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Journal of Microbiology and Related Research

JMRR

July - December 2016
Volume 2, Number 2

Contents

Original Articles

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Original Article

Journal of Microbiology and Related Research
Volume 2 Number 2, July - December 2016
DOI: http://dx.doi.org/10.21088/jmrr.2395.6623.2216.1

C-Reactive Protein (CRP) in Early Diagnosis of Neonatal Septicemia

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Received on 06.08.2016, **Accepted on** 17.08.2016

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Abstract

A study was conducted to evaluate C-reactive protein(CRP) as a screening tool for neonatal sepsis. CRP is one of the most studied and most used laboratory tests for neonatal sepsis. As part of the acute phase reaction to infection, it plays a central role in the humoral response to bacterial invasion. CRP is useful for monitoring the response to treatment and guiding antibiotic therapy. 75 neonates with risk factors and clinical features suggestive of CRP sepsis were selected as per operational definition and fulfilling the inclusion and exclusion criteria. Detailed physical examination was carried out. Blood sample for culture and CRP was taken from all neonates. CRP performed by semi quantitative latex agglutination method. Positive culture were the 'gold standard' against which the performance of CRP, abnormal white blood cell count (WBC) and platelet count were compared. Among 75 septic screens, 40 (53.33%) neonates had positive cultures. The sensitivity, specificity and positive predictive value of CRP was 92.5%, 89.74%, 90.24% respectively. Abnormal platelet count had lowest specificity(62.86%) and sensitivity(37.5%) among them. CRP assay is a valuable adjuvent in screening for neonatal sepsis, complementing clinical decision-making.

Keywords: C-Reactive Protein; Acute Phase Reactant; Neonatal Sepsis.

Introduction

Neonatal septicemia remains a significant cause of neonatal morbidity and mortality [1]. The most important risk factors for neonatal sepsis are prematurity, low birth weight, invasive medical procedures and prolonged hospitalization. Neonatal sepsis presents in diverse ways. It may present with fever, poor feeding, abdominal distention, diarrhea, tachypnea, oliguria, tachycardia or bradycardia, hypotension, irritability, seizures, bulging fontanelle or bleeding [2]. Although various hematological indices had been utilized to screen for sepsis, most were neither highly sensitive nor specific and were commonly affected by perinatal factors like maternal hypertension, asphyxia and hemolytic disease. C-reactive protein (CRP) has been used as an acute phase reactant to diagnose and follow the course of infection in neonates. Its advantages that its very low serum levels in normal infants, a rapid rise within 12 to 24 hours of sepsis and a large incremental increase thereafter [3]. C reactive protein production is a very early and sensitive response to most forms of microbial infection [4]. There is great interest in rapid diagnostic tests that are able to safely distinguish

infected from uninfected newborns, especially in the early phase of the disease [5,6]. Although blood culture is gold standard for diagnosis of neonatal sepsis and allows targeted antimicrobial therapy, its result may be delayed for up to 48 hours and it may yield negative results in many cases of septic shock. Also, contamination rates are high due to the technical difficulty of obtaining a sterile sample from small babies. On the other hand, unnecessary antibiotics increase the risk of drug side effects and contribute to emergence of microbial resistance [2].

Measurement of CRP allows rapid identification of infected patients, does not require a sterile sample, and a normal value may help in early exclusion of infection. Serial measurements of CRP have a prognostic value, and show the effectiveness of antibiotic therapy [2].

Materials and Methods

The present study was conducted in the Department of Microbiology, SRTR Medical College, Ambajogai from December 2014 to May 2015. 75 suspected septic neonates were included with the age group of first 28 days (4 weeks) of life in study.

For identification of suspected neonatal sepsis, two or more of the following clinical features were used: Respiratory and cardiovascular compromise, metabolic and neurologic changes. Exclusion criteria include, age at the time of admission is greater than 28 days, neonates who received antibiotic dose prior to septic setup and neonate diagnosed to have congenital malformation. Blood samples were drawn prior to administration of antibiotic therapy for blood culture by trained staff with all aseptic precaution in blood culture bottle and should be observed for 5 days for culture growth and after that they are reported as sterile, complete hemogram and routine biochemical investigations including glucose level [3].

CRP value was estimated by semi quantitative latex agglutination slide method with CRP kit manu-factured by BEACON Diagnostics Pvt. Ltd. as per instructions in the manual provided by company. Results are given as negative, 0.6mg/dl, 1.2mg/dl etc. by serial dilution of serum of patients. Total leukocyte count and indices were counted on a cell counter.

Results

During study period, total 75 neonates admitted in neonatal intensive care unit were studied. They were divided into 3 groups based on clinical features and blood culture reports- proven sepsis, probable sepsis, clinically sepsis.

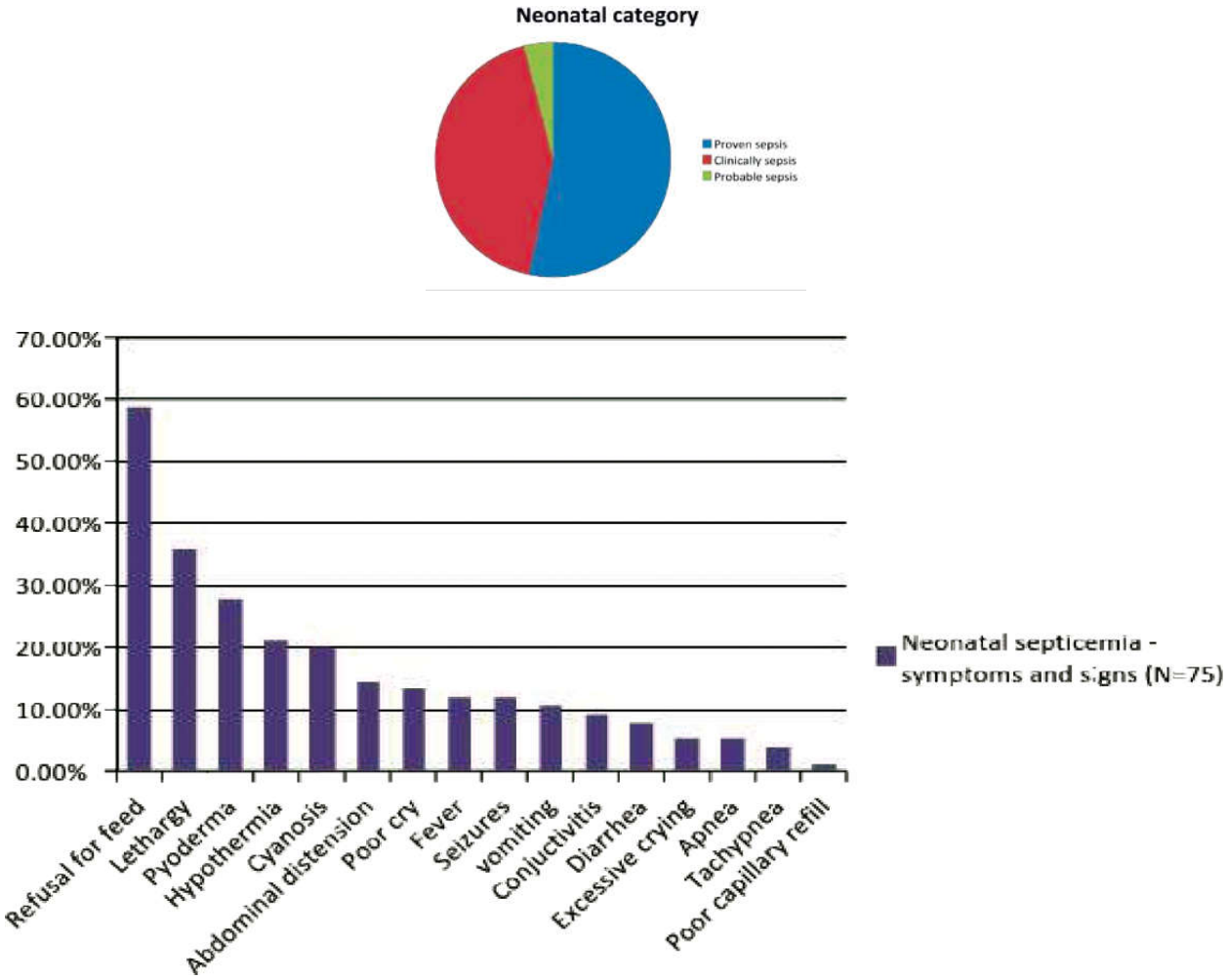
Table 1: Relation between birth weight and sepsis

Sr. No.	Neonatal category	Low birth weight (<2500 gm)	Normal birth weight (>2500 gm)
1	Proven sepsis (%)	72.5% (29/40)	27.5% (11/40)
2	Probable sepsis (%)	66.67% (2/3)	33.33% (1/3)
3	Clinically sepsis (%)	71.88% (23/32)	28.13% (9/32)

Table 2: Neonatal septicemia - symptoms and signs (N=75)

Symptoms*	N	(%)
Refusal for feed	44	58.67
Lethargy	27	36
Poor cry	10	13.33
Diarrhea	6	8
Vomiting	3	4
Fever	5	6.67
Excessive crying	2	2.67
Signs*	N	(%)
Pyoderma	21	28
Hypothermia	16	21.33
Cyanosis	15	20
Abdominal distension	11	14.67
Seizures	9	12
Conjunctivitis	7	9.33
Vomiting	5	6.67
Fever	4	5.33
Apnea	4	5.33
Tachypnea	3	4
Excessive crying	2	2.67
Poor capillary refill	1	1.33

*More than one sign or symptom were present together



Proven Sepsis: These are the patients among suspected neonatal sepsis in which blood culture confirms sepsis or there is definite evidence of localized infection.

Probable Sepsis: These are the suspected septic patient with CRP and/or hematological parameters suggestive of septicemia but negative culture.

Clinically Sepsis: These are the suspected septic patient with CRP <0.6 mg/dl, almost normal hematological parameters and sterile blood culture.

Out of 75 total cases 40 cases were proven sepsis, 3 cases have probable sepsis and rest of 32 cases having clinically sepsis. Study confirms that low birth weight babies are prone to develop neonatal sepsis as compared with normal birth weight.

Refusal for feed, lethargy and pyoderma were the main presenting features followed by poor cry, hypothermia and cyanosis. In this study, commonest organism for neonatal sepsis was coagulase negative Staphylococcus followed by *Klbsiella pneumonia, E.coli, Pseudomonas aeruginosa* and *Acinetobacter.*

Sensitivity of CRP, abnormal WBC Count, Platelet Count were 92.5%, 37.5%, 22.5% respectively. Specificity of CRP, abnormal WBC Count, Platelet Count were 89.74%, 62.86%, 42.86%. Positive predictive value of CRP, WBC Count, Platelet Count were 90.24%, 83.33%, 31.03%.

Table 3: Sensitivity, Specificity, Positive predictive value

Investigation	Sensitivity	Specificity	Positive predictive value
CRP >= 0.6mg/dl	92.5%	89.74%	90.24%
Abnormal WBC Count (<5000/ml or >20000/ml)	37.5%	62.86%	83.33%
Platelet Count (<1.51/cumm)	22.5%	42.86%	31.03%

Discussion

Neonatal septicemia is very common in the present Indian set-up. The disease has got high morbidity and mortality but it is unfortunate that none of laboratory parameters available till are rapid, specific, sensitive, cheap and simple enough to confirm the diagnosis and to asses the prognosis or therapeutic response in this condition. The present work was concluded to assess the efficacy and reliability of CRP in neonatal septicemia and values of the CRP as a tool of prognosis in neonatal septicemia. CRP production is very early and sensitive response to most form of microbial infections(7).

Comparison of the performance of CRP and abnormal hematology was thus made against well defined 'gold standard'. Majority of infecting organisms were Staphylococcus, which form a leading cause of nosocomial infections in the susceptible neonate. The calculation of both sensitivity and specificity depend on knowing which infants were already septic when CRP assay was performed. Platelet counts had the lowest sensitivity (22.5%) and lowest specificity (42.86%) among the indices. These indices render them less valuable than CRP for screening purpose, as abnormal hematology may be affected by non-septic processes like steroid treatment as part for chronic lung disease. Commonly used anti-inflammatory or immunosuppressive drugs including steroids, unless these drugs affect actively of underlying diseases do not affect the CRP response.

Sensitivity, specificity and positive predictive value for CRP were 92.5, 89.74 and 90.24% respectively; while for WBC Count, Platelet Count they were markedly less.

A good response of treatment was assessed by rapid fall in CRP level whereas, insignificant rise of CRP suggested that either the treatment was inadequate or some complications had developed.

Conclusion

C-reactive protein has high sensitivity and specificity for establishing the diagnosis of neonatal septicemia which is comparable with other indices. With the added benefit of early test result availability, it is highly recommendable that it should be used routinely in the evaluation of neonates with any features suggestive of sepsis to reliably include or exclude the diagnosis of neonatal septicemia.

CRP levels are useful in monitoring the course of neonatal septicemia. It provides an early indication of response of treatment. It can help in decision of initiating or discontinuing antibiotic therapy. The persistence or insignificant decline of serum CRP with treatment signifies about inadequate treatment or development of complications.

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Original Article

Journal of Microbiology and Related Research
Volume 2 Number 2, July - December 2016
DOI: http://dx.doi.org/10.21088/jmrr.2395.6623.2216.2

Impact of Different Chemical Agents on Reproductive Potential of Male Mice Challenged By *Escherichia Coli*

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Received on 08.11.2016, **Accepted on** 16.11.2016

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Abstract

The role of chemical agents in aggravation or amelioration of infertility is widely acknowledged, however, their impact in cases of microbial induced infertility is yet to be deciphered. Further, *Escherichia coli* is known to reduce the reproductive potential of male mice therefor in the light of present information, this work was carried out to study the effect of the chemical agents *viz*. carnitine, tamoxifen and nictotine on *E. coli* induced infertility. For this, *in vitro* effect of carnitine/tamoxifen/nicotine was assessed on human spermatozoa when coincubated with *E. coli*. The results showed that carnitine and a tamoxifen at lower concentrations enhanced the motility and viability of sperms but at higher concentrations they proved to be toxic. Nicotine on the other hand was found to negatively influence the motility and viability of spermatozoa at all concentrations. For *in vivo* relevance of these results, male Balb/c mice were intraperitoneally inoculated with these chemical compounds challenged intravasaly with *E. coli*. The results revealed that carnitine and tamoxifen were able to ameliorate the negative impact of *E. coli* on male reproductive potential but nicotine aggravated the same. Therefore, it could be speculated that a healthier lifestyle has the ability to avert the microbial induced decreased reproductive potential.

Keywords: *Escherichia coli;* Tamoxifen; Nicotine.

Introduction

Male infertility is defined as the failure to induce pregnancy within one year of regular intercourse in the fertile phase of menstrual cycle without the use of any contraception. The male factor is either the only cause or a contributing factor leading to infertility in about 40% of infertile couples. Broad and evident causes of male infertility include congenital or acquired conditions such as varicocele, genetic abnormalities, immunological problems, endocrine disturbances or hormonal problems, environmental factors, altered lifestyle, sperm antibodies, urogenital abnormalities such as testicular failure and infection of the genital tract (Louis *et al*, 2013; Agarwal *et al*, 2014). Amongst these, male genitourinary tract infections are attributed to 15% cases of male infertility that leads to compromised sperm cell function or deterioration of the whole spermatogenic process causing qualitative and quantitative sperm alterations (Urata *et al*, 2001; Sanocka-Maciejewska *et al*, 2005; Pellati *et al*, 2008). Various experimental and investigational studies have pointed out the reduced reproductive potential due to occurrence of bacteria

in semen (Moretti *et al*, 2009).

The most commonly isolated microorganism in the males with genital tract infections or semen contamination is *E. coli* causing prostatitis and epididymitis. It can impair sperm motility by agglutination and clumping or by releasing extracellular factors (Teague *et al*, 1971). It can damage the acrosomal function (Diemer *et al*, 2000; Diemer *et al*, 2003) and also known to induce apoptosis (Villegas *et al*., 2005). *Escherichia coli* and *Enterococci* have been found to be the foremost microorganisms with the maximum negative impact on sperm motility and morphology (Naessens *et al*, 1986; Hillier *et al*, 1990; Ombelet *et al*, 1997).

Besides infection, lifestyle factors hypothesized to play a role in development of infertility, have generated a considerable amount of interest. Recent evidence suggests that various lifestyle factors such as the age at which to start a family, nutrition, weight, exercise, stress, occupational and environmental exposures, cigarette smoking, tobacco, illicit drug use, alcohol and caffeine consumption can have substantial effects on male fertility (Sharma *et al*, 2013). The primary active component of tobacco is nicotine. Nicotine has shown deleterious effects on all levels of male reproductive system by interfering with the function of each component causing genetic and epigenetic alterations, oxidative stress, reduced male secondary sexual characteristics and infertility. It has also been associated to the production of abnormal sperm cells with deformed heads (Sunanda *et al*, 2014). It can also induce variations in the quantity and positioning of normal axoneme, a basic structural organ of motile cilia and flagella, impairing the flagellar movement and thus, causing the sperm motility pathologies (Zavos *et al*, 1998; Yeung *et al*, 2009).

