
| | | | | | |
| **Sex** | | | | | |
| Male | 130 | 6(4.62) | 5(3.85) | 0.016199 | 0.307574 |
| Female | 70 | 10(14.29) | 5(7.14) | | |
| **Total** | **200** | **16(8.0)** | **10(5.0)** | | |
| | | | | | |

*Table 2 :* Norovirus and Astrovirus clinical sign/symptom in relation to sex

| Sign and Symptom | No. of Sample | Norovirus positive | | Astrovirus positive | | p-value of M / F |
|---|---|---|---|---|---|---|
| | | Male (M) | Female (F) | Male | Female | |
| Fever (F) only | 67 | 1 | 2 | 1 | 1 | 0.42018 |
| Vomiting (V) only | 23 | 2 | 2 | 1 | 0 | |
| Fever and Vomiting | 39 | 1 | 4 | 3 | 3 | |
| Abdominal Cramp | 31 | 2 | 2 | 0 | 1 | |
| Mucoid/bloody stool | 40 | 0 | 0 | 0 | 0 | |

## IV. Discussion

The results from the present study suggest that norovirus and astrovirus contribute significantly to the disease burden of childhood diarrhea in Borno state, Nigeria.

The norovirus prevalence of 8% (Table 1) is similar to 8% obtained in Owo, Nigeria (19) but lower than the prevalence (21%) found for children in the United States of America (28), and that reported in a pooled analysis of studies conducted in seven developing countries (12.1%), spanning from Malawi to Thailand to Peru (29). Also, the figure in the present study was lower than 37.5% found for children in Nigeria (17). The prevalence of astrovirus in Borno state of Nigeria found in this study was 5% (Table 1). It is within the prevalence range of 2%–16% of human astrovirus (HAstV) infection reported among children hospitalized with diarrhea and 5%–17% in community studies that used either EIA or RT-PCR analysis (21-23). The prevalence of astrovirus in this study was observed to be similar to 5% prevalence in northwest Nigeria (26); and 4.9% prevalence reported in Mexico, it was lower than 10.8% reported in the United States, and 16% prevalence in Nasarawa Nigeria (27).

These disparities in norovirus and astrovirus prevalence across different studies may have been caused by different reasons. One reason may be due to the period samples were collected relative to the duration of diarrhea. Norovirus and astrovirus shedding generally peak within the first week of illness but can last for nearly two months (30). This reflects how the duration of illness can affect the outcome of each study because samples collected after the peak period of viral shedding will, as expected, present a possible outright negative or false negative result thereby impacting on the prevalence to be reported. In this study, the duration of illness of patients was not recorded implying that some samples might have been collected perhaps after peak period of infection. This limitation is similar to a report in 2006 which neglected to list limits in the duration of illness (31). Another factor contributing to the disparities among results was the variance in age-based inclusion criteria among the various study populations. In the present study, children had to be less than five years of age, which is the age range most affected by diarrhea.

Sex stratification of the children sampled revealed that the proportion of males (6/130) positive for norovirus antigen relative to female (10/70) was found to be significant ($P < 0.016199$; Table 1). However, the prevalence of male (6/130: 4.62%) positive for norovirus antigen was found to be lower than that of female (10/70: 14.29%). This is contrary to previous study which had reported a greater male susceptibility rate. The greater susceptibility of male to norovirus infection had been attributed to genetic and immunological factors (32). Susceptibility to infection with human Astrovirus showed no association to gender ($P = 0.307574$; Table 1). Overall, the occurrences of infection between the sexes indicate that either male or female could be



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infected. This information, if and when corroborated by other studies, can serve to guide possible vaccination policy in future to target children not more 5 years old.

In the present study, the age-based prevalence of norovirus in Borno state was found to be significant (P=0.0001; Table 1) affecting a greater proportion (9/16) of children less than or equal to age of 2 years. This finding is similar to that of other studies (28,33). Interestingly, 3/9 of these children were of age 7-12 month (Table 1). Possible reason for this observation is behavioural. Since the virus transmission is through fecal-oral route, fecal-contaminated items picked from the ground into the mouth by crawling children could serve as potential mechanical vector. For astrovirus, age-based prevalence was also significant (P=0. 0.013321; Table 1). We also observed in this study that the number of children less than or equal to age of 2 years positive for Astrovirus antigen were more (7/10) than children older than age 2 (3/10) (Table 1). Above reasons are applicable. However, from Table 1, for both norovirus and Astrovirus, the number of infected individual declined from age above 2 years. Boosted immunity and reduced tendency to consume soiled edibles might be attributable. This assertion has yet to be proven scientifically, though.

The most recurring clinical symptom observed among both sexes for norovirus and astrovirus (Table 2) respectively was fever with vomiting (5/39 and 6/39 respectively) though it was not significant between male and female (P=0.42018). Since vomiting (with or without fever) featured prominently as a clinical symptom of infection with both viruses, it implies that the sequelae will be dehydration. Although this study did not determine the association of norovirus and astrovirus diarrhea with dehydration, a previous study in Zaria, Nigeria, reported a rotavirus prevalence of 21% among those not dehydrated and 78.4% among those that were dehydrated (34). Norovirus and astrovirus are also implicated in diarrhea cases; therefore one could infer that the sequelae of infection among the children in this study would also be dehydration arising from vomiting. Hence, the ensuing dehydration warrants public enlightenment/education on the use of oral rehydration solution in order to reduce number of deaths due to possible severe dehydration.

## V. Conclusion

This study has established the presence of norovirus and astrovirus in diarrheic stools of children in the Borno state. The prevalences obtained in this study reveal that, though it is under-reported, it could be a debilitating childhood disease due to dehydration. And since definitive diagnosis/examination is hardly done to ascertain causes of death in this part of the developing world, deaths which have been reported to be due to diarrhea related causes in neonates and children might have been caused by either or both of these enteric pathogens. Due to limited funds, we could not undertake immediate molecular characterization of the positive samples.