Reducing the number of infertile couples has become a topic of discussion; therefore, there is an urgent need of multidimensional therapeutic approach to cure and manage male infertility. Since oxidative stress has also been linked to development of male subfertility, treatment approaches that reduce oxidative stress are required (Martinez *et al*, 2007). A wide range of therapies consisting of vitamins such as vitamin A, vitamin C, vitamin E and coenzyme Q_{10} and component including phosphotidylcholine, kallikrein and carnitines have been used to neutralize the lipoperoxidative injury (Lanzafame, 2009). Carnitines are highly polar compounds that are widely scattered in nature. Human requirements for carnitine are fulfilled through endogenous biosynthesis and diet (Bieber, 1988). They are highly concentrated within the epididymis and spermatozoa of the male genital tract. It is known to supply energy to spermatozoa, protect cell membrane and DNA against reactive oxygen species-induced damage (Zhou, 2007). Further, anti-estrogens are the oldest and most frequently suggested forms of therapy for the idiopathic infertility. Tamoxifen has been postulated to produce tissue-specific estrogenic and antiestrogenic effects (Smith and O' Malley, 1999). These drugs hinder the negative feedback of the estrogen on the hypothalamus and pituitary, increasing endogenous gonadotropin secretion. Anti-estrogen therapy has stated to escalate the FSH and LH secretion directly from the pituitary, thereby stimulating spermatogenesis.

In an earlier work done in our laboratory, *E. coli* capable of causing sperm agglutination *in vitro* was isolated from the semen samples of the patients attending the infertility clinic.Intravaginal colonization of the female mice with this strain led to infertility (Kaur and Prabha, 2014). Thus, interest was developed to study the role of this *E. coli* strain in male infertility. Moreover, various authors have reported that some chemical compounds can either aggravate or ameliorate the infertility. Therefore, present study was sought to assess the relationship between *E. coli* infection, chemical compounds and male infertility.

Materials and Methods

Microorganism

The clinical isolate of *Escherichia coli* used in the current work was previously isolated in our laboratory from the semen samples of infertile males undergoing semen analysis at the special infertility clinic at the Department of Urology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. This strain was capable of causing 100% spermagglutination *in vitro.* The strain was preserved in 40% glycerol stocks and kept in reserve at 60^{o}C.

Experimental Animals

In the present study, sexually mature, 5-6 week old male (25 ± 5g) BALB/c mice were used. Mice were housed in propylene cages (430×270×150 mm^3, six animals per cage) (Gharib Naseri, 2003) at 20-25^{o}C, bedded with clean rice husk in well aerated animal room of the Department of Microbiology, Panjab University, Chandigarh. All the animals

received standard pellet diet and water ad libitum. Animals were acclimatized to the new housing and experimental conditions for atleast one week. The experimental protocols were approved by the Institutional Animals Ethics Committee of the Panjab University Chandigarh (Registration No. 51/ 1999/CPC/SEA) and performed in accordance with the guidelines of the committee for the Purpose of Control & Supervision of Experiments on Animals (CPCSEA), Government of India, on animal experimentation.

Spermatozoa from Human Samples

Semen samples were procured from males turning up at infertility clinic at the Department of Urology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, by masturbation into a sterile plastic specimen cup after an advised abstinence period of 48h.Only those ejaculates corroborating normal semen parameters as per WHO protocols were used (WHO, 2010).

In vitro effect of carnitine/tamoxifen/nicotine on human spermatozoa on coincubation with E. coli

To evaluate the effect of these chemical compounds on human spermatozoa on coincubation with *E. coli*, the following set of reaction mixtures were made

- Semen sample(100µl)+ PBS (100µl)
- Semen sample(100µl) + *E. coli*(100µl)
- Semen sample(100µl)+ PBS (100µl)+ Carnitine (2.5mg, 5mg, 10mg and 20mg)
- Semen Sample (100µl) + *E. coli* (100µl) + Carnitine (2.5mg, 5mg, 10mg or 20mg)
- Semen sample(100µl) +PBS(100µl) + Tamoxifen (5mg, 10mg, 20mg or 50mg)
- Semen Sample (100µl) + *E. coli* (100µl) + Tamoxifen (5mg, 10mg, 20mg or 50mg)
- Semen sample(100µl) +PBS (100µl) + Nicotine (0.1mg, 0.5mg, 0.75 mg or 1.0 mg)
- Semen Sample (100µl) + *E. coli* (100µl) + Nicotine (0.1mg, 0.5mg, 0.75 mg or 1.0 mg)

The reaction mixtures were then kept for incubation at 37°C. After incubation, a wet preparation was made using 10 µl of each mixture and observed at 400X magnification under light microscope.

Impact of intraperitoneal inoculation of carnitine/ tamoxifen/nicotine on reproductive potential of male mice challenged with *E. coli*

Preparation of Inoculum

The sperm agglutinating *E.coli* was grown in LB under shaking conditions (150 rpm) for 24 h at 37°C. After incubation, the culture broth was centrifuged at 4°C at 10,000 rpm for 10 min. The pellet so obtained was washed twice with Phosphate Buffer Saline (PBS) (50mM, pH 7.2). The pellet was then suspended in same buffer to achieve a final concentration of 10^8cfu/20µl.

Experimental Design

In order to assess the role of chemical compounds on reproductive potential of male mice challenged with sperm agglutinating *E. coli*, male Balb/c mice (n=24) were divided into eight groups and given following treatments.

- Group I: PBS (Control)
- Group II: 10^8 cfu of sperm agglutinating *E. coli*
- Group III: 5mg of carnitine per 25g of mice.
- Group IV: 5mg carnitine per 25g of mice challenged by *E. coli*
- Group V: 0.5 mg of tamoxifen per 25g of mice
- Group VI:0.5 mg of tamoxifen per 25g of mice challenged by *E. coli*
- Group VII: 0.1mg of nicotine per 25g of mice
- Group VIII: 0.1mg of nicotine per 25g of mice challenged by *E. coli*

The administered volume for each mouse was 20µl. Each chemical agent *viz.* carnitine/tamoxifen/ nicotine was intraperitoneally administered at 24 hour intervals for 14 consecutive days whereas *E. coli* was administered as a single dose intravasally.

Intravasal Inoculation

Mice were anesthetized with ketamine (75mg/ kg) and xylazine (12mg/kg)) and under aseptic conditions the right testis and epididymis were exteriorized via a vertical incision of the scrotum. The inoculum (20 µl) was instilled into the lumen of the right vas deferens by using a 27-gauge needle towards the direction of epididymis. Incisions were closed with 3-0 silk suture and animals were housed individually in isolated propylene cages to prevent transmission of the organism. There was no mortality due to the surgical procedure and the animals revived quickly (Figure 1).

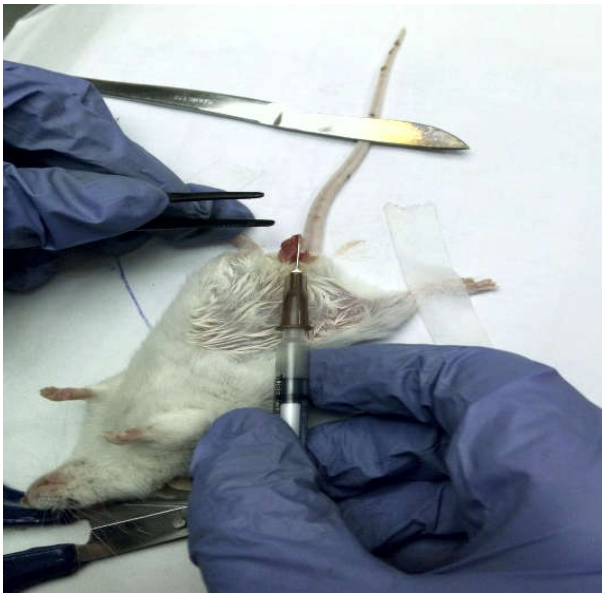

Fig. 1: Photograph of intravasal inoculation

Weight Profile

Mice from each group were weighed prior the establishment of experiment and on day 14 before sacrificing them to evaluate any effect on the weight.

Autopsy Schedule

On day 14, mice were autopsied from each group. The reproductive (caudal epididymis, vas deferens, testes) and non-reproductive organs (bladder, kidneys, liver and spleen) were removed aseptically. Fuether following procedures were carried out

- Evaluation of Seminal parameters/ Sperm analysis
- Calculation of tissue somatic indices
- Viable Bacterial load determination/ Organ culture
- Reisolation of the administered microorganism
- Tissue histology

Evaluation of Seminal Parameters/ Sperm Analysis

Sperm number, motility and morphology are traditionally used as markers of male fertility. Therefore, these seminal parameters were examined. The characteristics of sperm like motility, morphology, viability and screening of the preparation for the presence of any cellular elements other than spermatozoa was also carried out.

Sperm Motility

For sperm motility evaluation, a fixed volume of 10µl of the sample obtained was delivered on a clean glass slide with a micropipette, covered by a cover slip (22mm x 22 mm) and examined under the light microscope at a magnification of 400X while evaluating different fields. For the purpose of this study, motility was classified as either motile or non-motile. After assessing different microscopic fields, the relative percentage of motile and immotile sperms was determined (Salisbury *et al.*, 1978).

$$\% \text{ Motile sperms} = \frac{\text{No. of motile sperms/field}}{\text{No. of total sperms/field}} \times 100$$

Viability Test

For sperm viability evaluation, the concept of using eosin to differentiate live and dead cells was used. For this, 0.5% (w/v) Eosin Y (Colour index 45380) was prepared by dissolving 0.5g of Eosin Y in 100 ml of 0.9% NaCl. Then, 10µl of semen sample was mixed with equal volume of 0.5% eosin on a microscopic slideusing a pipette tip by swirling the sample on the slide.The suspension was covered with a 22mm x 22mm coverslip and left undisturbed for 30 s and observed under the light microscope (Olympus India Pvt. Ltd.) at 400X. The live and dead spermatozoa were counted in number of fields and percentage viability was calculated.

Calculation of Tissue Somatic Indices (TSI%)

Twenty-four hours after the last dose administration, mice in each group were sacrificed by cervical dislocation. Both the reproductive and non-reproductive organs were removed aseptically from the mouse with the help of a dissection kit. They were then freed from the adherent tissues and blood, grossly examined and weighed. The TSI (percent organ weight in relation to body weight) was calculated according to the equation

$$\text{Tissue index} = \frac{\text{Weight of the tissue in grams}}{\text{Weight of the body in grams}} \times 100$$

Viable Bacterial Count Determination

One half of the organs dissected under sterile conditions were weighed and used to determine the viable bacterial load. The organs were immersed in 500 µl PBS (50mM, pH 7.2) in separate eppendorfs. These organs were homogenized manually in a pestle containing phosphate buffer saline solution to form a mixture. 100µl of this mixture was spread on LA and incubated at 37°C for 24h. After

incubation, the number of colonies were counted and the cfu/g/tissue was calculated.

Re-Isolation of Microorganisms

The isolates so obtained were streaked on selective media i.e. Eosin Methylene Blue Agar, to recover microorganism present in the reproductive and non-reproductive organs viz vas deferens (left and right), testes (left and right), cauda epididymis, bladder, liver, spleen and kidneys (left and right) after 14 days of inoculation with sperm agglutinating *E.coli*.

Tissue Histology

In parallel, other half of the reproductive and non reproductive organs which were previously collected were examined for any histopathological changes. Tissues were fixed in 10% buffered formalin, processed for histological analysis and were examined under 40X and 100X objective using bright field microscope (Olympus India Pvt. Ltd.). The slides were then photographed by Nikon camera fitted on the microscope.

Results

Microorganisms

The sperm agglutinating strain of *E. coli* isolated from semen samples of infertile males undergoing semen analysis, already available in the laboratory, was used in the present study.

In vitro effect of carnitine/tamoxifen/nicotine on human spermatozoa coincubated with *E. coli*

The impact of different concentrations (2.5, 5, 10, 20 and 50mg) of carnitine was examined on human sperm parameters *in vitro*.

The results revealed that in comparison to motility (39%) and viability (51.2%) of control group (PBS), carnitine resulted in an increase in sperm motility and viability. At a concentration of 2.5, 5, 10 and 20 mg, carnitine enhanced the sperm motility to 43.75, 53.3, 47.05 and 43.75% respectively. A noteworthy observation in the form of toxicity was observed at a concentration of 50mg where sperm motility reduced to zero.

A pattern similar to that of motility was observed in case of viability wherein the percent viability increased to 58.75, 66.67, 63.75 and 53.75 at a concentration of 2.5, 5, 10 and 20mg, respectively, whereas it reduced to zero at a concentration of 50mg.

However, the sperm morphology was normal in both test and control groups as no decapitation or curling of tail was observed (Table 1).

When the effect of tamoxifen was assessed on human sperm parameters, a pattern identical to that of carnitine was observed where at a concentration of 5, 10 and 20mg, percentage of motile spermatozoa increased to 43.7, 50 and 43.75; and percentage viability increased to 66.5, 55.5 and 68.75, respectively. However, at a higher concentration of 50mg, both the parameters reduced to zero (Table 2).

Table 1: Impact of coincubation of carnitine and *E. coli* on sperm parameters *in vitro*

Parameters	PBS	*E. coli*	Carnitine (mg)					*E. coli* + Carnitine (mg)			
			2.5	5	10	20	50	5	10	20	50
Motility%	39	0(A)	43.75	53.3	47.05	43.75	0	0(A)	0 (A)	0 (A)	0 (A)
Viability%	51.2	**	58.75	66.67	63.75	53.75	0	**	**	**	**
Morphology	N	N	N	N	N	N	N	N	N	N	N

** could not be determined due to agglutination of spermatozoa
N : Normal
A : Agglutination

Table 2: Impact of coincubation of tamoxifen and *E. coli* on sperm parameters *in vitro*

Parameters	PBS	*E. coli*	Tamoxifen (mg)				*E. coli*+ Tamoxifen (mg)			
			5	10	20	50	5	10	20	50
Motility%	37	0(A)	43.7	50	43.75	0	0(A)	0(A)	0(A)	0(A)
Viability%	50	**	66.6	55.5	68.75	0	**	**	**	**
Morphology	N	N	N	N	N	N	N	N	N	N

* could not be determined due to agglutination of spermatozoa
N : Normal
A : Agglutination

Table 3: Impact of coincubation of nicotine and *E. coli* on sperm parameters *in vitro*

Parameters	PBS	*E. coli*	Nicotine (mg)				*E. coli*+ Nicotine (mg)			
			0.1	0.5	0.75	1	0.1	0.5	0.75	1
Motility%	71	0(A)	37.03	47.1	32.6	30.3	0(A)	0(A)	0(A)	0(A)
Viability%	83.2	**	65.4	52.8	48.3	57.2	**	**	**	**
Morphology	N	N	N	N	N	N	N	N	N	N

** could not be determined due to agglutination of spermatozoa
N : Normal
A : Agglutination

Table 4: Seminal parameters of male mice after treatment with spermagglutinating *E. coli* followed by intraperitoneal administration of chemical compounds (carnitine, tamoxifen and nicotine)

Organs	Parameters	PBS	*E. coli*	Carnitine only	Carnitine+ *E. coli*	Tamoxifen only	Tamoxifen + *E. coli*	Nicotine only	Nicotine+ *E. coli*
Left vas deferens	Total count (x 10^6 /ml)	39	9	40.88	11.83	19.6	13.8	17.3	ND
	% Motility	30.11	8.57	68.19	77	55	58	22.69	ND
	% Viability	42.7	23.8	72.1	77.4	66	64	36.2	ND
Right vas deferens	Total count (x 10^6 /ml)	50	0	23.37	0	8.7	1	12.28	0
	% Motility	58.8	0	59.35	0	47	0	20.9	0
	%Viability	62.7	0	67.3	0	51	0	22.4	0

While evaluating the effect of different concentrations of nicotine (0.1, 0.5, 0.75, 1mg) on the seminal parameters, results indicated that sperm motility declined from 71% in case of PBS control to 37.03 (0.1mg), 47.1 (0.5mg) and 32.6 (0.75mg), 30.3 (1mg). Similarly, percent viability also reduced from 83.2 (PBS) to 65.4 (0.1mg), 52.8 (0.5mg), 48.3(0.75mg) and 57.2 (1mg) (Table 3).

In order to study the effect of carnitine/ tamoxifen/nicotine co-incubated with *E. coli*, semen sample was mixed with *E. coli* and varied concentrations of either of the three chemical agents. None of the parameters could be evaluated since *E .coli* caused agglutination of human spermatozoa indicating that these chemical agents were unable to exhibit any effect in the presence of *E. coli.*

Impact of intraperitoneal inoculation of carnitine/ tamoxifen/nicotine on reproductive potential of male mice challenged with *E. coli.*

The effect of chemical compounds (carnitine/ tamoxifen/nicotine),administered intraperitoneally, on reproductive potential of male mice challenged with *E. coli* intravasally was determined in terms of body weight, seminal parameters, bacterial load and histopathological changes.

Weight Profile

For assessment of treatment related changes in body weight, the initial (day 1) and final (day 14) body weights of the animals were recorded. Results revealed that the control group receiving PBS showed 6.6% increase in weight, however, *E. coli* inoculated group showed a decrease of 6.8% in the body weight.

Further, in case of the mice inoculated with carnitine, an increase of 10.3% in body weight was observed. Interestingly, an increase of 6.6% in body weight was noticed when carnitine was inoculated in mice challenged with *E. coli.*

Body weight of mice administered with tamoxifen only, 12.5% increase in weight was seen but in mice challenged with *E. coli,* tamoxifen resulted in 11% reduction in body weight.

In contrast, nicotine treated mice showed a decrease in body weight by 5% whereas enhanced decrease in body weight (28%) was recorded when mice challenged with *E. coli* were administered with nicotine (Figure 2).

Tissue Somatic Index (TSI)

The TSI of various reproductive and non-reproductive organs was examined in all the groups. Results revealed that in all the groups, there were no changes in the TSI values of reproductive organs and the non reproductive organs except for left and right vas deferens of mice inoculated with both *E. coli* and nicotine where TSI values reduced to 0.005 in comparison to 0.02 in control (Figure 3,4). Reduction in the TSI values of right and left vas is indicative of diminished reproductive vigour of the mice as a result of *E. coli* infection.