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# Prevalence, Isolation of Bacteria and Risk Factors of Mastitis of Dairy Cattle in Selected Zones of Oromia Regional States, Ethiopia

By Dinaol Belina, Yimer Muktar, Adem Hiko, Nateneal Tamerat, Tadesse Kebede, Tarekegn Wondimu & Jelalu Kemal

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*Abstract-* A cross sectional study was conducted on a total of 471 cross and pure borana breed dairy cattle to determine prevalence of clinical and subclinical mastitis using CMT in selected districts of North Showa and Borana zones of pastoral area from April 2012 to February 2014. The overall mastitis prevalence was 237(50.3%). The cow level prevalence was 9.5% clinical and 40.7% were subclinical cases. Of 1884 quarters examined 10(0.5%) quarters were blind teats and quarters 550(50.2%) were showed mastitis. High score CMT positive milk sample were investigated using standard microbiological techniques. Identification of bacterial isolates revealed that 10 types of bacterial isolates were identified. The isolated bacteria were Staphylococcus aureus and CNS 20 (37.7%), *Diplococcus* spp 2 (3.8%), *Corynebacterium pseudotuberclosis* 3(5.8%), *Corynebacterium bovis* 1(1.7%), *Micrococcus* spp 1(1.9%), *Pseudomonas* spp 1(1.9%), *Bacillus* spp 1(1.9%), *E. coli* 3(5.7%) *Proteus* spp 1(1.9%). Different risk factors like parity number, farming hygiene, animal origin and husbandry type were considered. Hygienic conditions and husbandry type were the most important potential risk for mastitis.

*Keywords:* *CMT, bacterial culture, mastitis, prevalence, bovine, borana, north showa.*

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# Prevalence, Isolation of Bacteria and Risk Factors of Mastitis of Dairy Cattle in Selected Zones of Oromia Regional States, Ethiopia

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*Abstract-* A cross sectional study was conducted on a total of 471 cross and pure borana breed dairy cattle to determine prevalence of clinical and subclinical mastitis using CMT in selected districts of North Showa and Borana zones of pastoral area from April 2012 to February 2014. The overall mastitis prevalence was 237(50.3%). The cow level prevalence was 9.5% clinical and 40.7% were subclinical cases. Of 1884 quarters examined 10(0.5%) quarters were blind teats and quarters 550(50.2%) were showed mastitis. High score CMT positive milk sample were investigated using standard microbiological techniques. Identification of bacterial isolates revealed that 10 types of bacterial isolates were identified. The isolated bacteria were *Staphylococcus aureus* and CNS 20 (37.7%), *Diplococcus* spp 2 (3.8%), *Corynebacterium pseudotuberclosis* 3(5.8%), *Corynebacterium bovis* 1(1.7%), *Micrococcus* spp 1(1.9%), *Pseudomonas* spp 1(1.9%), *Bacillus* spp 1(1.9%), *E. coli* 3(5.7%) *Proteus* spp 1(1.9%). Different risk factors like parity number, farming hygiene, animal origin and husbandry type were considered. Hygienic conditions and husbandry type were the most important potential risk for mastitis. Statistically, cattle from North Showa were highly infected than those from Borana zone ($p < 0.05$). Farmers and herd managers should give great attention for hygiene condition and husbandry type and further investigation should be conducted especially in the pastoral area.

*Keywords: CMT, bacterial culture, mastitis, prevalence, bovine, borana, north showa.*

## I. Introduction

Ethiopia holds large potential for dairy development due to its large livestock population and the favorable climate for improved and high yielding breeds. Ethiopia has the largest livestock population among African countries (CSA, 2008).However, compared to other countries in Africa, Ethiopians consume less dairy products. Moreover, the quality and quantity of milk in the country deteriorates because of various causes. Given the considerable potential for generation of income and employment, the development of small holder dairy sector in Ethiopia, has a promising future and can contributes significantly to poverty alleviation improved nutrition in the country. According to Ahmed *et al.* (2007), milk production during the 1990s expanded at an annual rate of 3.0% compared to 1.63- 1.66% during the preceding three decades, with the expected growth in income, increased urbanization and improved policy environment (Kelay, 2002).The central highlands, mainly Selalle, are the major dairying areas, and as a result they are the main sources of milk for Addis Ababa, Ethiopia's capital and main urban population center, where 8% of its inhabitants live. Furthermore, farmers in Selalle are conscious of the milk market and produce milk for commercial sale, unlike the majority of Ethiopian farmers,who produce milk for home use. In Selalle farmers keep high-yield cross bred (zebu * Holstein) and Holstein dairy cattle mainly for milk production alongside native zebu breeds (Ameni *et al.,* 2007). Study by Kasim *et al.* (2012) indicated pure Borana breed are more productive than other local breeds in Ethiopia. However, milk production often does not satisfy the country's requirements due to a multitude of factors among them udder infection is the one (Erskine, 2001).

Mastitis is an inflammation of mammary gland, primary resulting from invasion of the mammary gland by pathogenic microorganisms through the teat canal resulting in physical, chemical, pathological and bacteriological changes in glandular tissues and milk. Many risk factors have been identified for clinical and subclinical mastitis in dairy animals such as breed, increased milk production, hygienic conditions, milking practices, age, parity, stage of lactation (Fox *et al*., 1995; Barnouin, and Chassagne, 2001).

The most common pathogen comprises contagious bacteria mainly *Staphylococcus aureus* and *Streptococcus agalactae* and environmental bacteria mainly coli forms and some species of streptococci that are commonly present in the environment (Radostitis *et al.,* 2007).Besides, mastitis may render milk unsuitable for human consumption or provide a mechanism for the spread of diseases like Tuberculosis, Streptococcal intoxication, Colibacillosis, streptococcal sore throat and Brucellosis to human

Bovine mastitis was reported as one of the most prevalent dairy health problems in Ethiopia including north Showa and Borana zones (Argaw and Tolosa,

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2008). Yet, the information on the prevalence of sub clinical mastitis in the areas is lacking and what available is fragments of information from cases of clinical mastitis that has been presented to veterinary clinic for the treatment. Therefore, the objectives of this investigation were: to determine the prevalence and major risk factors associated with clinical and sub clinical mastitis at herd, cow and quarter level in small holder and pastoral area dairy cattle.

## II. Materials and Methods

*a) Study area*

This study was carried out in North Shoa Zone of the Oromia Regional state in central Ethiopia and Borana pastoral and agro pastoral zone from April 2012 to February 2014. NorthShoa Zone is located in central Ethiopia 126 km north west of Addis Ababa in Oromia Regional state. It covers 1,174,500 hectare of land from which 40% is crop land, 25% is grazing land, 13% is forest and bush area, 7% is construction area and 15% is unproductive land. It's minimum and maximum temperatures vary from 11.5-29°C and 17.5-35°C, respectively. It gets bimodal rainfall that ranges from 651-1115mmi.e. from February-May (short rainy season) and from June- October (long rainy season) (North Shoa Agricultural Department, 2013).In particular Girarjarso, Debrelibanos and Wachale districts of North Showa were our study area.42% of the area is highland that is suitable for crop cultivation and livestock husbandry and the herd structure is characterized by a higher number of cows. Sample collection areas will be in 10 km radius of the milk collection units along the main road in both sides.

The study also conducted in Miyo District of Borana zone. The district is located at 717 Kms south of Addis Ababa. This district is characterized by pastoral and agro-pastoral production systems. The livestock species reared within the district are cattle (61,023), goat (72,224), sheep (14,567), camel (15,672), equines (12,613) and poultry (11,236). The rainfall pattern is bimodal in nature with average annual rainfall around 700mm. The average annual temperature is 22 °C. The altitude of the district ranges from 1300-1520 m.a.s.l (CSA, 2008).

*b) Study Population*

The study were conducted on a total of 144 heads of cross (Zebu and HF) breeds(of lactating cows kept under small holder dairy herds kept under extensive, semi- intensive and intensive husbandry practice in Salale of North Showa, and on327 pure Borana breed characterized by both meat and milk production merits.

*c) Study design*

A cross sectional study was conducted on small scale holder dairy farms in North Showa, and pastoral and agro pastoral area in Borana of Southern Ethiopia. Data on each cow was collected in a format designed for this purpose. The data sheet mainly focused to address associated risk factors with the occurrence of bovine mastitis. Risk factors considered were cow history, housing system, milking practice, hygiene, parity number, stage of lactation, husbandry type and other management practices in the study area.

*d) Sample Size determination*

The sample size was calculated according to the formula given by Thrusfield (2007). In Salale of North Showa it is calculated by taking (89.54%) prevalence from previous report by Argaw and Tolosa (2008).In Miyo of Borana considering the previous prevalence 59.3% in and around Yabello district (Kasim *et al.,* 2012), a total of 374 lactating dairy cows were proposed to be sampled but, attributable to the2010/11 drought shock of the zone only 327 heads of cows were included.

*e) Sampling Procedure*

To include cows from small scale holder dairy farms in selected districts of North Showarandom sampling method was employed to select the individual dairy cow. Selection was done by judgment mainly following accessibility to the main road in 10 km radius which is supplying their milk to eight primary farmer milk cooperatives in the three study districts. In Miyo of Borana purposive sampling was used to test lactating dairy cows available within the district due to the fact that the number of cows within the district are limited as a result 2010/11 drought shock in the zone.

*f) Study Methodology*

i. *Clinical Inspection of the Udder*

The clinical inspection of the udder was done in the following way. The udder was first examined visually and then by palpation to detect fibrosis, inflammatory swellings, visible injury, atrophy of the tissue, and swelling of supra mammary lymph nodes. The size and consistency of mammary quarters were inspected for the presence of any abnormalities, such as disproportional symmetry, swelling, firmness, and blindness. Information relating to the previous health history of the mammary quarters and causes of blindness was obtained from interviews with owners. Viscosity and appearance of milk secretion from each quarter were examined for the presence of clots, flakes, blood, and watery secretions (Quinn *et al.,* 2002).

*g) Laboratory test*

i. *California mastitis test (CMT)*

The California mastitis reagent was used to screen cows with subclinical mastitis when milk sample is collected according to the procedures of National Mastitis Council (NMC, 1999). Milk sampled according to National Mastitis Council, (NMC, 1999) was subjected to California mastitis reagent to screen subclinical

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mastitis described by (Quinn *et al.,* 2002).This test is based on increased number of leucocytes and increased alkalinity in milk due to mastitis (as an indirect measurement of leucocytes). 0.5ml of milk from each quarter is taken in plastic peddle cups and added to equal quantity of CMT reagent solution and mix well by circular movement of peddles mixed on a horizontal plane. The result of the test indicated on the basis of gel formation .Depending on clinical inspection and CMT results, cases were categorized as either positive or negative. Positive cases were further categorized as clinical and sub-clinical mastitis. The interpretation (grades) of the CMT was evocated and the results was graded as 0 for special chemical concentrated commercially prepared negative and trace 1, 2 and 3, for positive (Quinn *et al.,* 2002).

*h) Microbial investigation of mastitis*

i. *Milk sample collection*

The milk sample was taken from cows not treated previously with either intra mammary or systematic antimicrobials agents. For good collection of sample the teat was wiped thoroughly with 70% ethyl alcohol. Procedures for collecting milk sample were according to (NMC, 1999; Quinn *et al*., 2002). Strict aseptic procedures were used when collecting milk samples in order to prevent contamination with the microorganisms present on the skin of cow's flanks, udder and teats, on the hands of the sampler, and in the barn environment. Teats towards sample collection were taken first and then the far ones. The first 3-4 streams of milk were discarded. The collecting vial was as near horizontal as possible and by turning the teat to a near horizontal position. The milk sample was held in an ice box and transported immediately to the laboratory for culturing.