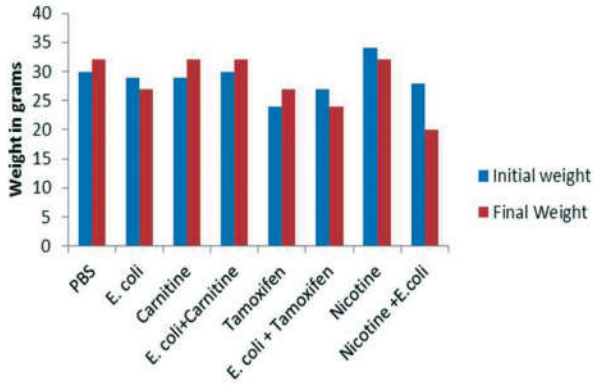

Fig. 2: Body weight profile of mice after administration with sperm agglutinating *E. coli* and various chemical compounds (carnitine/ tamoxifen/ nicotine)

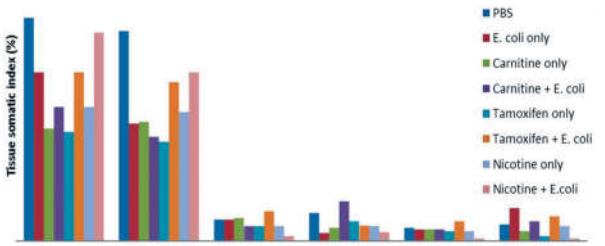

Fig. 3: Tissue somatic indices (TSI) of reproductive organs of mice administered with various chemical agents (carnitine/ tamoxifen/ nicotine) and *E. coli*

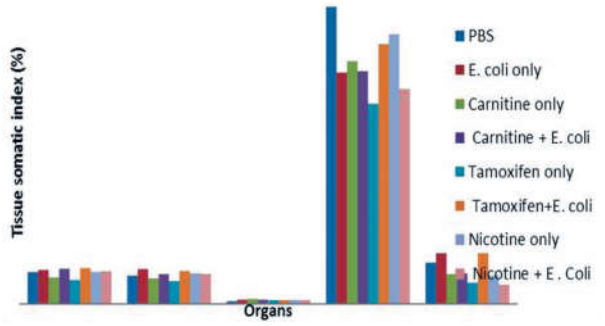

Fig. 4: Tissue somatic indices (TSI) of non-reproductive organs of mice administered with various chemical agents (carnitine/ tamoxifen/ nicotine) and *E. coli*

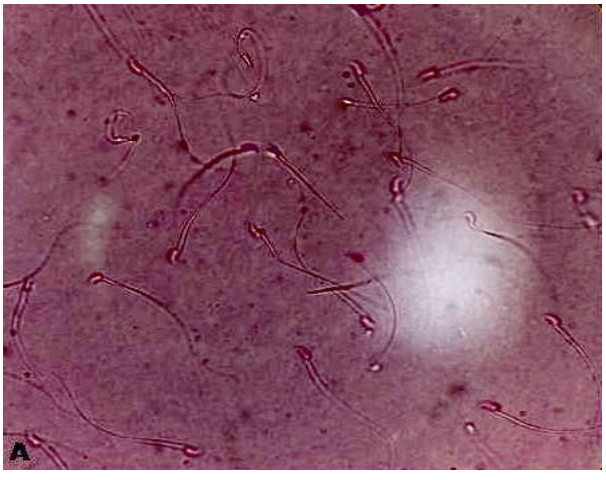

Fig. 5:

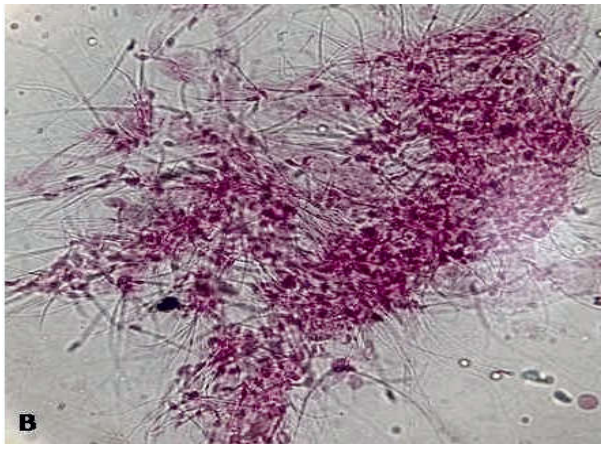

Fig. 5: Light micrographs of A) control group receiving PBS showing normal spermatozoa, (B) group administered with *E. coli* showing sperm agglutination

Evaluation of Seminal Parameters

On day 14, all the animals were sacrificed for the evaluation of seminal parameters *viz.* sperm count, viability and morphology. Spermatozoa were extracted separately from left (non-treated) and right (treated) vas deferens. The results showed that in control (PBS) group, the total sperm count, motility and viability in the left vas deferens was found to be approximately 39 x 10^6/ml, 30.11% and 42.7% respectively whereas in the right vas deferens, the corresponding values were 50 x 10^6/ml, 58.8% and 62.7% respectively. Upon challenging mice with *E. coli*, the count, motility and viability in left vas deferens decreased to 9 x 10^6/ml, 8.57% and 23.8%whereas the values reduced to zero in the right vas deferens (Table 5, Figure 5).

When the seminal parameters of the mice receiving carnitine were examined, the total sperm count in left vas deferens was comparable to that of PBS control whereas it decreased to 23.37 x 10^6/ml in right vas deferens from 50x 10^6/ml in control. Further, motility and viability increased both in case of left (68.19%, 72.1%) and right vas deferens (59.35% and 67.3%) in comparison to control. However, *E. coli* challenged mice upon administration with carnitine resulted in decreased sperm count (11.83x 10^6/ml) and enhanced motility (77%) and viability (77.4%) in left vas deferens. Interestingly, the total sperm count in right vas deferens declined to zero (Table 4).

Upon assessing the effect of tamoxifen on seminal parameters, it was observed that total sperm count decreased to 19.6 x 10^6/ml and 8 x 10^6/ml in left and right vas deferens, respectively, as compared to control. Further, motility and viability in left vas deferens increased to 55% and 66% respectively, whereas right side showed diminished motility and

viability (47 and 51%, respectively) as compared to control receiving PBS. When tamoxifen was administered in mice challenged with *E. coli*, it was found that motility and viability (58 and 64%) in left vas deferens were comparable to control. But in case of right vas deferens (infection side) the effect of *E. coli* was more pronounced with complete loss of spermatozoa (Table 4).

In contrast, intraperitoneal infusion of nicotine affected sperm parameters negatively and reduced the total sperm count, motility and viability in both left (17.3 x 10^6/ml, 22.69%, 36.2%) and right (12.28 x 10^6/ml, 20.9%, 22.4%) vas deferens respectively. The deteriorative effect of nicotine on seminal parameters was aggravated when administered in mice challenged with *E. coli*. None of the parameters could be determined in the left vas deferens due to sperm agglutination whereas azoospermia was observed in the right vas deferens (Table 4).

Viable Bacterial Load Determination

Bacterial load was determined for both reproductive as well as non-reproductive organs of the mice in all the groups in terms of log cfu/g of tissue. The groups receiving PBS did not show the presence of any bacterial isolate inferring that the control mice were free from any microorganism. When *E. coli* was intravasally inoculated into the right vas deferens, bacterial load estimated from the right set of reproductive organs showed that bacteria could be isolated from vas deferens, caudal epididymis and testis with a $\log_{10}$ cfu of 4.5, 8.02 and 5.1 respectively. Interestingly, the bacteria could also invade and colonize left side since $\log_{10}$ cfu invas deferens, caudal epididymis and testis was found to be 4.4, 7.5 and 7.8.

When carnitine was intraperitoneally inoculated, all the organs were found to be bacteriologically sterile. However, upon intraperitoneal inoculation of carnitine in mice challenged with *E. coli*, higher bacterial counts of the order of 10.9, 10.3, 10.5 $\log_{10}$ cfu were present in right set of reproductive organs *viz.* testis, cauda and vas deferens, respectively. Also, the bacteria traversed to left vas deferens where it was present in higher numbers with $\log_{10}$ cfu of 11.2, whereas a comparatively lower number of bacteria could be isolated from left testis (4.9 $\log_{10}$ cfu) and left cauda (5$\log_{10}$ cfu).

Similarly, in case of tamoxifen, all the organs were culture negative. On the other hand, treatment with tamoxifen in presence of *E. coli*, resulted in isolation of bacteria from both left set of reproductive organs *viz.* vas deferens (7.3 $\log_{10}$ cfu), cauda (4.4 $\log_{10}$ cfu) and testis (5.2 $\log_{10}$ cfu) as well as right set *viz.* vas deferens (8.4 $\log_{10}$ cfu), cauda (9.4 $\log_{10}$ cfu), testis (8.7 $\log_{10}$ cfu).

In the same way, when bacterial load was calculated from group of mice receiving nicotine, none of the organs showed any bacterial growth. Although, when mice were intravasally inoculated with *E. coli* followed by nicotine, it was seen that

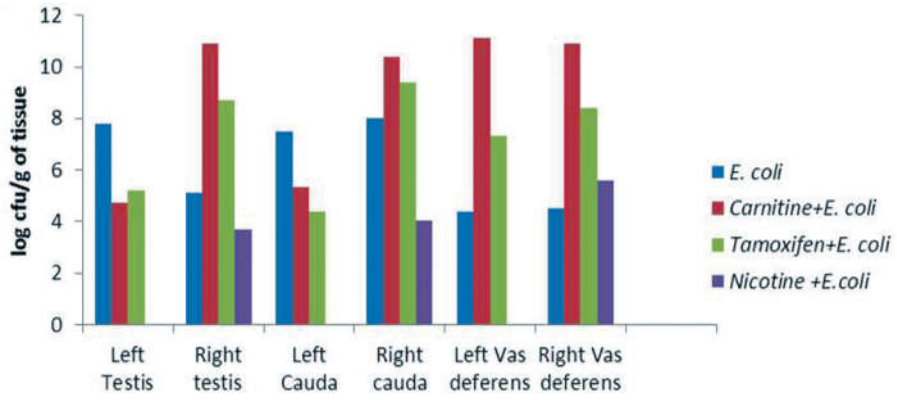

Fig. 6: Bacterial load from reproductive organs of mice after intravasal inoculation with spermagglutinating *E. coli* in the presence of Carnitine/ Tamoxifen/ Nicotine

right vas deferens, cauda and testis were culture positive with a $\log_{10}$ cfu5.6, 4.03 and 3.7, respectively (Figure 6).

Bacterial enumeration was also done in non reproductive organs of all the groups.The organs of the group of mice receiving PBS were culture negative. However, on intravasal inoculation with *E. coli* into the lumen of right vas deferens, the results showed that bacteria could be isolated from all the non reproductive organs with $\log_{10}$ cfu of 6.3 (left kidney), 6.9 (right kidney), 5.8 (liver), 7.02 (spleen) and 7.24 (bladder).

Moreover, no *in vivo* bacterial population could be enumerated in group of mice receiving either

carnitine, tamoxifen or nicotine. On the contrary, the increased $\log_{10}$ cfu of 9.83 (left kidney), 10.68 (right kidney), 10.61 (liver), 10.55 (spleen) and 11.09 (bladder) in carnitine and $\log_{10}$ cfu 7.4 (left kidney), 7.3 (right kidney), 6.1 (liver), 7.8 (spleen) and 9.9 (bladder) in tamoxifen was observed upon infusion with spermagglutinating *E. coli.* However, bacteria failed to colonise in liver and spleen whereas low bacterial count was observed in rest of the organs *viz.* left kidney ($\log_{10}$ cfu 3.4), right kidney ($\log_{10}$ cfu

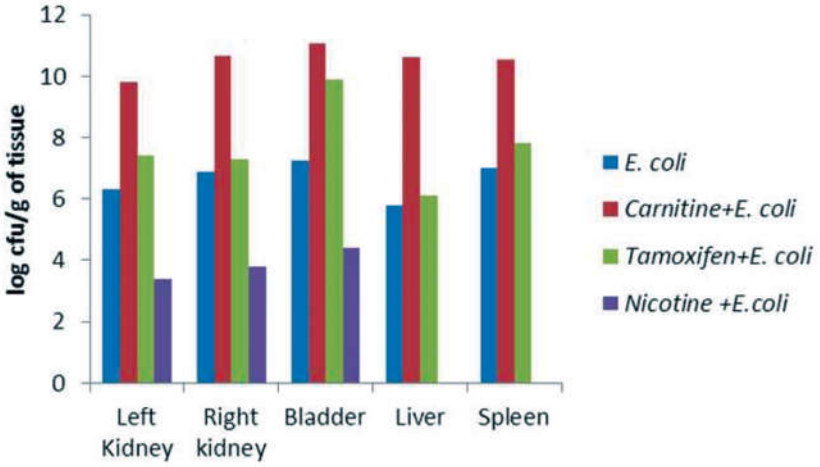

Fig. 7: Bacterial load from non reproductive organs of mice after inoculation with spermagglutinating *E. coli*, Carnitine + *E. coli*, Tamoxifen + *E. coli*, Nicotine + *E. coli*

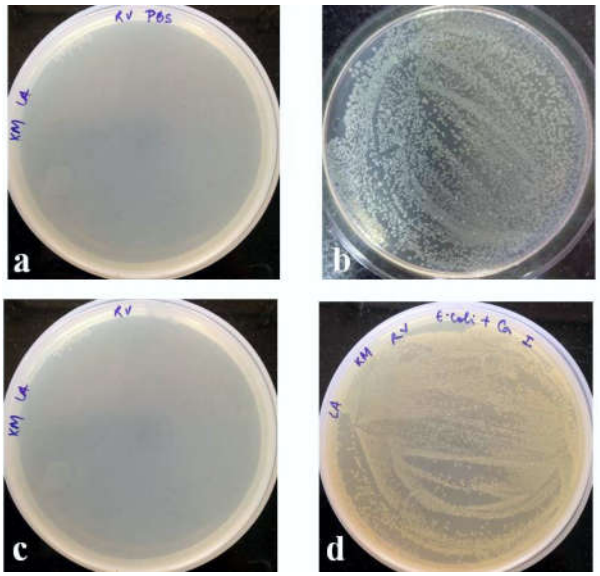

Fig. 8: Representative photographs of bacterial load from the homogenates of mice administered with a) PBS (control), b) spermagglutinating *E. coli*, c) Carnitine, d) *E. coli* followed by Carnitine

3.8) and bladder ($\log_{10}$ cfu 4.4) of mice administered with nicotine in the presence of *E. coli* (Figure 7, 8).

Recovery of Bacteria

The bacterial isolates, so obtained, were streaked on Eosin Methylene Blue agar plates. The group of mice inoculated with PBS/ carnitine/ tamoxifen/ nicotine were culture negative whereas green metallic sheen, confirming presence of *E. coli*, was

Fig. 9: Representative photograph of reisolation of spermagglutinating *E. coli* from various reproductive and non reproductive organs

observed in all the remaining groups where *E. coli* was administered alone or in presence of any of the chemical agents (Figure 9).

Histopathological Examination

Histopathological examination was carried out to observe any changes in the reproductive and non reproductive organs upon treatment with *E. coli* and the chemical agents. Sections of various reproductive and non reproductive organs (both left and right) of the control group receiving PBS showed normal histology. Testis showed normal tubules with usual spermatogenesis, caudal epididymis revealed normal epididymal tubules with adequate number of spermatozoa and vas deferens showed the presence of normal columnar epithelium. In case of non-reproductive organs,

spleen displayed regular morphology with clear distinction between red and white pulp; kidneys presented typical glomeruli and tubules and bladder showed transitional epithelial lining with normal muscle wall. The morphology of liver revealed normal cell cords, vascular channels and sinusoids with Kupffer cells (Figure 10).

However, in the mice administered with spermagglutinating *E. coli,* right vas deferens as well as right cauda displayed severe inflammation both within the mucosa and along the outer wall. The right testis also showed inflammation with hypospermatogenesis. However, all the reproductive organs of left side exhibited normal

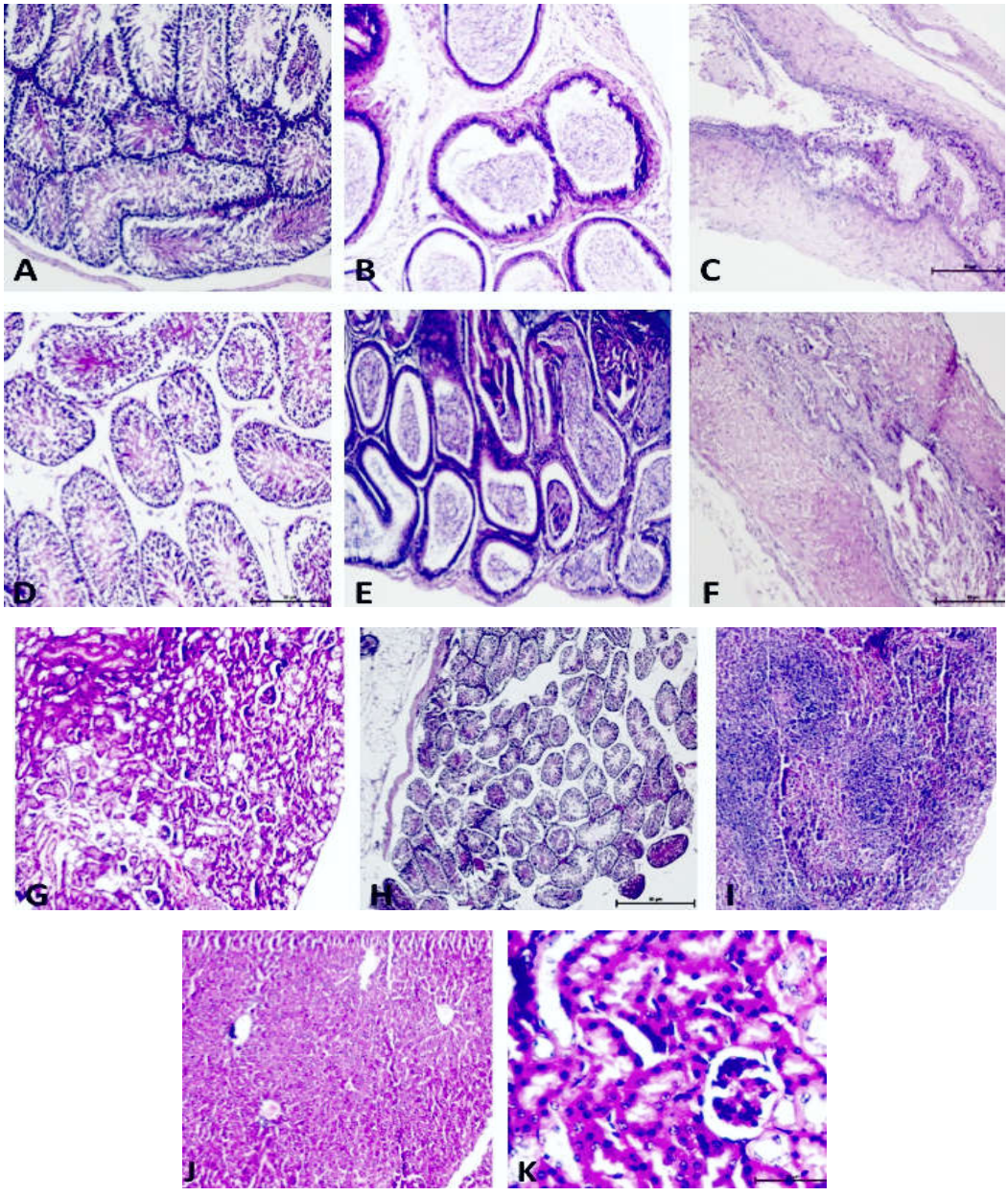

Fig. 10: Light micrographs of histopathological examination of various reproductive and non reproductive organs of control mice A) Left testis, B) Left cauda, C) Left vas deferens, D) Right testis, E) Right cauda, F) Right vas deferens, G) Right kidney, H) Left kidney, I) spleen, J) Liver and K) bladder

morphology. In case of non reproductive organs, kidneys and liver were normal but spleen was enlarged and appeared reactive with mild excess of lymphocytes in red pulp (Figure 11).

When the histopathological examination was done in group of mice receiving carnitine/ tamoxifen/ nicotine, all the reproductive and non reproductive organs showed normal tissue histology. However, when these compounds were

inoculated in the presence of *E. coli,* an altered histology was observed. In case of reproductive organs, the vas deferens and cauda of right side were inflammed whereas the right testis displayed maturation defect with cluster of spermatids in the tubules. However, the left set of organs were quite

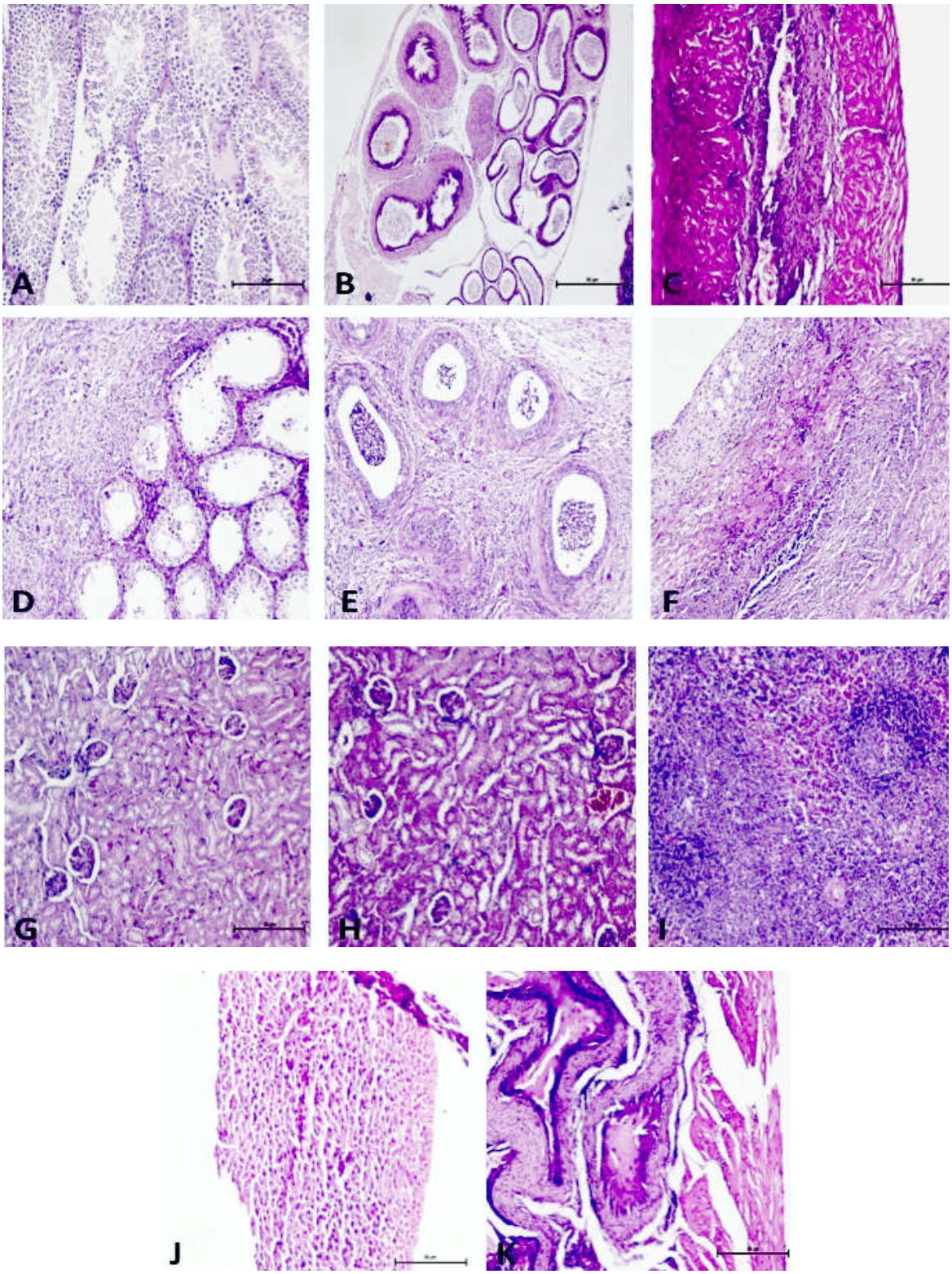

Fig. 11: Light micrographs of histopathological examination of various reproductive and non reproductive organs of mice inoculated with *E. coli* A) Left testis, B) Left cauda, C) Left vas deferens, D) Right testis, E) Right cauda, F) Right vas deferens, G) Right kidney, H) Left kidney, I) spleen, J) Liver and K) bladder

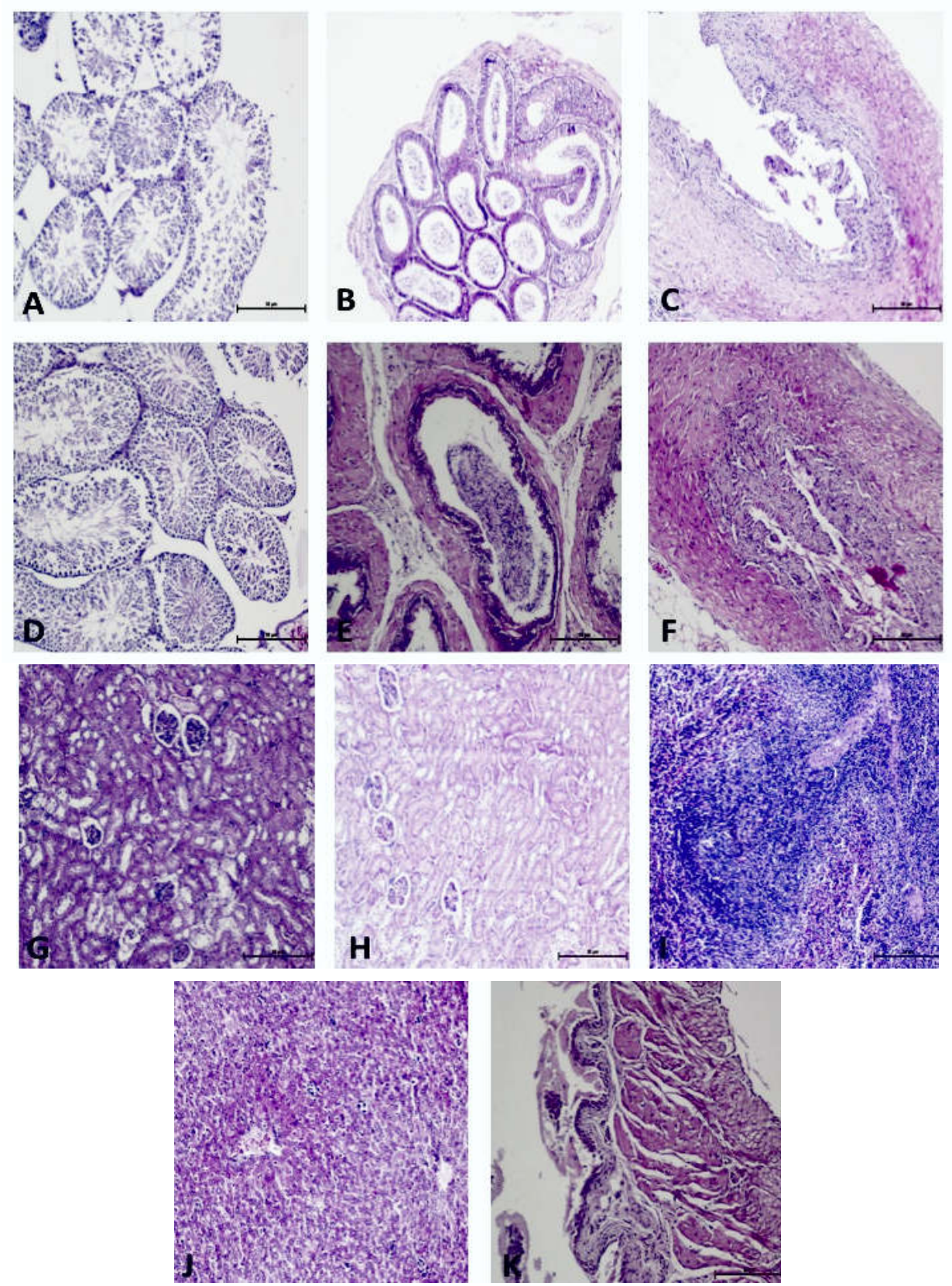

Fig. 12: Representative photomicrographs of histopathological examination of various reproductive and non reproductive organs of mice receiving carnitine/ tamoxifen/ nicotine A) Left testis, B) Left cauda, C) Left vas deferens, D) Right testis, E) Right cauda, F) Right vas deferens, G) Right kidney, H) Left kidney, I) spleen, J) Liver and K) bladder

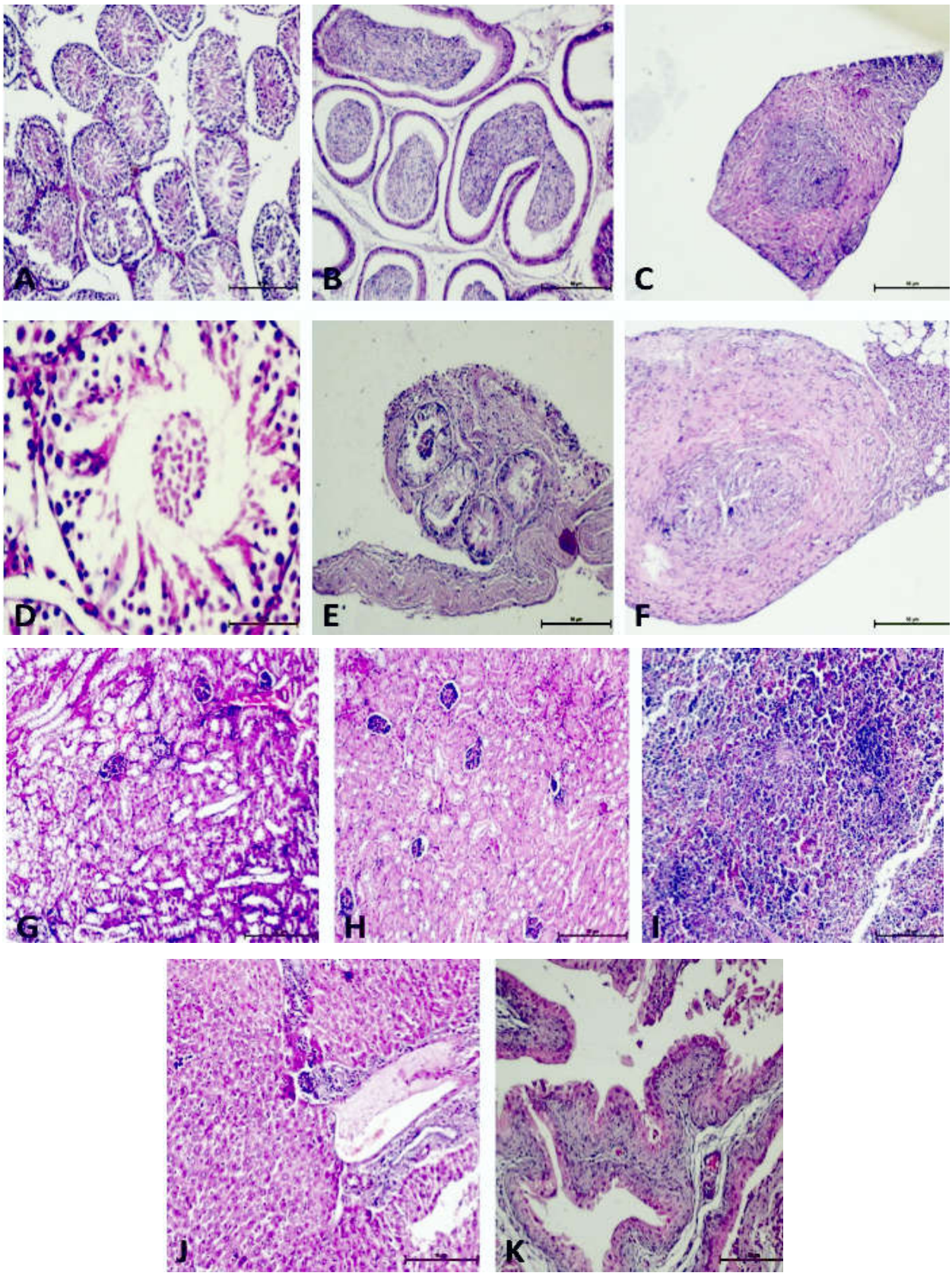

Fig. 13: Representative photomicrographs of histopathological examination of various reproductive and non reproductive organs of mice receiving carnitine/ tamoxifen/ nicotine in presence of *E. coli* A) Left testis, B) Left cauda, C) Left vas deferens, D) Right testis, E) Right cauda, F) Right vas deferens, G) Right kidney, H) Left kidney, I) spleen, J) Liver and K) bladder

normal. In case of non reproductive organs, both the kidneys and bladder showed normal histology while spleen was expanded and liver showed the presence of mild reactive lymphocytes in the lobules (Figure 12, 13).

Discussion

Among bacterial species that interact with spermatozoa are the causative agents of genitourinary infections such as *Escherichia coli*, *Ureaplasma urealyticum, Mycoplasma hominis* and *Chlamydia trachomatis* (Huwe *et al,* 1998 and Cunningham *et al,* 2008). *E. coli* undoubtedly represents the most frequently isolated microorganism in infections of genitourinary tract (Liu *et al,* 2002). It appears to affect different sites of male reproductive tract and can be accounted for upto 65-80% cases of infections (Pellati *et al,* 2008). *E. coli* can alter sperm parameters by either direct attachment or by producing certain soluble factors. Moreover, it can lead to various morphological defects and thereby, a decrease in the fertilizing potential of the spermatozoa. In addition to urogenital tract infections, there has been mounting apprehension about the role of substances in the environment to disturb male reproductive potential. Agents in this group that can affect male fertility comprise alcohol, tobacco, smoking and illicit drugs etc. (Pasqualotto *et al,* 2004). Several reports have demonstrated that smoking has a considerable negative influence on sperm production, motility, and morphology. It can also affect rapidly dividing germ cells in testis. This deteriorative effect of smoking on sperm parameters has been attributed to nicotine (Harlev *et al,* 2015).