*i) Bacteriological isolation and biochemical characterization*

Culturing of milk sample was performed according to microbiological producers of (Quinn *et al*., 2002 and Radostits *et al*., 2007) for the diagnosis of bovine mastitis. Briefly, a loopful of milk sample collected from each infected quarter was inoculated separately on to MacConkey agar and blood agar base enriched with 5% defibrinated sheep blood. The inoculated plates were then incubated aerobically at $37^0$C for 24 to 48 hours. Identification of the bacteria on primary culture was made on the basis of colony morphology, hemolytic characteristics, Gram stain reaction including shape and arrangements of the bacteria, catalase and O-F tests. Staphylococci were identified based on Catalase test, growth characteristics on Mannitol salt agar and purple agar and tube coagulase test. Gram negative isolates grown on MacConkey agar were identified based on growth characteristics on MacConkey agar, Oxidase reaction, Catalase test, triple sugar iron (TSI) agar ,the "IMViC" (indole ,methyl red, Voges-Proskaur, and citrate) test (Quinn *et al.,* 2002).

*j) Data Management*

The collected data about history, clinical inspection, CMT, and result of bacterial culture were evaluated using SPSS soft- ware (SPSS 20.0 version) with Chi-square ($\chi 2$) and $p < 0.05$ was considered statistically significant.

## III. Results

Of a total 471 examined lactating cows the overall prevalence of mastitis in the study areas was 237 (50.3%).The result showed that the prevalence of clinical and subclinical mastitis were 9.5 % and 40.7%, respectively. The result also indicated prevalence of bovine mastitis in north Showa zone is higher as compared to the Borana zone (Table.1).

*Table 1 :* Prevalence of Bovine Mastitis in Borana and North Showa zone

| Variable | Levels | No. examined | Prevalence (%) | | | P value |
|---|---|---|---|---|---|---|
| | | | Clinical | Sub clinical | Subtotal | |
| Zone | Borana | 327 | 40(12.2) | 70(21.4) | 110(33.6) | 0.00 |
| | North Showa | 144 | 5(3.5) | 122(84.7) | 127(88.2) | |
| Districts/PA | Mio | 68 | 10(14.7) | 16(23.5) | 26 (38.2) | 0.00 |
| | Baha | 80 | 6(7.5) | 20(25.0) | 26(32.5) | |
| | Boku-luboma | 83 | 12(14.4) | 14(16.9) | 26(31.3) | |
| | Hiddi | 35 | 3(8.6) | 5(14.3) | 8(22.9) | |
| | Dhokisu | 61 | 9(14.7) | 15(24.6) | 24(39.3) | |
| | G/ Jarso | 54 | 2(3.7) | 50(92.60 | 52(96.3) | |
| | D/Libanos | 49 | 2(4.1) | 45(91.8) | 47(95.9) | |
| | Wuchale | 41 | 1(2.4) | 27(65.9) | 28(68.3) | |
| | **Total** | **471** | **45(9.5)** | **192(40.7)** | **237(50.3)** | |



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Out of 1884 quarters examined 10(0.5%) quarters were blind, leaving 1884 functional quarters. The prevalence of mastitis on quarter bases was 550 (29.2%). The result showed that higher infection rate in hindquarters as compared to the front quarters (Table 2).

*Table 2 :* The Prevalence of Mastitis at quarter level

| Quarter | No. examined | No. positive (%) | | | No. blind |
|---|---|---|---|---|---|
| | | clinical | subclinical | Sub total | |
| Right front | 471 | 24(17.8) | 111(82.2) | 135(28.9) | 4(0.8) |
| | | $X^2$=199.9, P=0.00 | | | |
| Right back | 471 | 23(16.3) | 118(83.7) | 141(30.1) | 2(0.4) |
| | | $X^2$=202.0, P=0.00 | | | |
| Left front | 471 | 23(17.2) | 111(82.8) | 134(28.6) | 3(0.6) |
| | | $X^2$=192.3, P=0.00 | | | |
| Left back | 471 | 21(15) | 119(85) | 140(29.8) | 1(0.2) |
| | | $X^2$=203.3, P=0.00 | | | |
| Total | 1884 | 91(16.5) | 459(83.5) | 550(29.2) | 10(0.5) |

The result showed that the effect of lactation stage and parity number were statistically insignificant (P>0.05) on the prevalence of bovine mastitis. However, husbandry type and hygienic condition were statistically have significant differences on the infection prevalence (P=0.00) (Table. 3).

*Table 3 :* prevalence of bovine mastitis based on lactation stage,husbandry practice, hygienic condition and parity number

| Risk factors | | Result | | | | P value |
|---|---|---|---|---|---|---|
| | | No. examined | No. positive (%) | | | |
| | | | clinical | subclinical | Sub total | |
| Lactation Stage | Early | 141 | 17(21.5) | 62(78.5) | 79(56.0) | |
| | Mid | 111 | 7(14.6) | 41(85.4) | 48(43.2) | 0.13 |
| | Late | 219 | 21(19.1) | 89(80.9) | 110(50.2) | |
| Husbandry | Extensive | 17 | 0(0) | 13(100) | 13(76.5) | |
| | Semi Intensive | 84 | 3(3.9) | 74(96.1) | 77(91.6) | |
| | Intensive | 43 | 2(5.4) | 35(94.6) | 37(86.0) | 0.00 |
| | Pastoral | 232 | 28(35.4) | 51(64.6) | 79(34.0) | |
| | Agropastoral | 95 | 12(38.7) | 19(61.3) | 31(32.6) | |
| Hygiene | Good | 74 | 2(3) | 66(97) | 68(91.9) | |
| | Medium | 52 | 1(3.2) | 30(96.8) | 31(59.6) | 0.00 |
| | Poor | 345 | 42(30.4) | 96(69.6) | 138(40.0) | |
| Parity number | 1-3 | 338 | 38(21.7) | 137(78.3) | 175(51.8) | |
| | 4-6 | 109 | 5(10.40 | 43(89.6) | 48(44.0) | 0.26 |
| | >6 | 24 | 2(14.3) | 12(85.7) | 14(58.3) | |
| | Total | 471 | 45(19) | 192(81.0) | 237(50.3) | |