Various infertility management choices have been recommended to enhance the sperm count, motility and viability that include general, medical and surgical methods. However, the alternative therapy (nutraceuticals), which is considered the harmless amongst these, has become more popular among the masses. It involves the use of multivitamins (antioxidants), L-carnitine (sperm vitalizers), tamoxifen (antiestrogen) etc. (Peyvandi *et al*, 2009). L- carnitine is present in the epididymis and helps in sperm metabolism and maturation (Agarwal and Said, 2005). It has also been reported to increase sperm concentration and total sperm count in men with asthenozoospermia. Also, it protects sperm membrane against the attack of reactive oxygen intermediates (Aram *et al,* 2012). Tamoxifen, non-steroidal anti-oestrogen drug has also been evaluated for empirical treatment of idiopathic male infertility. It appears to have a beneficial effect on endocrinal outcomes and it has been found to improve sperm count, motility and functional sperm fraction (Nada *et al,* 2015). Therefore, this experimental study was designed to investigate the correlation between *E. coli* infection, chemical compounds and male reproductive potential.Many microorganisms have been greatly acknowledged to impede sperm parameters and thereby reducing the fertilizing potential of males. Thus, the clinical isolate of *E. coli* capable of causing 100% spermagglutination was used in the present study.

Reports from earlier studies have demonstrated that supplementation with various agents like sugars and ions can improve or reduce the sperm motility *in vitro*.

On similar grounds, when the impact of carnitine, tamoxifen and nicotine was studied on sperm parameters, it was observed that at lower concentrations carnitine and tamoxifen can enhance sperm motility and viability. These results are in concordance with the findings of Sariozkan *et al,* 2014 wherein they have shown that carnitine and glutamine significantly increase the percentage of motile spermatozoa. However at higher concentrations, carnitine and tamoxifen tend to inhibit the motility and viability of spermatozoa. Rai and Vijayalaxmi (2001) have also reported the significant increase in the number of abnormal sperms at higher doses of tamoxifen indicating towards the genotoxicity of these drugs. The nicotine at different concentrations (d"50mg) was found to reduce sperm motility and viability *in vitro*. Findings by Oyeyipo *et al,* 2014, have also shown a significant reduction in sperm motility and viability on exposure of nicotine at a concentration of e"5mM.

In an attempt to study the *in vitro* effect of coincubation of carnitine/tamoxifen/nicotine and *E. coli* on sperm parameters, semen sample was coincubated with these compounds and *E. coli*. The results showed that tamoxifen, carnitine and nicotine were not able to recuperate the alterations in sperm parameters caused by *E. coli*. These results are in concordance with earlier studies done in our lab wherein chelators like sodium citrate and EDTA were unsuccessful in inhibiting immobilization of spermatozoa induced by *E. coli* (Kaur and Prabha, 2014). In contrast Fraga *et al.* (1991) and Dawson *et al.* (1992) have reported dietary supplementation of Vitamin C improves the sperm quality and has a beneficial effect on the integrity of sperm DNA in male smokers.

To study the *in vivo* relevance of these results, *E.*

coli challenged male BALB/c mice were administered intraperitoneally with carnitine/ tamoxifen/nicotine. Mice were sacrificed on day 14 and their impact on male reproductive potential was determined in terms of body weight profile, tissue somatic indices, seminal parameters, bacterial load and histopathological analysis.

Understanding the body weight profile is important for good health, therefore, body weights of the mice in each group were estimated. The results showed normal weight profile in group receiving PBS, carnitine and tamoxifen. However, intravasal inoculation with *E. coli* led to decrease in body weight. Similar results have been reported by van Heeckeren (2000), wherein they have correlated weight loss with the inoculation of mice with *Pseudomonas.* Zhu *et al.* (2012) while studying the role of macrophages in bacterial infections has also reported loss in weight upon infection with bacteria. When carnitine was administered in *E. coli* challenged mice, it mitigated the effect of *E. coli*, thereby, resulting in increased body weight. However, in case *E. coli* challenged mice, tamoxifen could not ameliorate the effect of *E. coli* on body weight, as decrease in body weight was observed. Wallen *et al,* (2002) have also reported altered body weight profiles in hypertensive female rats that used tamoxifen. On the other hand, nicotine led to decrease in weight when administered alone or with spermagglutinating *E. coli*. Similar results are also available in literature stating that nicotine has undoubtedly been the most effective long-term weight control drug in use over the past century (McGovern and Benowitz, 2011).

To investigate the functional status of the various reproductive and non reproductive organs under various experimental conditions, the TSI was calculated. No remarkable variations were observed in TSI values of all the organs except vas deferens of *E. coli* challenged mice inoculated with nicotine. This was consistent with the results obtained by Reddy *et al,* (2011) wherein oral administration of nisin revealed no significant treatment related changes in TSI of different organs. In an earlier work done in our laboratory, no changes in TSI values were observed when female mice were intravaginally inoculated with spermagglutinating *E. coli* (Kaur and Prabha, 2014). This study indicates that only after substantial damage to the tissues, apparent changes in the organ weight could be observed. In a previous study carried by Sharma *et al.* (1993), they have also demonstrated that tamoxifen did not affect the weights of reproductive organs at a dose of 40g per kg when given for 90 days.

Various spermiogram parameters, such as altered sperm count, motility and viability have been used for diagnosis of silent genital tract infections (Shimoya *et al,* 1993). Therefore in the current study the impact of sperm agglutinating *E. coli* and chemical compounds was checked on seminal parameters. *E. coli* was found to deteriorate the quality of seminal parameters by reducing sperm count, motility and viability in left side and decreased to zero on the right side. Similar findings have also been reported by Demir *et al* (2007), who have witnessed a decrease in sperm concentration when *E. coli* was inoculated into right ductus vas deferens.

Further when carnitine and tamoxifen were intraperitoneally inoculated, they led to enhancement of sperm motility and viability on both left and right sides. However, in *E. coli* challenged mice, carnitine and tamoxifen improved sperm motility and viability on left side but failed to ameliorate the damage to seminal parameters done by *E. coli* on the right side. Various studies have demonstrated the efficacy of L carnitine in treating male infertility due to idiopathic or microbial infections by increasing sperm count, motility and semen volume significantly (Adel *et al,* 2009; Moradi *et al,* 2010). Authors have suggested supplementation with carnitine improves sperm quality in testis of mice exposed to physical insults such as heat and radiation (Chi Ming *et al,* 2004).

In case of tamoxifen, various arguments have been proposed in favour of and against the effectiveness of tamoxifen in improving sperm quality. Nada *et al,* (2015) reported improvement in sperm concentration and morphological parameters but not in sperm motility on treatment with tamoxifen. On the other hand, Motrich *et al,* (2007) stated that tamoxifen treatment significantly alters sperm quality thereby compromising fertility ability of rats.

Furthermore, nicotine when administered alone led to decrease in seminal parameters; its negative effect was intensified when inoculated in mice challenged by *E. coli.* The deleterious impact of nicotine has also been highlighted by Oyeyipo *et al.* (2013), who have shown that on daily administration of 1mg/kg of nicotine for 4 weeks significantly decreased the progressive motility of the sperms in a dose dependent manner.

The bacterial load was monitored by quantitative culture of the homogenates of organs to assess the colonization of bacteria. The results showed that all organs of the mice in groups receiving PBS,

carnitine, tamoxifen or nicotine were found to be sterile. However, in the group of mice inoculated intravasally with *E. coli*, although the inoculation was done in right vas deferens, the bacterial isolates were recovered from the organs of left side as well. Moreover, addition of carnitine and tamoxifen increased the number of bacteria isolated from different reproductive and non-reproductive organs. This increase can be attributed to the antioxidant property of carnitine which protects bacterium against oxidative stress thereby helping in its proliferation. Beumar *et al* (1994) and Atroshi *et al* (1998) have also observed the positive effect of carnitine on growth of *Listeria monocytogenes* and *E. coli* respectively. When nicotine was administered in *E. coli* challenged group, low counts in different organs were observed with complete absence in left testis, liver and spleen. Baek *et al.* (2012) in their study have suggested that nicotine might exert an inhibitory effect on the growth of *Porphyromonas gingivalis.*

To further elucidate that these detrimental effects were due to *E. coli,* the isolates so obtained were subjected to identification by their growth on EMB agar. All the isolates showed presence of green sheen thereby indicating the presence of *E. coli.* Similar results have also been reported by Jantos *et al* (1998) who have successfully recovered *Chlamydia psittaci* from different organs of male rats after intravasal inoculation.

In order to evaluate any morphological alterations in reproductive and non reproductive organs induced by colonization of *E. coli,* histopathological examination was carried out. The results show that control group had normal histology whereas right testis, cauda and vas deferens showed severe inflammation on intravasal inoculation with *E. coli.* However, organs on left side were normal. Non-reproductive organs also displayed usual morphology except spleen. Jantos *et al.* (1998) have also shown prominent swelling of cauda epididymis in rats sacrificed on day 14 post inoculation with *C. psittaci.* Also, in testis histological alterations were characterized by mild to severe reduction in spermatogenesis and focal intratubular and interstitial infiltration of mononuclear cells.

In case of group treated with carnitine, tamoxifen and nicotine usual morphology of the organs were observed. When carnitine, tamoxifen and nicotine were intraperitoeally administered in *E. coli* challenged mice, inflammation was observed in reproductive organs of right side in all these cases, however, reproductive organs were normal on left side. Amongst the non- reproductive organs, bladder and kidneys displayed normal morphology whereas liver and spleen were enlarged and mildly reactive. These results are in concordance with histological examination of tamoxifen treated testis where marked disorganization of the cytoarchitecture of the tubules and obliteration of the lumen was observed (Sharma *et al,* 1993). Similar reports are also available in case of nicotine where, the group of rats when exposed to nicotine showed in testis there was thickening of tunica propria and junctional specializations between the Sertoli cells were degenerated (Aydos *et al,* 2001).

Conclusion

From the results, it can be concluded that, carnitine and tamoxifen could ameliorate the adverse effect on reproductive potential of male mice challenged with *E. coli* whereas nicotine aggravated the same. Hence, it can be suggested that adopting a healthier lifestyle may prevent infertility induced by microorganisms.

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Original Article

Journal of Microbiology and Related Research
Volume 2 Number 2, July - December 2016
DOI: http://dx.doi.org/10.21088/jmrr.2395.6623.2216.3

Detection and Molecular Characterization of Indian Isolates of Canine Parvovirus in Fecal Samples

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Received on 05.10.2016, **Accepted on** 12.10.2016

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Abstract

The canine parvovirus which causes myocarditis and enteritis is considered to be a very serious pathogen causing mortality in pups. The original strain CPV2 is found to be continuously evolving and presently three antigenic strains CPV-2a, CPV-2b and CPV-2c found to be circulating worldwide. In the present study 52 fecal samples were collected from 1-2 month old pups showing clinical signs of CPV from Hisar (India) and screened using vp2 gene specific semi-nested polymerase chain reaction assay. Three fecal samples were found positive for CPV infection. The PCR products were allowed for nucleic acid sequencing and nucleotide sequences were deposited to GenBank with accession numbers KC012923, KC012928 and KC012932. The sequence analysis using various bioinformatics tools revealed that all the three isolates belong to CPV-2a genotype and are much closer to other CPV-2a genotype from India and China. The present study showed that the predominant genotype running in India is CPV2a. Therefore, CPV2a strain should be incorporated in vaccine formulation.

Keywords: CPV; CPV-2a; PCR; Genotype.

Introduction

Canine parvovirus (CPV) was first identified in USA in 1978 and was designated as CPV type 2 (CPV-2) (Appel et al., 1979). The CPV-2 was genetically distinct from previously recognized parvovirus of dogs i.e., minute virus of canines (Binn et al., 1970). After its emergence, CPV-2 became globally endemic in domestic and wild canid population (Parrish et al., 1988). It is assumed that CPV-2 was originated from Feline panleukopaenia virus (FPV) as host variant in late 1970 (Truyen et al., 1998). However, CPV-2 is still continuously emerging with nucleotide changes where as some sort of genetic stability is maintained by FPV (Decaro et al., 2008). CPV-2 is a serious pathogen which causes diarrhea and myocarditis mainly in young dogs (Perez et al., 2007). However, virus is also detected in cats (Decaro et al., 2010) and wolves (Mech et al., 2012). CPV-2 belongs to family *parvoviridae*, subfamily *parvovirinae* and genus parvovirus (Nandi et al., 2011). It has single stranded DNA genome having negative polarity with a whole genome size of 5.2kb. It has got two open reading frames which encodes for non-structural (NS) as well as structural virus protein (VP). VP proteins consist of VP1, VP2 and VP3. VP2 is major protein (80% of capsid protein), translated from an in frame ATG codon which is present within VP1 open reading frame (Rai et al., 2006). All the epitope neutralizing antibodies are found within VP2 region. However, a T cell epitope is found

within VP1 region (Rai et al., 2004). Thus, any changes in amino acid residue of VP2 protein may seriously alter the biological characteristics of virus (Parrish and Carmichael, 1986).

After a few years of its emergence CPV-2 has completely replaced by CPV-2a which may cause disease in both cats and dogs (Truyen, 1996). CPV-2a virus differ from CPV-2 by amino acid changes in the capsid protein (VP2) at positions 87 (Met to Leu), 300 (Gly to Ala), 305 (Tyr to Asp) and 555 (Val to Ile) (Castro et al., 2010). Later on, a new antigenic variant of CPV i.e. CPV-2b was emerged which is now circulating along with CPV-2a among dog population around the world. The CPV-2b differs from CPV-2a in two amino acid changes at 426 (Asn to Asp) and at 555 (Ile to Val) (Decaro et al., 2006). A third variant i.e. CPV-2c was also discovered in Italy in 2000 which has a mutation at position 426 (Asp to Glu) in main neutralizing epitope of capsid (Buonavoglia et al., 2001). The CPV-2c variant is widely distributed in Italy and is co-circulating along with CPV-2a and CPV-2b variant (Martella et al., 2004). The molecular epidemiological survey suggest that CPV-2a is predominantly found in Italy (Martella et al., 2006), India (Chinchkar et al., 2006) and Korea (Kang et al., 2008). Similarly, CPV-2b is predominantly circulating in USA, Japan, Brazil, Switzerland, Taiwan and South Africa (Shoorijeh et al., 2011). Both CPV2a and CPV2b are equally circulating in UK, Germany, Spain and Australia (Shoorijeh et al., 2011). CPV-2c is found to be circulating in Italy, (Martella et al., 2004), Vietnam, Spain, Germany, United Kingdom (Perez et al., 2007) and India (Nandi et al., 2010a).

Although, Canine parvovirus is a major infectious disease of pups, very few researches has been carried out in India with regard to various genotype circulating in different part of India. In present study we have carried out molecular genotyping of CPV-2 circulating in India so that better information can be generated for future development of vaccine against canine parvovirus.

Materials and Methods

Viral Samples

A total of 52 faecal samples from 1-2 month old pups showing various symptoms such as gastroenteritis, off smelling diarrhea with blood were collected from University Veterinary clinic of college of veterinary sciences, Hisar from July 2011 to May 2012. The fecal samples were collected in screw capped sterile cotton swab containing 0.5ml of 0.1M phosphate buffer saline (PBS).

DNA Extraction

DNA was extracted from the faecal sample by using DNAzol kit (Invitrogen, USA) according to the manufacturer's instructions. The extracted DNA was stored at -20°C for further molecular biology study.

Polymerase Chain Reaction

The samples were screened using semi nested PCR assay with published primers (Sakulwira et al., 2001). The semi nested polymerase chain reactions were performed by standardized protocol with some modifications (Sakulwira et al., 2001). In short 2µl of extracted DNA was added to a reaction mixture containing 1.25U of Taq DNA polymerase, 200µM dNTP mix, 1mM $MgCl_2$, 5 Pmol of both forward and reverse primers. The total reaction volume was made to 12.5µl by adding 9.3µ1 of nuclease free water. Primer pair: P1 (5′-TCCAGCAGCTATGAGATC-3′; nt no. 3342-3360) and P2 (5′-GATCTGTGGTAGCAATAC-3′; nt no. 4570-4588) were used for the first round amplification. For second amplification round P1 and P3 (5′-GATCTGTTGGTAGCAATAC-3′; nt no. 4070-4088) were used. The first amplification round consists of an initial denaturation step of 95^0C for 5 minute followed by denaturation at 95^0C for 30seconds, annealing at 52^0C for 50 seconds and elongation at 72^0C for 1 minute. The whole cycle repeated for 30 times and a final elongation is done at 72^0C for 10 minutes. For the second amplification step 2µl of the first PCR product were added to the reaction mixture and amplification was performed in a manner identical to that of the first step. The PCR amplicon of 747bp were visualized under UV trans-illuminator after 1% agarose gel electrophoresis.

Sequencing PCR Product

The PCR product obtained was purified with QIAquick PCR Purification Kit (Quiagen, USA) as per manufacturer's instruction. The purified PCR products were subjected to nucleotide sequencing using Genetic Analyser 3130XL (ABI, USA) machine in Department of Animal Biotechnology, LUVAS, Hisar.

Bioinformatics Analysis

The nucleotide sequence data obtained was

allowed for GenBank database search using online BlastN 2.5.0+ (Zhang et al., 2000) for similarity search with other nucleotide sequences available in GenBank. The nucleotide sequences of both ends (forward and reverse) were assembled and contig is prepared using Bioedit v7.2.5 programme (Hall, 1999). The Bioedit v7.2.5 programme was also used for multiple sequence alignment and percent nucleotide identity calculation of our CVP isolates with other isolates from Gen Bank. The phylogenetic analysis of our CPV sequences, along with other sequences from GenBank were done using Mega 5 programme (Tamura et al., 2011). The multiple sequence alignment of deduced amino acid sequences of our CPV isolates along with other CPV from genebank were done using Bioedit v7.2.5 programme (Hall, 1999).

Results and Discussion

The CPV2 was emerged in late 1970 as a host variant of Feline Panleukopenia Virus (FPV). Later on it was evolved into different variants due to continuous mutation in the nucleotide sequences which codes for capsid genes. This has raised a worldwide concern regarding the health of cats and dogs. CPV can be detected in feces by electron microscopy but only few institutes have got this facility in India. The virus can also be isolated in canine and feline kidney cells and can be confirmed by the characteristic cytopathic effect produced and also by polymerase chain reaction. The viral antigen can also be detected in clinical specimens by ELISA (Phukan et al., 2005), haemagglutination-haeminhibition tests (HA-HI), (Senda et al., 1986), nucleic acid hybridization assay (Waldvogel et al., 1992). The polymerase chain reaction were found to be 10 fold higher sensitive than others in detecting CPV in clinical samples (Truyen, 2000). The present study used PCR and found to be very effective in detecting the virus in fecal sample.

In our study a total of 52 fecal samples from 1-2 month old pups were collected. The samples were allowed for further processing and viral DNA was extracted using a commercially available DNAzol kit (Invitrogen, USA). The DNA from fecal samples was allowed for semi nested PCR using previously published primers (Sakulwira et al., 2001). The agarose gel electrophoresis revealed that three isolates from fecal samples (P4/10/HSR, P53/11/HSR and P58/11/HSR) showed specific amplification of 747 bp (Figure 1). However, remaining samples did not show any amplification. The PCR products of P4/10/HSR, P53/11/HSR and P58/11/HSR samples were purified and allowed for nucleic acid sequencing. The BlastN 2.5.0+ search revealed that these isolates showed maximum identity of >99% with nucleotide sequences of CPV-2a isolates from different regions of the world. Thus, these isolates were characterized as CPV-2a. The nucleotide sequences of P4/10/HSR, P53/11/HSR and P58/11/HSR isolates were deposited to GenBank database and Accession numbers KC012923.1, KC012928.1 and KC012932.1 respectively were assigned. These isolates showed nucleotide (nt) and deduced amino acid (aa) identity of 98.8-99.8/ 98.3-100% (nt/aa) among themselves which suggests that these isolates are distinct from each other. The multiple sequence alignment using Bioedit v7.2.5 programme showed that P4/10/HSR, P53/11/HSR and P58/11/HSR isolates have >99/ 98% (nt/aa) identity with several isolates of CPV-2a from different regions of the world.

The phylogenetic analysis of nucleotide sequences of P4/10/HSR, P53/11/HSR and P58/ 11/HSR isolates along with several other isolates from different regions of the world showed that P4/ 10/HSR and P53/11/HSR isolates form a separate close cluster with CPV-2a isolates from China (CPV/CN/JL1/2013 and CPV/CN/SD6/2014) (Wang et al., 2016) and India (Faizabad) (Doley et al., 2014) (Figure 2). However, these isolates were slightly distantly related with a CPV-2b isolate from France (04S23). Similarly, P58/11/HSR isolate was formed a separate close cluster with several CPV-2a isolates from India (WBD1 and NAG3) and China (CPV/CN/SD10/2014, CPV/CN/SD19/2014 and CPV/CN/SD18/2014) (Wang et al., 2016).

Several variants of CPV2 such as CPV2a, CPV2b and CPV2c were detected in India by different researcher (Nandi et al., 2010a, b; Mohan Raj et al., 2010). The nucleotide sequence based study revealed that CPV-2a is predominant genotype in India. With regard to the pathogenicity of the canine parvovirus the studies are showing conflicting results. Some studies suggest that CPV2b is milder pathogen when compared to other variants; however both CPV2a and CPV2b have been detected in animals with severe diarrhea (Castro et al., 2010). The CPV2c has been associated with severe hemorrhagic enteritis and mortality (Buonovoglia et al., 2001).

Since the virus has been continuously evolved to new variants like CPV-2a, CPV-2b and CPV-2c, the newer strains/isolates should be incorporated in vaccines. Since the present study has shown that predominant genotype running in India and

especially in Hisar region is CPV-2a, vaccines should be prepared keeping in view of the genotypes running regionally (Perez et al., 2007). Although, genotype specific vaccine is better than other vaccines for genotype specific vaccines against CPV, some study showed that CPV2 can give cross protection to the newly evolved CPV variants (Spibey et al., 2008). However, more detailed studies are required in this aspect. Also detailed epidemiological studies has to be performed regionally every year in order to determine which genotypes are running currently, emergence of new genotypes etc.

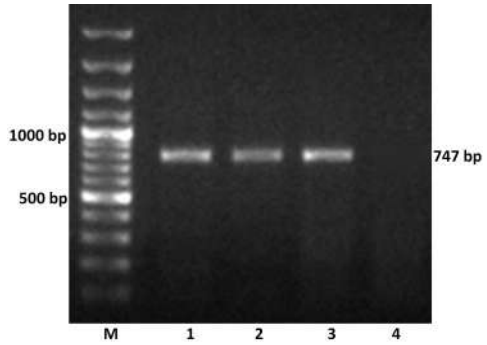

Fig. 1: Agarose gel electrophoresis of semi-nested PCR of vp2 gene of Canine Parvovirus samples showing 747 bp PCR amplicon. Lane M: Marker 1000bp: Lane 1: P4/10/HSR; Lane 2: P53/11/HSR; Lane 3: P58/11/HSR; Lane 4: Nuclease free water negative control

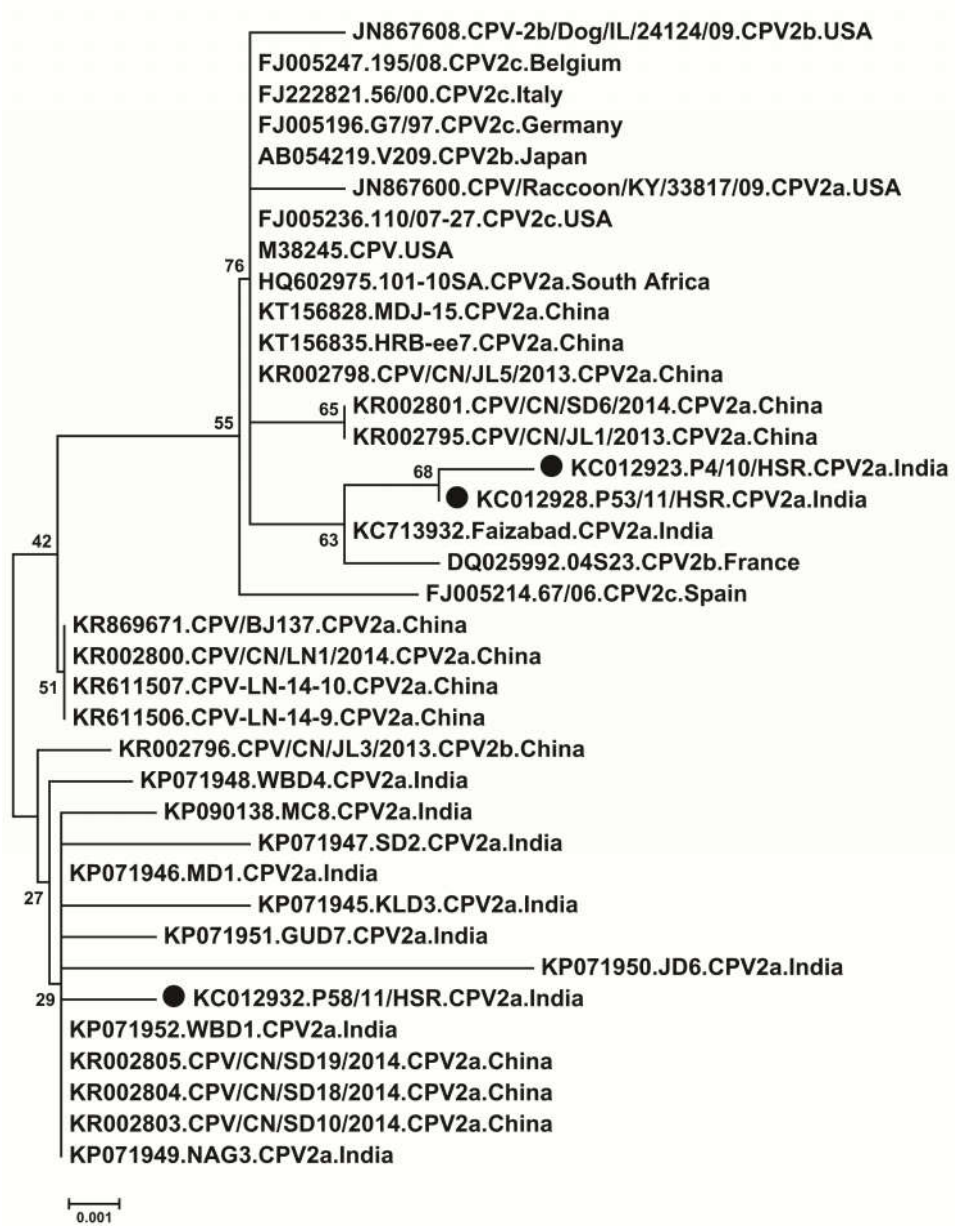

Fig. 2: Vp2 gene nucleotide sequence based phylogenetic analysis of P4/10/HSR, P53/11/HSR and P58/11/HSR isolates along with other global isolates of Canine Parvovirus 2. Phylogenetic tree was constructed using p-distance determinant of neighbor joining algorithm of Mega 5 programme with 1000 bootstrap values. ● = Isolates used in present study

Conclusions

The PCR followed by nucleic acid sequencing is a sensitive technique for molecular detection of CPV from fecal samples. The nucleic acid sequence analysis of fecal samples revealed that these isolates belong to CPV-2a genotype. The sequence analysis further revealed that P4/10/HSR, P53/11/HSR and P58/11/HSR isolates are very similar with CPV-2a isolates from India and China. The present study recommends that new vaccine should be made available in the market incorporating at least CPV-2a genotype since it is predominant genotype circulating in Hisar. Also proper awareness programs should be conducted among the dog owners for preventing the spread of the disease. Proper surveillance of CPV should be done in a regular manner to identify whether new strains are circulating or not since the sequence analysis has shown that genetic mutations are continuously occurring in virus.

Acknowledgements

The authors are thankful to the Department of Animal Biotechnology, LLR University of Veterinary and Animal Sciences, Hisar for providing infrastructural facility.

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Original Article

Journal of Microbiology and Related Research
Volume 2 Number 2, July - December 2016
DOI: http://dx.doi.org/10.21088/jmrr.2395.6623.2216.4

The Role of Protease as Detergents and Disinfectants in Instrument Cleaning and Reprocessing

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Received on 03.11.2016, **Accepted on** 12.11.2016

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Abstract

Preventing infections in patients undergoing surgical procedures is a primary goal for all members of the healthcare team. This is especially important in today's dynamic healthcare environment, in the face of newly recognized pathogens, well-known microorganisms that have become resistant to treatment modalities, and the economic pressures to reduce. Healthcare-associated infections. A key infection control practice for reducing the likelihood of a surgical site infection is proper reprocessing of surgical instruments. In its 2008 Guideline for Disinfection and Sterilization in Healthcare Facilities, the Centers for Disease Control and Prevention (CDC) notes that failure to properly disinfect and sterilize equipment carries not only the risk associated with breach of host barriers, but also the risk for person-to-person transmission as well as transmission of environmental pathogens; furthermore, thorough cleaning is required before disinfection and sterilization because inorganic and organic materials that remain on the surfaces of instruments interfere with the effectiveness of these processes. Proteases are the most important type of enzyme to look for when choosing an enzymatic detergent for medical use because there is a high content of protein in most body fluids (including blood, tissue and mucous) which cannot be easily removed with regular detergents/surfactants and water, proteases break down protein into individual amino acids or short string of amino acid. Amino acid and peptides are much more soluble in water and will float away the surface of the instrument. In the present study the slaughterhouse drainage sample collected was screened for presence of bacteria, which can utilize blood protein as their protein source. The samples were serially diluted on blood agar. From these plates, depending upon their morphological, microscopical and physiological characters, the isolates were suspected to be *Bacillus cereus.* The colonies were confirmed by cultivating on selective media. On PEMBA (Polymyxin pyruvate egg-yolk mannitol bromothymol blue agar) media the isolated colonies were peacock blue in colour. This confirms the presence of *Bacillus cereus*. The isolates have been designated as *Bacillus cereus* KLM1 KLM2, KLM3 and KLM4 Of these KLM4 isolate was chosen as potential producer of protease enzyme depending upon its zone of lysis on fibrin plates and studied further for production of protease enzyme and its compatibility for the use of enzymatic detergent preparation for the cleaning surgical instruments.

Keywords: Protease; Zymography; *Bacillus Cereus KLM4.*

Introduction

Surgery is a complex process performed by employing various surgical instruments. During surgery, the surgical instruments invariably come in contact with blood of the patients. If such instruments are not properly washed, it leads to contamination and foul smelling due to microbial degradation of blood finally paving way to transmission of diseases to other patients and health care personnel. Hence, in order to prepare surgical instruments and other medical devices for reuse, they must be cleaned with proper solutions. Cleaning not only avoids the transmission of diseases, it also forms an important aspect for the maintenance of hygiene and safety of surgical instruments. Usually the surgical instruments are washed or cleaned by sterilization or by using chemical steriliants. However, sterilization cannot be used for thermo sensitive surgical instruments; similarly chemical steriliants can not remove the microbes that usually trapped behind the bioburden that is encrusted on or within surgical instruments. Therefore, this has spurred researchers to expand their efforts to identify new technologies and products that employ novel cleaning solution for the removal of bioburden from the surgical instruments. One of such alternative steps is the use of "Biodetergents or Biocleaners". When selecting detergents and other cleaning agents for use in healthcare facilities, it is also important to remember that the agent should be compatible with the medical device to be cleaned, and also with the materials used in the cleaning equipment itself.16 For example, the chemicals should not cause corrosion in any type of automated cleaner (e.g., ultrasonic cleaner, washer disinfectors, or washer sterilizers); and they should not promote electrolytic action between the equipment and the items being cleaned. In addition, any chemical should be easily removable from the item by rinsing it with readily available water with specific properties so that the item does not retain residual chemicals in amounts that could potentially be harmful to patients, damage the device itself, or create other hazardous situations. Therefore in the present study an attempt has been made to extract enzyme from microorganisms, which can act against the bioburden laden on surgical instruments.

Materials and Methods

During the present study of investigation the slaughterhouse drainage samples were collected and samples were transported to the laboratory in sterile peptone broth. About 1 ml of sample was mixed thoroughly in 100 ml of saline, the suspension was serially diluted, and 0.1 ml of each of the sample was inoculated on blood agar plates and incubated at 37°C. About 100 isolates, were exhibited clear zone (near the vicinity of the colony, visible by naked eyes were chosen and preserved on blood agar slants for further determination of haemolytic activity, morphological, microscopical and physiological characters. Though the blood agar media gives a clear picture of haemolytic (fibrinolytic) enzymes releasing organisms, fibrin plates were used to confirm the isolates. The fibrinolytic activities of selected colonies were determined by the plasminogen fibrin plate method as described by Astrup and Mullertz (1952). Further, the isolates that exhibited which have given more than 2 mm zone of lysis were chosen and designated KLM1, KLM2, KLM3 and KLM4. Amongst the four isolates KLM4 exhibited more zone of lysis and as such was chosen to characterization through morphological, microscopical and physiological characters. Blood agar media was chosen for detailed morphological study of fibrinolytic enzyme releasing organism to characterize them. To study the nature and structure of the isolate the standard methods described by Collins and Lyne (1995) were used. The microscopic observations of the isolates were used as per standard method described by Collins and Lyne (1995). All the biochemical examinations were carried out as described by Collins and Lyne (1995).

Casein Hydrolysis

The milk agar containing casein as protein source was used to demonstrate the secretion of exoenzymes. The medium inoculated with isolates and incubated. Following incubation for 24 hours, plates were examined for clear zone near the vicinity of the colony.

Gelatin Hydrolysis

To 10ml of gelatin medium, a loopful of isolates to be tested were inoculated and incubated for 37°C for 48 hours. The extent of liquefaction was noted after keeping the tubes at 4°C for 30 minutes.

Oxidase Test

The ability of each isolate to produce oxidase can be determined by the addition of test reagent P-

amino dimethylamine oxalate to colonies grown on agar plate medium. The addition of the reagent turns the colonies black colouration, represents the positive test.

Fermentation Studies

Production of protease from *B. cereus* KLM4 was carried out in a medium containing the following composition of (g/lt) Peptone 5.0; yeast extract 5.0; skimmed milk 250.ml; agar12.0 and maintained at 37°C for 48 hours in a shaker incubator (140 rpm). The pH of the medium adjusted with 1N NaOH or 1N HCl. Samples were drawn every 24 hr. Protease activity was estimated as per Adinarayana (2003).

Recovery of Protease Enzyme

After completion of fermentation, the whole fermentation broth was centrifuged at 10,000 rpm at 4°C and clear supernatant was recovered. The crude enzyme supernatant was subjected to further studies and it also showed a clear zone of lysis on blood agar.

Partial Purification of Protease

The culture fluid was centrifuged at 12000 rpm 30 min at 4°C. The supernatant was adjusted to 3.5% saturation with ammonium sulphate. The precipitate formed by standing overnight at 4°C was removed by centrifugation. The supernatant was adjusted to 80% saturation with solid ammonium sulphate and allowed to stand overnight. The precipitate was collected by centrifugation, dissolved in small volume of sodium borate-sodium hydroxide buffer (pH 11.0) and dialyzed (Feng, 2000).