From the CMT positive samples, 63 milk samples were cultured and 53 bacteria were cultured on blood, nutrient agar for gram positive bacteria and Macconkey agar for gram negative aerobically. *S. aureus* and CNS (coagulase negative staphylococci), were the most isolated followed by *C. pseudotuberclosis* and *E. coli* and the other such as *Diplodocus* Spp., *C. bovis, Micrococcus* Spp., *Bacillus* Spp., and *Pseudomonas* Spp. were rarely isolated as causative agents of mastitis. (Table 4).

*Table 4 :* Prevalence of isolated bacteria in the study areas in (2012-2014).

| Bacteria | Frequency | Proportion% |
|---|---|---|
| *S. aureus* | 20 | 37.7 |
| CNS | 20 | 37.7 |
| *Diplococcus species* | 2 | 3.8 |
| *C.pseudotuberclosis* | 3 | 5.7 |
| *C. bovis* | 1 | 1.9 |
| *Micrococcus* species | 1 | 1.9 |
| *Pseudomonas* | 1 | 1.9 |
| *Bacillus* species | 1 | 1.9 |
| *E .coli* | 3 | 5.7 |
| *Proteus* species | 1 | 1.9 |



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## IV. Discussion

Mastitis prevalence at cow level was found 50.3% which is highly disagreed with the report of Kifle and Tadele (2000) who reported 89.5% from North showa. This finding was also lower than the reports of Tariku *et al* (2011), Mekibib *et al* (2010) and Matios *et al* (2008) which were reported 75.2%, 71.3% and 65.5% respectively. However, it is slightly comparable with the finding of Birru (1989) who reported 43.5% from of Ethiopia. Mastitis is a multi –factorial disease and this difference may be due to herd size, agro- ecological and different managemental systems. In addition the present study shows the prevalence of mastitis is statistically higher ($p<0.05$) in north Showa than in Borana zone. As compared to the other districts/PAs of the study areaprevalenceof mastitis is highly important in w/jarso of the North showa and this may due to poor sanitation of the barn floor and use of organic bedding. Generally, the overall prevalence of mastitis reported from North Showa in the present study was higher than most of reports in the country and this could be due to major farmer depending on cross breed dairy cattle, absence of balanced diet feeding and lack of awareness about the disease.

From 1884 examined quarters, 550(50.2%) were CMT positive and 10(0.5%) quarters were blind. This finding is lower than that obtained by Kifle and Tadele (2000) and Birehanu (2008) as they reported 63.1% and 52.4% respectively at quarter level. Mekibib *et al.* (2010) reported that the prevalence of blind teat was 14% which is far higher than the present finding.

The prevalence of clinical mastitis at cow level was 9.5% and this is comparable with the reports of Molalegne *et al* (2010), Bedada and Hiko (2011) and Demelash *et al* (2005) who reported 10.3%, 10% and 11.9% respectively in different parts of Ethiopia. The current finding is little higher than the reports of Husien *et al* (1999) and Bishi (1998) who reported 5.7% and 5.3% respectively in different parts of the country. This may be due to concurrent disease involvement, interaction of several risk factors relating with animal and virulence of causative organism. In our study the rate of sub clinical mastitis is higher in pastoral production system than agro-pastoral (Table 2) attributable to higher number of animals in this system than agro-pastoral.

In this study, the prevalence of subclinical mastitis was 40.7% and this is lower than that reported by Argaw and Tolosa (2008) 89.5%. In addition, it is nearly similar to that founded by The finding is Bishi (1998) and Ahmed *et al*. (2007) However, our finding is higher than Mekibib *et al* (2010) who reported 25.2%. The high prevalence of subclinical mastitis may be due to improper milking hygiene, poor housing system, lack of post milking teat dipping.

Lactation stage showed statistically insignificant effect on the occurrence of mastitis $P>0.05$) which contradicts with the report of Birru (1989).Parity number has also no difference for occurrences of bovine mastitis.

In our study husbandry and hygiene showed statistically significant effect on the prevalence of mastitis ($p<0.05$). This could be due to different farming system, managemental practice and may be attributed to occurance of contagious mastitis and inability of control and physiology effect.

From the isolated bacteria the most dominant in the study area were CNS and *staphylococcus aureus* (37% for each) and the predominant causes of clinical and sub clinical mastitis in the area. This finding is little different from the finding of Molalegn *et al* (2010) who reported, (51.9%) of CNS. This study is compatible with the study of Mekibib *et al* (2010) and Abdella (1996) who reported (47.1%) and (31%) of *S. aures* respectively. In addition Tariku *et al.* (2011) reported 39.44% of *staphylococcus* species responsible for mastitis. However, the current finding is much lower than Workineh *et al.* (2002) who reported 70.5% of *Staphylococcus* species. The difference might be resulted from lack of effective udder washing and drying, inter cow hand washing and disaffection in the route of the area.