Enzyme Assay

One ml of 1% casein solution was inoculated with 1 ml of appropriately diluted enzyme for 20 min at 30°C. Reaction was stopped by addition of 4 ml of 5% trichloracetic acid. The tubes centrifuged after that 3000-x g for 10 min and the degraded products were measured by modified Lowry's method (Sandeep Kaur *et al.,* 2001). The absorbance was measured at 280 nm. A standard curve was generated using solutions of 0-50 mg tyrosine. One unit of protease activity was defined as the amount of enzyme, which liberated 1 mg tyrosine in 1 min at 60°C.

Zymography of Partially Purified Enzyme

The zymography partially purified enzyme as carried out as per the method described by Beaton (1997) (Plate 1).

Composition of Commercial Detergent

Following is the composition of the commercial detergent used in the present study (Table 1).

Table 1: Composition of enzyme detergent

Constituent	Composition (%)
Sodium tripolyphosphate (water softener, loosens dirt)	38.0
Sodium alkane sulphonate (surfactant)	25.0
Sodium perborate tetrahydrate (oxidizing agent)	25.0
Soap (Sodium alkane carboxylates)	3.0
Sodium sulphate (filter, water softener)	2.5
Sodium carboxymethyl cellulose (dirt suspending agent)	1.6
Sodium metasilicate (binder, loosens dirt)	1.0
Bacillus protease (3% active)	0.8
Fluorescent brighteners	0.3
Foam – controlling agents	Trace
Perfume	Trace
Water	to 100%

Evaluation of Partially Purified Enzyme for Washing Performance

Application of protease (5000 m/ml) as a detergent additive was studied on white cotton cloth pieces (4x4 cm) stained with blood. The stained cloth pieces were taken in separate trays. The following sets were prepared and studied.

1. Tray with distilled water (100 ml) + Blood stained cloth
2. Tray with distilled water (100 ml) + Blood stained cloth + 1 ml of commercial detergent (7 mg/ml).
3. Tray with distilled water (100 ml) + Blood stained cloth + commercial detergent + partially purified enzyme.
4. Tray with distilled water (100 ml) + Blood

stained cloth + partially purified enzyme.

The above trays were incubated at 60°C for 30 minutes. At regular intervals of 5 minutes, cloth pieces were taken out from each set, rinsed with water, and dried, and visual examination (Plate 2) of various pieces was carried out. Untreated cloth pieces stained with blood were taken as control (Adinarayana, 2003).

Preparation of the Enzyme Detergent

To the above-referred commercial detergent composition, the crude enzyme obtained was added at the rate of 7-ml/kg detergents. Thus prepared enzyme detergent has been presently designated as *MICRODET* (Plate 3).

Evaluation of Washing Performance of the Enzyme Detergent Microdet on Surgical Instruments

Various blood stained surgical instruments were taken and subjected to washing for 20 minutes period at 60°C by commercial detergent and also the microdet. Visual observation was made for the extent of bloodstain removal from the instruments after 20 minutes (Plate 4).

Comparative evaluation of washing performance of Microdet with commercial detergents.

Washing performance of the microdet was compared with the capacity of the commercial branded detergents like Doctor, Surf Excel, Rin Supreme, Rin Shakti, Super Nirma, Henko, Nirma, Active Wheel, 555, Tide, 501, Double Dog, Aeriel, Trishul, Hipolene and Fena (Plate 5).

Compatibility of Enzyme with Commercial Branded Detergents

Various commercial detergents, including Surf Excel, Surf, Ariel, Nirma, 501 Bar Soap, Snow White, Rin Shakthi, Dettol Liquid Soap and Tide (7mg ml-1) were incubated with 5000 m/ml units of partially purified enzyme at 40°C for 5 hours. Aliquots withdrawn at intervals of 60 min and the residual activity were determined under standard assay condition. Enzymes samples incubated in the absence of detergents served as control (Kamal Kumar, 2004).

Results

The results on colony morphology of the isolates obtained from slaughterhouse drainage on blood agar are presented in Table 2. The perusal of results indicated that the size of the colony obtained from slaughterhouse drainage measured 4 mm. The shape of the colony obtained is irregular and round. All the colonies isolated from slaughterhouse drainage are convex, smooth feathery, creamy and b-haemolytic. The results on microscopic characters of the isolates obtained from slaughterhouse drainage are presented in Table 3. The perusal of the results indicated that the colonies from slaughterhouse drainage have shown gram positive, non-motile, rod shaped, cells in chains and free and presence of endospores. Results on the biochemical reactions of the isolates studied from slaughterhouse drainage are presented in Table 4. The data presented in Table 4 indicated catalase, b-haemolysis and endospore positive, whereas glucose fermentation, acid from mannitol growth in 6.5% NaCl, oxidase and litmus coagulation negative.

The results on zone of lysis of haemolytics isolated on fibrin plates are presented in Table 5. Perusal of the results indicated that the zone of lysis of isolates occurred in the range of 45.2 to 5.6 mm. The maximum zone lysis occurred is 5.6 mm by isolate KLM4.

Protease Production by Isolate KLM4

The results on the production of protease from isolate KLM4. The results revealed that the production of protease enzyme increased up to 48 hours of fermentation. Thereafter the decreased in the enzyme activity was observed. The maximum enzyme production was observed at 48 hours of fermentation.

Zymography

The results on zymography of isolate KLM4 are presented in Plate 1. The results revealed that the clear areas were interpreted as representing areas of protease activity and whole dark areas represent areas where protease activity is absent.

Enzyme Partial Purification and Assay

The results are presented in Table 6. It is observed that the crude enzyme presented an activity of 0.103 IU / ml / min while the partially purified enzyme showed an activity of 0.116 IU / ml / min.

Evaluation of Partially Purified Enzyme for Washing Performance

The results are presented in Plate 2. The results

reveal that the blood stain on the cloth piece remained as it was even after 30 minutes rinsing in the controls and commercial detergent. Blood stain was totally removed from the cloth after rinsing it with a combination of detergent and enzyme for a period of 20 minutes, whereas it was removed after 25 minutes when rinsed with crude enzyme alone.

Evaluation of Washing Performance of the Enzyme Detergent Microdet on Surgical Instruments

The results of the study to evaluate the washing performance of the enzyme detergent microdet on the surgical instruments are presented in Plates 4. After an incubation of 20 minutes, stains were not removed completely with detergents alone, while the combination of the enzyme with commercial detergent (Microdet) removed the bloodstains from the surgical instruments very effectively.

Comparative Evaluation of Washing Performance of Microdet with Commercial Branded Detergents

Of all the branded detergents, Tide and Aerial removed bloodstains after 20 minutes rinsing on par with the microdet (i.e., commercial detergent supplemented with crude enzyme) (Plate 5).

Compatibility of Crude Enzyme with Commercial Branded Detergents

The results of the studies involving the compatibility of the crude enzyme obtained in the present study along with the commercial branded detergents. It was observed that the enzyme activity was retained at only 50% at the end of 5 hours when incubated with the commercial branded detergents, except in the presence of Tide and Aerial and also in our new enzyme wherein it was retained at slightly higher rate of 60.

Table 2: Colony characters of isolated strains on blood agar

Sl. No.	Bacterial Strain	Source of Isolation	Colony Characters
1.	KLM1	Drainage of slaughter house	4mm, irregular, convex, smooth, feathery, creamy and β - hemolytic
2.	KLM2	Drainage of slaughter house	4mm, irregular, convex, smooth, feathery, creamy and β - hemolytic
3.	KLM3	Drainage of slaughter house	4mm, irregular, convex, smooth, feathery, creamy and β - hemolytic
4.	KLM4	Drainage of slaughter house	4mm, irregular, convex, smooth, feathery, creamy and β - hemolytic

Table 3: Microscopic characters of isolated strains

Sl. No.	Strain type	Mobility	Gram reaction	Shape	Arrangement	Endospore
1.	KLM1	Motile	Gram +VE	Rods	Free	Present
2.	KLM2	Motile	Gram +VE	Rods	Free	Present
3.	KLM3	Motile	Gram +VE	Rods	Free	Present
4.	KLM4	Motile	Gram +VE	Rods	Free	Present

Table 4: Biochemical reaction of isolated strains

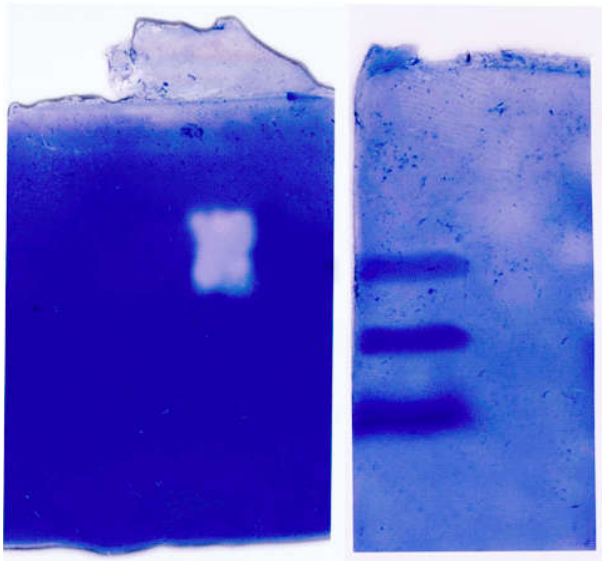
Sl. No.	Strain type	Catalyse	Glucose fermentation	Acid from mannitol	Growth in 6.5% NaCl	Acid from glycerol	Endspore	Oxidase	Heaemolysis	Litmus coagulate
1.	KLM1	+	-	-	-	-	+	-	β	-
2.	KLM2	+	-	-	-	-	+	-	β	-
3.	KLM3	+	-	-	-	-	+	-	β	-
4.	KLM4	+	-	-	-	-	+	-	β	-

Table 5: Diameter of Zone (in mm) formed by isolated strains on fibrin Plate.

Sl. No.	Purification steps	Total Enzyme activity (IU)	Total protein (mg)	Specific activity
1.	Crude enzyme	0.103	0.9	0.114
2.	Ammonium solution (70%)	0.116	0.68	0.170

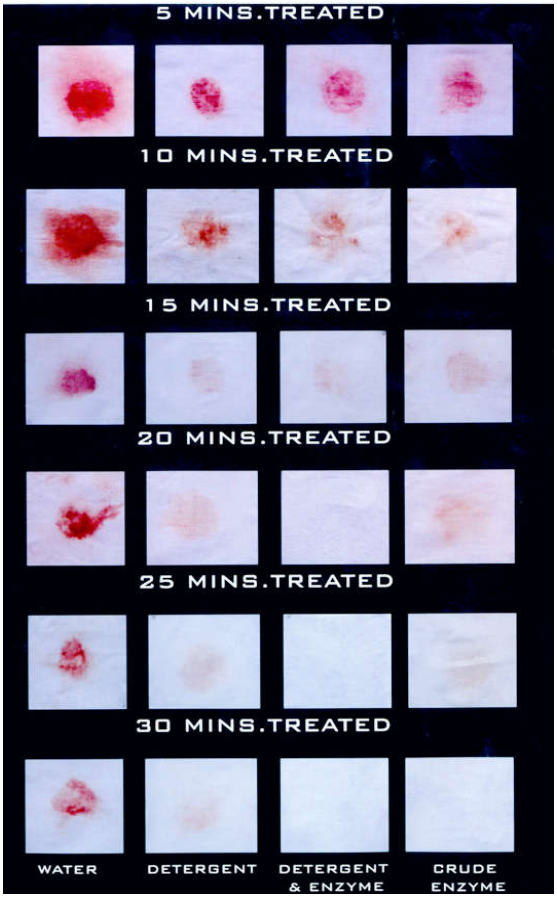
Table 6: Partial Purification and assay of Enzyme

Sl. No.	Bacterial strain	Source of Isolation	Zone of Lysis (mm)
1.	KLM1	Slaughter house drainage	5.2
2.	KLM2	Slaughter house drainage	5.2
3.	KLM3	Slaughter house drainage	4.0
4.	KLM4	Slaughter house drainage	5.6

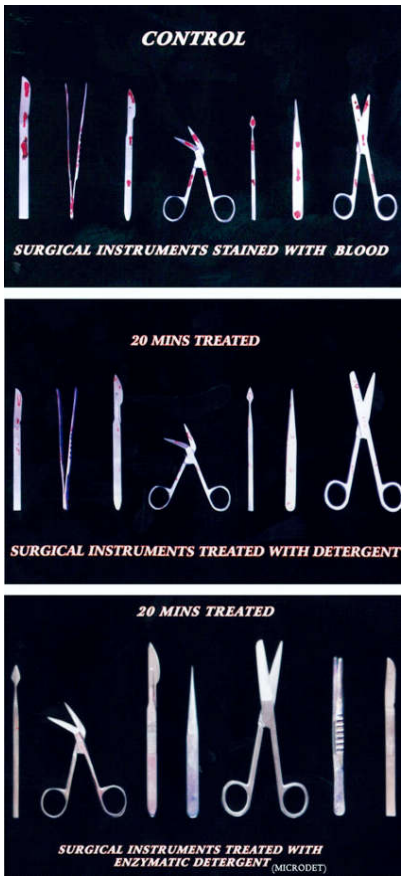


Platte. 1: Zymography of partially purified enzyme

Platte. 3: Microdet detergent



Platte. 2: Evaluation of partially purified enzyme for washing



Platte. 4: Evaluation of washing performance of the enzyme detergent micredet on surgical instrument

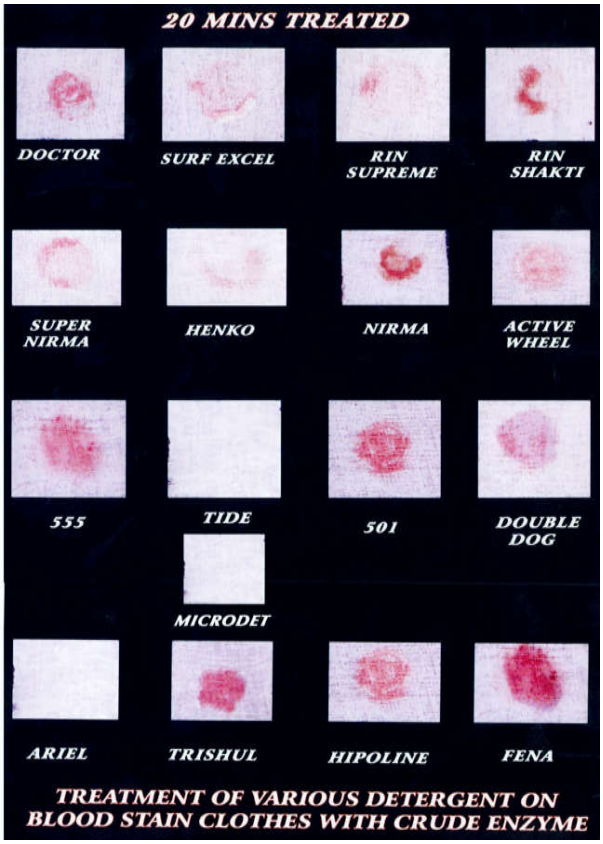


Plate 5: Comparative evaluation of washing performance of microdot with commercial detergent

Discussion

A variety of enzyme product has been developed for use in so-called "biological or enzymatic detergents" to enhance the removal of bioburden from surgical instruments. They are three basic types enzymes used in detergents: proteases, amylases and lipases. Proteases are the most important type of enzyme to look for when choosing an enzymatic detergent for medical use because there is a high content of protein in most body fluids (including blood, tissue and mucous) which cannot be easily removed with regular detergents/ surfactants and water, proteases break down protein into individual amino acids or short string of amino acid. Amino acid and peptides are much more soluble in water and will float away the surface of the instrument. The incorporation of enzymes into a detergent for endoscope cleaning has several benefits in addition to enhancing overall cleaning performance. Clogging of endoscope channel is virtually eliminated with the appropriate enzymatic detergents. This reduces the need for costly routine maintenance and results in large saving. As a result, the quality of the images is improved. Properly formulated enzymatic detergents are non-corrosive and do not attack any metal surfaces on medical instruments and will effectively work in mild conditions without damaging valves, rubber gaskets or any surface of the flexible fiber optic endoscope or other medical instruments. Therefore, in the present study, an attempt is made to develop enzymatic detergent for cleaning of surgical instruments. In the present study the slaughterhouse drainage sample collected was screened for presence of bacteria, which can utilize blood protein as their protein source. The samples were serially diluted on blood agar. From these plates, depending upon their morphological, microscopical and physiological characters, the isolates were suspected to be *Bacillus cereus.* The colonies were confirmed by cultivating on selective media.

On PEMBA (Polymyxin pyruvate egg-yolk mannitol bromothymol blue agar) media the isolated colonies were peacock blue in colour. This confirms the presence of *Bacillus cereus.* The isolates have been designated as *Bacillus cereus* KLM1, KLM2, KLM3 and KLM4 Of these KLM4 isolate was chosen as potential producer of protease enzyme depending upon its zone of lysis on fibrin plates and studied further for production of protease enzyme and its compatibility for the use of enzymatic detergent preparation for the cleaning surgical instruments. The protease used in a detergent formulation should have a high level of activity over a broad range of pH and temperature. One of the drawbacks of enzyme recovered from thermopiles is non-stability to pH and temperature. Thus its desirable to search for new protease with novel properties like thermo and alkaline stable.In the present study we examined the efficiency of an enzyme, recovered from *Bacillus cereus* KLM4 for stability to pH and temperature. The results observed on the studies of the effect of temperature on enzyme activity. The maximum enzyme activity was observed at 60°C. Thereafter decrease in the activity was observed. The enzyme in the presence of its substrate was more active below the 60°C. It's known that temperature increase the reaction velocity and also affects the rate of enzyme activity. At high temperature its adverse effect become significance as the reaction proceeds Thus the residual enzyme activity detected less at temperature higher than 60°C might be due to the stability effect of its substrate (Ahmed F. Abdel – Fattah 1983) The temperature optima of 60°C was also reported by Adinarayana (2003) for maximum enzyme activity.The results on the effect of pH on the enzyme activity. The observation revealed that the optimum pH recorded was at pH 10. Indicated

that the enzyme even active at alkaline pH, which is most desirable quality for the enzyme used in detergent formulation (Ruchi Oberio *et al.*, 2001; Sangita, 1993 and Adil Anwar, 2004).The similar optimal ranges were also found and reported Adinarayana (2003) Kamal Kumar (2004) and Sandeep Kaur (2001).Optimal ranges were also found and reported Adinarayana (2003) Kamal Kumar (2004) and Sandeep Kaur (2001).