The other isolated bacteria were *E. coli* and *C. pseudotuberclosis (*5.56 % for each) and *Diplococcus* species 3.8%. This finding is lower than Hunder *et al.* (2005) who reported 14.2% of *C. Pseudotuberclosis* around Sebeta. Rarely isolated bacteria were *C. bovis, Baccilus spp, Micrococcus spp, Proteus spp* and *Pseudomonas spps* with equal ration of 1.9% for each. *B. cereus* and *B. subtilis* are saprophytic *Bacillus species* and the only mastitis causing pathogens (Radostits *et al.,* 2007).This result is in agreement with Tariku *et al* (2011) who reported (3.3%) *Micrococcus spp* and (2%) *C. bovis.*

In the current study the hind quarter have high mastitic prevalence and this may due to the hindquarter is more prone surface when the animal lay down touches hind leg in contact with contagious bacteria and to greater production capacity of hind quarter (Radostits*et al.,* 1994), likelihood of fecal accumulation, environmental contamination and difficulty of cleaning.

## V. Conclusion and Recommendation

The overall prevalence of cattle mastitis is high as compared to most of previous reports in different parts of Ethiopia. Hygienic conditions and husbandry type were the most important potential risk for mastitis. The major isolated bacteria in this study were CNS and *S. aureus.* Farmers and herd manager are only concerned with clinical from of mastitis and often are unawareness of the status of subclinical infection in the



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herd. The farmers have no enough understanding about effect of sanitation on the occurrence of the disease. Relaying on the above conclusion the following recommendation is for warded:

- Farmers and herd managers should give great attention for hygiene condition and husbandry type
- Periodic monitoring of infection status of the udder should be undertaken and positive animals should be treated using appropriate drug
- Careful milking practice and hygienic condition should be applied and the use of dry towel or sponge at least for each cow should be practiced
- Contaminated washing water and inappropriate bedding material that predispose to animals to mastitis should be avoided.
- Further investigation should be conducted on the area

*Conflicts of interest*

The authors have none to declare

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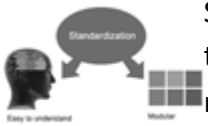



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"

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2. Ethical Guidelines,

3. Submission of Manuscripts,

4. Manuscript's Category,

5. Structure and Format of Manuscript,

6. After Acceptance.

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**1. Choosing the topic:** In most cases, the topic is searched by the interest of author but it can be also suggested by the guides. You can have several topics and then you can judge that in which topic or subject you are finding yourself most comfortable. This can be done by asking several questions to yourself, like Will I be able to carry our search in this area? Will I find all necessary recourses to accomplish the search? Will I be able to find all information in this field area? If the answer of these types of questions will be "Yes" then you can choose that topic. In most of the cases, you may have to conduct the surveys and have to visit several places because this field is related to Computer Science and Information Technology. Also, you may have to do a lot of work to find all rise and falls regarding the various data of that subject. Sometimes, detailed information plays a vital role, instead of short information.

**2. Evaluators are human:** First thing to remember that evaluators are also human being. They are not only meant for rejecting a paper. They are here to evaluate your paper. So, present your Best.

**3. Think Like Evaluators:** If you are in a confusion or getting demotivated that your paper will be accepted by evaluators or not, then think and try to evaluate your paper like an Evaluator. Try to understand that what an evaluator wants in your research paper and automatically you will have your answer.

**4. Make blueprints of paper:** The outline is the plan or framework that will help you to arrange your thoughts. It will make your paper logical. But remember that all points of your outline must be related to the topic you have chosen.

**5. Ask your Guides:** If you are having any difficulty in your research, then do not hesitate to share your difficulty to your guide (if you have any). They will surely help you out and resolve your doubts. If you can't clarify what exactly you require for your work then ask the supervisor to help you with the alternative. He might also provide you the list of essential readings.

**6. Use of computer is recommended:** As you are doing research in the field of Computer Science, then this point is quite obvious.

**7. Use right software:** Always use good quality software packages. If you are not capable to judge good software then you can lose quality of your paper unknowingly. There are various software programs available to help you, which you can get through Internet.

**8. Use the Internet for help:** An excellent start for your paper can be by using the Google. It is an excellent search engine, where you can have your doubts resolved. You may also read some answers for the frequent question how to write my research paper or find model research paper. From the internet library you can download books. If you have all required books make important reading selecting and analyzing the specified information. Then put together research paper sketch out.

**9. Use and get big pictures:** Always use encyclopedias, Wikipedia to get pictures so that you can go into the depth.

**10. Bookmarks are useful:** When you read any book or magazine, you generally use bookmarks, right! It is a good habit, which helps to not to lose your continuity. You should always use bookmarks while searching on Internet also, which will make your search easier.

**11. Revise what you wrote:** When you write anything, always read it, summarize it and then finalize it.

**12. Make all efforts:** Make all efforts to mention what you are going to write in your paper. That means always have a good start. Try to mention everything in introduction, that what is the need of a particular research paper. Polish your work by good skill of writing and always give an evaluator, what he wants.

**13. Have backups:** When you are going to do any important thing like making research paper, you should always have backup copies of it either in your computer or in paper. This will help you to not to lose any of your important.

**14. Produce good diagrams of your own:** Always try to include good charts or diagrams in your paper to improve quality. Using several and unnecessary diagrams will degrade the quality of your paper by creating "hotchpotch." So always, try to make and include those diagrams, which are made by your own to improve readability and understandability of your paper.

**15. Use of direct quotes:** When you do research relevant to literature, history or current affairs then use of quotes become essential but if study is relevant to science then use of quotes is not preferable.

**16. Use proper verb tense:** Use proper verb tenses in your paper. Use past tense, to present those events that happened. Use present tense to indicate events that are going on. Use future tense to indicate future happening events. Use of improper and wrong tenses will confuse the evaluator. Avoid the sentences that are incomplete.

**17. Never use online paper:** If you are getting any paper on Internet, then never use it as your research paper because it might be possible that evaluator has already seen it or maybe it is outdated version.

**18. Pick a good study spot:** To do your research studies always try to pick a spot, which is quiet. Every spot is not for studies. Spot that suits you choose it and proceed further.

**19. Know what you know:** Always try to know, what you know by making objectives. Else, you will be confused and cannot achieve your target.

**20. Use good quality grammar:** Always use a good quality grammar and use words that will throw positive impact on evaluator. Use of good quality grammar does not mean to use tough words, that for each word the evaluator has to go through dictionary. Do not start sentence with a conjunction. Do not fragment sentences. Eliminate one-word sentences. Ignore passive voice. Do not ever use a big word when a diminutive one would suffice. Verbs have to be in agreement with their subjects. Prepositions are not expressions to finish sentences with. It is incorrect to ever divide an infinitive. Avoid clichés like the disease. Also, always shun irritating alliteration. Use language that is simple and straight forward. put together a neat summary.

**21. Arrangement of information:** Each section of the main body should start with an opening sentence and there should be a changeover at the end of the section. Give only valid and powerful arguments to your topic. You may also maintain your arguments with records.

**22. Never start in last minute:** Always start at right time and give enough time to research work. Leaving everything to the last minute will degrade your paper and spoil your work.

**23. Multitasking in research is not good:** Doing several things at the same time proves bad habit in case of research activity. Research is an area, where everything has a particular time slot. Divide your research work in parts and do particular part in particular time slot.

**24. Never copy others' work:** Never copy others' work and give it your name because if evaluator has seen it anywhere you will be in trouble.

**25. Take proper rest and food:** No matter how many hours you spend for your research activity, if you are not taking care of your health then all your efforts will be in vain. For a quality research, study is must, and this can be done by taking proper rest and food.

**26. Go for seminars:** Attend seminars if the topic is relevant to your research area. Utilize all your resources.



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**27. Refresh your mind after intervals:** Try to give rest to your mind by listening to soft music or by sleeping in intervals. This will also improve your memory.

**28. Make colleagues:** Always try to make colleagues. No matter how sharper or intelligent you are, if you make colleagues you can have several ideas, which will be helpful for your research.

**29. Think technically:** Always think technically. If anything happens, then search its reasons, its benefits, and demerits.

**30. Think and then print:** When you will go to print your paper, notice that tables are not be split, headings are not detached from their descriptions, and page sequence is maintained.

**31. Adding unnecessary information:** Do not add unnecessary information, like, I have used MS Excel to draw graph. Do not add irrelevant and inappropriate material. These all will create superfluous. Foreign terminology and phrases are not apropos. One should NEVER take a broad view. Analogy in script is like feathers on a snake. Not at all use a large word when a very small one would be sufficient. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Amplification is a billion times of inferior quality than sarcasm.

**32. Never oversimplify everything:** To add material in your research paper, never go for oversimplification. This will definitely irritate the evaluator. Be more or less specific. Also too, by no means, ever use rhythmic redundancies. Contractions aren't essential and shouldn't be there used. Comparisons are as terrible as clichés. Give up ampersands and abbreviations, and so on. Remove commas, that are, not necessary. Parenthetical words however should be together with this in commas. Understatement is all the time the complete best way to put onward earth-shaking thoughts. Give a detailed literary review.

**33. Report concluded results:** Use concluded results. From raw data, filter the results and then conclude your studies based on measurements and observations taken. Significant figures and appropriate number of decimal places should be used. Parenthetical remarks are prohibitive. Proofread carefully at final stage. In the end give outline to your arguments. Spot out perspectives of further study of this subject. Justify your conclusion by at the bottom of them with sufficient justifications and examples.

**34. After conclusion:** Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print to the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects in your research.

## Informal Guidelines of Research Paper Writing

**Key points to remember:**

- Submit all work in its final form.
- Write your paper in the form, which is presented in the guidelines using the template.
- Please note the criterion for grading the final paper by peer-reviewers.

**Final Points:**

A purpose of organizing a research paper is to let people to interpret your effort selectively. The journal requires the following sections, submitted in the order listed, each section to start on a new page.

The introduction will be compiled from reference matter and will reflect the design processes or outline of basis that direct you to make study. As you will carry out the process of study, the method and process section will be constructed as like that. The result segment will show related statistics in nearly sequential order and will direct the reviewers next to the similar intellectual paths throughout the data that you took to carry out your study. The discussion section will provide understanding of the data and projections as to the implication of the results. The use of good quality references all through the paper will give the effort trustworthiness by representing an alertness of prior workings.

Writing a research paper is not an easy job no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record keeping are the only means to make straightforward the progression.

**General style:**

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear

· Adhere to recommended page limits

Mistakes to evade

- Insertion a title at the foot of a page with the subsequent text on the next page
- Separating a table/chart or figure - impound each figure/table to a single page
- Submitting a manuscript with pages out of sequence

In every sections of your document

· Use standard writing style including articles ("a", "the," etc.)

· Keep on paying attention on the research topic of the paper

· Use paragraphs to split each significant point (excluding for the abstract)

· Align the primary line of each section

· Present your points in sound order

· Use present tense to report well accepted

· Use past tense to describe specific results

· Shun familiar wording, don't address the reviewer directly, and don't use slang, slang language, or superlatives

· Shun use of extra pictures - include only those figures essential to presenting results

**Title Page:**

Choose a revealing title. It should be short. It should not have non-standard acronyms or abbreviations. It should not exceed two printed lines. It should include the name(s) and address (es) of all authors.



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**Abstract:**

The summary should be two hundred words or less. It should briefly and clearly explain the key findings reported in the manuscript--must have precise statistics. It should not have abnormal acronyms or abbreviations. It should be logical in itself. Shun citing references at this point.

An abstract is a brief distinct paragraph summary of finished work or work in development. In a minute or less a reviewer can be taught the foundation behind the study, common approach to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Yet, use comprehensive sentences and do not let go readability for briefness. You can maintain it succinct by phrasing sentences so that they provide more than lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study, with the subsequent elements in any summary. Try to maintain the initial two items to no more than one ruling each.

- Reason of the study - theory, overall issue, purpose
- Fundamental goal
- To the point depiction of the research
- Consequences, including definite statistics - if the consequences are quantitative in nature, account quantitative data; results of any numerical analysis should be reported
- Significant conclusions or questions that track from the research(es)

Approach:

- Single section, and succinct
- As a outline of job done, it is always written in past tense
- A conceptual should situate on its own, and not submit to any other part of the paper such as a form or table
- Center on shortening results - bound background information to a verdict or two, if completely necessary
- What you account in an conceptual must be regular with what you reported in the manuscript
- Exact spelling, clearness of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else

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The **Introduction** should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable to comprehend and calculate the purpose of your study without having to submit to other works. The basis for the study should be offered. Give most important references but shun difficult to make a comprehensive appraisal of the topic. In the introduction, describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will have no attention in your result. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here. Following approach can create a valuable beginning:

- Explain the value (significance) of the study
- Shield the model - why did you employ this particular system or method? What is its compensation? You strength remark on its appropriateness from a abstract point of vision as well as point out sensible reasons for using it.
- Present a justification. Status your particular theory (es) or aim(s), and describe the logic that led you to choose them.
- Very for a short time explain the tentative propose and how it skilled the declared objectives.

Approach:

- Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done.
- Sort out your thoughts; manufacture one key point with every section. If you make the four points listed above, you will need a least of four paragraphs.

- Present surroundings information only as desirable in order hold up a situation. The reviewer does not desire to read the whole thing you know about a topic.
- Shape the theory/purpose specifically - do not take a broad view.
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**Procedures (Methods and Materials):**

This part is supposed to be the easiest to carve if you have good skills. A sound written Procedures segment allows a capable scientist to replacement your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt for the least amount of information that would permit another capable scientist to spare your outcome but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section. When a technique is used that has been well described in another object, mention the specific item describing a way but draw the basic principle while stating the situation. The purpose is to text all particular resources and broad procedures, so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step by step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

- Explain materials individually only if the study is so complex that it saves liberty this way.
- Embrace particular materials, and any tools or provisions that are not frequently found in laboratories.
- Do not take in frequently found.
- If use of a definite type of tools.
- Materials may be reported in a part section or else they may be recognized along with your measures.

Methods:

- Report the method (not particulars of each process that engaged the same methodology)
- Describe the method entirely
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures
- Simplify - details how procedures were completed not how they were exclusively performed on a particular day.
- If well known procedures were used, account the procedure by name, possibly with reference, and that's all.

Approach:

- It is embarrassed or not possible to use vigorous voice when documenting methods with no using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result when script up the methods most authors use third person passive voice.
- Use standard style in this and in every other part of the paper - avoid familiar lists, and use full sentences.

What to keep away from

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings - save it for the argument.
- Leave out information that is immaterial to a third party.

**Results:**

The principle of a results segment is to present and demonstrate your conclusion. Create this part a entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Carry on to be to the point, by means of statistics and tables, if suitable, to present consequences most efficiently.You must obviously differentiate material that would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matter should not be submitted at all except requested by the instructor.



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Content

- Sum up your conclusion in text and demonstrate them, if suitable, with figures and tables.
- In manuscript, explain each of your consequences, point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation an exacting study.
- Explain results of control experiments and comprise remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or in manuscript form.

What to stay away from

- Do not discuss or infer your outcome, report surroundings information, or try to explain anything.
- Not at all, take in raw data or intermediate calculations in a research manuscript.
- Do not present the similar data more than once.
- Manuscript should complement any figures or tables, not duplicate the identical information.
- Never confuse figures with tables - there is a difference.

Approach

- As forever, use past tense when you submit to your results, and put the whole thing in a reasonable order.
- Put figures and tables, appropriately numbered, in order at the end of the report
- If you desire, you may place your figures and tables properly within the text of your results part.

Figures and tables

- If you put figures and tables at the end of the details, make certain that they are visibly distinguished from any attach appendix materials, such as raw facts
- Despite of position, each figure must be numbered one after the other and complete with subtitle
- In spite of position, each table must be titled, numbered one after the other and complete with heading
- All figure and table must be adequately complete that it could situate on its own, divide from text

**Discussion:**

The Discussion is expected the trickiest segment to write and describe. A lot of papers submitted for journal are discarded based on problems with the Discussion. There is no head of state for how long a argument should be. Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implication of the study. The purpose here is to offer an understanding of your results and hold up for all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of result should be visibly described. Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved with prospect, and let it drop at that.

- Make a decision if each premise is supported, discarded, or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."
- Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work
- You may propose future guidelines, such as how the experiment might be personalized to accomplish a new idea.
- Give details all of your remarks as much as possible, focus on mechanisms.
- Make a decision if the tentative design sufficiently addressed the theory, and whether or not it was correctly restricted.
- Try to present substitute explanations if sensible alternatives be present.
- One research will not counter an overall question, so maintain the large picture in mind, where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

- When you refer to information, differentiate data generated by your own studies from available information
- Submit to work done by specific persons (including you) in past tense.
- Submit to generally acknowledged facts and main beliefs in present tense.

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| Topics | Grades | | |
|---|---|---|---|
| | **A-B** | **C-D** | **E-F** |
| ***Abstract*** | Clear and concise with appropriate content, Correct format. 200 words or below | Unclear summary and no specific data, Incorrect form<br><br>Above 200 words | No specific data with ambiguous information<br><br>Above 250 words |
| ***Introduction*** | Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited | Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter | Out of place depth and content, hazy format |
| ***Methods and Procedures*** | Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads | Difficult to comprehend with embarrassed text, too much explanation but completed | Incorrect and unorganized structure with hazy meaning |
| ***Result*** | Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake | Complete and embarrassed text, difficult to comprehend | Irregular format with wrong facts and figures |
| ***Discussion*** | Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited | Wordy, unclear conclusion, spurious | Conclusion is not cited, unorganized, difficult to comprehend |
| ***References*** | Complete and correct format, well organized | Beside the point, Incomplete | Wrong format and structuring |

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