Summary and Conclusion

Based on various biochemical properties the protease isolated from *Bacillus cereus* KLM4 is thermostable protease. It is stable at alkaline pH at high temperatures, and in presence of commercial and local detergents. These properties indicate the possibilities for use of the protease in the manufacture of surgical cleaning detergent industry. Therefore, the enzyme obtained from *Bacillus cereus* KLM4 has been used in the preparation Microdet. The Microdet under the present study showed promising results in the removal of bloodstains from the surgical instruments. However, the economics of its production for commercial exploitation has to be worked out.

Acknowledgements

This work was supported in Gulbarga University, Gulbarga. Department of studies and Research. Department of Microbiology.

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Original Article

Journal of Microbiology and Related Research
Volume 2 Number 2, July - December 2016
DOI: http://dx.doi.org/10.21088/jmrr.2395.6623.2216.5

Study of Antibiogram and Resistance Mechanism of Staph. Aureus in Clinical Isolates from Stand alone Diagnostic Centre in Central Madhya Pradesh

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Received on 18.07.2016, **Accepted on** 23.07.2016

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Abstract

Background: In recent years,Staph.aureus,both coagulase positive and negative,have shown resistance to commonly used antibiotics used to treat infections.Over the last two decades,methicillin resistant strains (MRSA) have also been on a rise specially in patients admitted to ICUs and immunocompromised patients.The drug resistance mechanism of MRSA has been studied extensively in the past decade.Our study aims to study the sensitivity pattern of Staph. aureus in different clinical specimens and to study thedifferent resistance mechanisms**.** *Objective:* The present retrospective study highlights the susceptibility pattern and resistance mechanism ofStaph.aureus in clinical specimens obtained in our Microbiology department from June 2015 to June 2016. *Materials and Methods:* This was a retrospective study of Staph.aureus isolates from different clinical specimens including urine, blood, pus, vaginal swab, semen, aural swab, BAL fluid, conjunctival swab etc obtained from out patients at our Diagnostic Clinic Microbiology department from June 2015 to June 2016 and reported in VItek II (Biomerieux) according to CLSI guidelines. A total of 278 samples were reviewed. *Results:* Out of278 Staph.aureus isolates grown in the lab from different clinical specimens the sensitivity pattern showed highest sensitivity for Tigecycline(91.7%),followed bypenicillin (91.3%), ,Gentamycin (80.9%),tetracycline (66%),levofloxacin(65.4%),Rifampicin(63.6%), Linezol id(63.3%), Daptomycin(50.3%), Vancomycin (46,7%), Teicoplanin (42%). 117 cases showed resistance to aminoglycosides by KAN (APH(3")-III) mechanism,Cefoxitin screen was positive in 166 patients,APH(3")-III in 117 patients,MecA gene in 113 cases,ANT(4')(4"))In 106 patients,acquired penicillinase in 77 ,SGA-SGB in 49 cases,efflux mechanism in 30 cases and inducible clindamycin resistance in 24 cases. *Conclusion:* According to our study Tigecycline, followed by Penicillin, Gentamycin ,Tetracycline, Levofloxacin and Linezolid are the antibiotics of choice for treating Staph aureus infections in the present scenario. With the introduction of automated equipments like Vitek II our understanding of the resistance mechanism is increasing.A larger study population would be required for a better understanding of resistance mechanisms.

Keywords: Staph Aureus; Vitec II; Resistance Mechanism; MRSA; Meca Gene.

Introduction

The genus Staphylococcus are ubiquitous gram positive, one micron in diameter, non –sporing cocci occurring in grape like clusters, singly or in pairs. They are facultative anaerobes, grow well on blood agar as golden yellow colonies [1]. Staphylococcus aureus causes boils, bronchopneumonia, carbuncles, diabetic foot, infection, furuncles, osteomyelitis, post operative infections, septicaemia and a host of other infections [2,3,4].

In recent years, Staph.aureus,both coagulase positive and negative, have shown resistance to commonly used antibiotics used to treat infections,it causes.Over the last two decades, methicillin resistant strains (MRSA) have also been on a rise specially in patients admitted to ICUs and immunocompromised patients [5].The drug resistance mechanism of MRSA has been studied extensively in the past decade.There are several other resistance mechanisms playing a role in resistance of antibiotics to Staph aureus. Our study aims to study the sensitivity pattern of Staph. aureus in different clinical specimens and to study the different resistance mechanisms**.**

Material and Methods

This was a retrospective study of Staph.aureus isolates in different clinical specimens including pus, sputum, urine, blood, aural swab, BAL fluid, conjunctival swab, CSF, Nasal swab, parotid fistulas, pleural fluid, Semen, synovial fluid,throat swab and vaginal swab obtained from out patients at our Clinic Microbiology department from June 2015 to June 2016 and reported in fully automated Vitek II (Biomerieux) according to CLSI guidelines. A total of 278 samples were reviewed. All samples whether urine, pus etc. were considered in the study. The patients were divided into four groups ie. Newborn(NB) to 20 years, 21-40 years ,41-60 years, 61 to 80 years and more than 80 years in both the sexes. The following points were taken into consideration for analysis:

- Age and sex of patients
- Staph.aureus i isolates
- Drug sensitivity pattern
- Resistance mechanism

Samples were processed and identified as per routine laboratory protocol. Identification and antibiotic sensitivity testing was done byVitecII (Biomerieux) according to clinical laboratory standard institute guidelines (CLSI guidelines)

Isolation and Identification

Urine samples were collected in universal container approx. 50 ml in amount and were inoculated using an inoculation loop of 10 ul volume calibration on MacConkey agar plates. Other specimens such as CSF, Sputum, and different body fluids collected in sufficient amount were inoculated on Blood and MacConkey agar plates using an inoculation loop. Blood samples collected in broth in a ratio of 1:5 (blood: broth) were incubated in BactT/Alert (Biomeriux) and then subcultured on blood and MacConkey agar plates on the basis of colony morphology , gram staining, motility P628 panel was selected for identification and sensitivity of the micro organism. Following criteria was used for identification of Staph. aureus

1. Colony morphology:-.1 micron diameter,golden yellow colonies
2. Grams Staining :- Gram positive cocci, size, uniformly stained , non sporing , non capsulated
3. Biochemical reaction:- performed on automated VitecII(Biomerieux)
4. Antimicrobial sensitivity tests:- performed on automated Vitec II(Biomerieux)

Results

The present study was conducted in total of 278 Staph aureus isolates from June 2015 to June 2016 through automated identification and sensitivity reporting by VitecII(Biomerieux).

The antimicrobial resistance pattern assessment revealed that out of 278 Staph. aureus isolates there were 60.4% males and 39.5% females.Male to female ratio was 1.52:1.Maximum patients were below 20 years of age(33%),followed by 21-40 years(30.5%), 19.5% in 21-40 years age group. 2.55% patients were above 80 years of age.Demographic data of patients is shown in Table 1.

Majority of Staph aureus isolates from pus(55%), followed by sputum(16.5%),throat swab (14.0%), blood(3.59%),semen(3.23%)urine(1.79%), conjunctival swab and CSF (1.07%), synovial fluid, pleural fluid and BAL fluid (0.7%) and lowest in parotid fistula, nasal and aural swab (0.35%).The data of Staph aureus isolated in different clinical specimens is shown in Table 2.

The sensitivity pattern showed highest sensitivity for Tigecycline (91.7%), followed by penicillin (91.3%), Gentamycin (80.9%), tetracycline (66%), levofloxacin (65.4%), Rifampicin (63.6%), Linezolid (63.3%), Daptomycin (50.3%), Vancomycin (46,7%), Teicoplanin (42%). Lowest sensitivity was found in ampicillin (2.15%)and amoxicillin (2.5%).Table 3 shows the MIC value and sensitivity pattern of antibiotics Ampicillin showed highest resistance (97.8%), followed by amoxicillin (97.5%), ofloxacin (89.57%), erythromycin(73.1%), ciprofloxacin (76.7%), ampicillin/sulbactum(71.6%), ceftriaxone and cefotaxime(71.3%)each, cefazoline (69.8%), Imipenem (70.9%).

Table 4 shows resistance pattern and MIC value of antibiotics.

117 cases showed resistance to aminoglycosides by KAN (APH(3')-III) mechanism. Out of these 117 cases, 61 cases were from pus, 16 from sputum,14 from throat swab, 7 from blood, 4 in urine, 3 in conjunctival swab and CSF, 2 in BAL fluid,one each in aural, nasal and vaginal swab, synovial and pleural fluid, parotid fistula and semen.

106 cases shows resistance to aminoglycosides by KAN TOB (ANT(4')(4")) mechanism.out of which 61 cases were from pus, 16 from sputum 14 from throat swab and seven from blood.

Resistance by acquired penicillinase mechanism to B-lactams was observed in 77 cases, with 53 cases in pus, 10 in throat swab, 6 in sputum, 4 in blood.

In the family of Macrolides/lincosamides/ streptogramins 30 cases showed resistance to antibiotics by efflux mechanism,out of which 18 were from pus isolates, 3 each from sputum and throat swab and 2 from blood.

Resistance to Streptogramins by SGA-SGB was observed in 49 cases, 30 from pus, 6 from sputum,5 each from blood and throat swab.

Inducible Clindamycin resistance was observed in 224 cases,18 in pus, 3 in throat swaband 2 in sputum.

Cefoxitin screen was found to be positive in 166 cases, 77 in pus, 36 in sputum, 24 in throat swab 9 in semen.

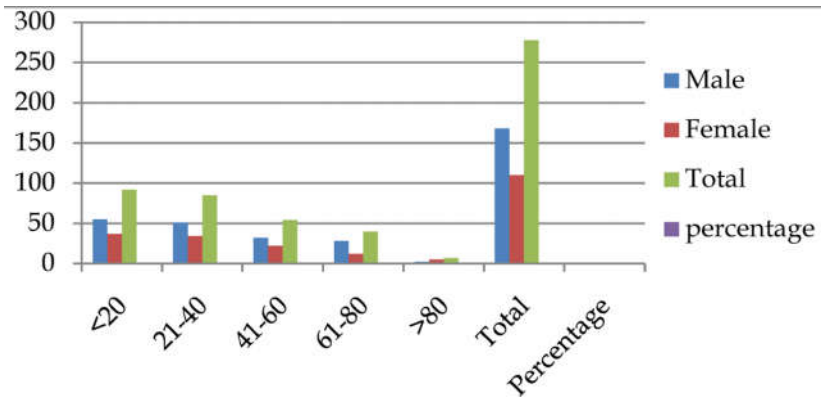
PBP (MecA) gene was observed to cause resistance in B lactam antibiotics in 113 patients,out of which 53 were from pus,16 from sputum13 from blood 11 from throat swab .

Cefoxitin screen was positive in 166 patients, APH(3")-III in 117 patients, MecA gene in 113 cases,ANT(4!)(4"))In 1q06 patients, acquired penicillinase in 77, SGA-SGB in 49 cases, efflux mechanism in 30 cases and inducible clindamycin resistance in 24 cases.

Table 5 shows the data of resistance mechanism in different clinical specimens.

Table 1: Demographic data of stapylococcus aureus

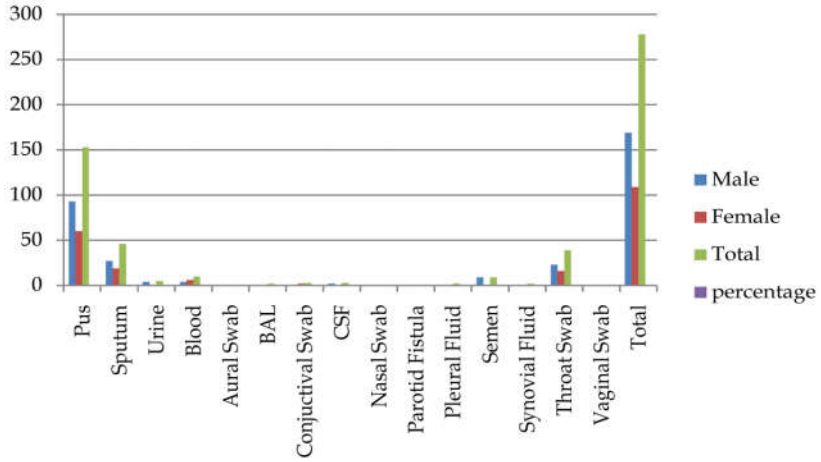
Age (Years)	Male	Female	Total
<20	55	37	92
21-40	51	34	85
41-60	32	22	54
61-80	28	12	40
>80	2	5	7
Total	168	110	278
Percentage	60.40%	39.50%	



Graph 1: Showing demographic data
Isolation of Staph.aureus in different clinical specimens

Table 2: Showing Staph Aureus isolates from different clinical specimens

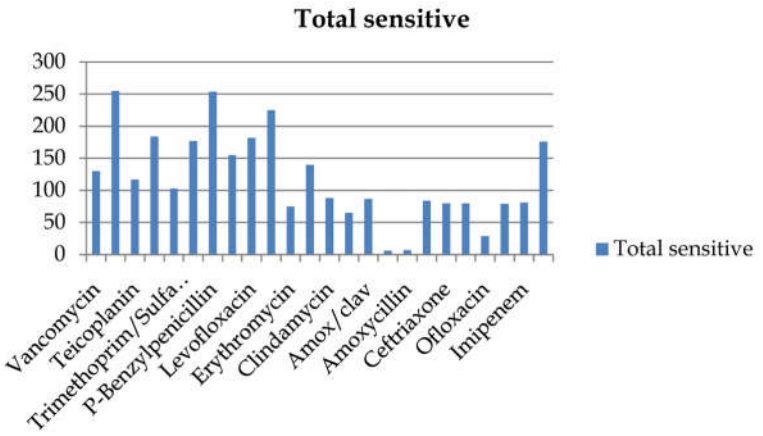
Specimen	Male	Female	Total	Percentage
Pus	93	60	153	55%
Sputum	27	19	46	16.50%
Urine	4	1	5	1.79%
Blood	4	6	10	3.59%
Aural Swab	1	0	1	0.35%
BAL	1	1	2	0.70%
Conjuctival Swab	1	2	3	1.07%
CSF	2	1	3	1.07%
Nasal Swab	1	0	1	0.35%
Parotid Fistula	1	0	1	0.35%
Pleural Fluid	1	1	2	0.70%
Semen	9	0	9	3.23%
Synovial Fluid	1	1	2	0.70%
Throat Swab	23	16	39	14.00%
Vaginal Swab	0	1	1	0.35%
Total	169	109	278	



Graph 2: Showing Staph Aureus isolates from different clinical specimens

Table 3: Sensitivity pattern of staphylococcus aureus in various clinical specimens

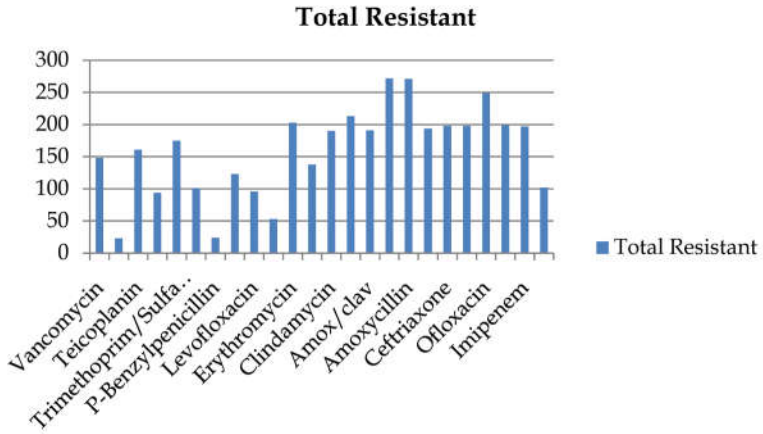
Antibiotic	Total sensitive	percentage	MIC Value
Vancomycin	130	46.70%	<=0.5
Tigecycline	255	91.70%	<=0.12
Teicoplanin	117	42%	<=0.5
Tetracycline	184	66%	<=1
Trimethoprim/Sulfamethoxazole	103	37%	<=10
Rifampicin	177	63.60%	<=0.03
P-Benzylpenicillin	254	91.30%	0.12,
Oxacillin	155	55.70%	<=0.25
Levofloxacin	182	65.40%	0.25
Gentamicin	225	80.90%	<=0.5
Erythromycin	75	26.90%	<=0.25
Daptomycin	140	50.30%	0.25,
Clindamycin	88	31.60%	0.25
Ciprofloxacin	65	23.30%	<=0.5
Amox/clav	87	31.20%	<= 0.5
Ampicillin	6	2.15%	<= 0.5
Amoxycillin	7	2.50%	<=0.25
Ceftazoline	84	30.20%	<=0.25
Ceftriaxone	80	28.70%	<=0.5
Cefotaxime	80	28.70%	<= 0.5
Ofloxacin	29	10.43%	<=0.25
Ampicillin+Sulbactum	79	28.40%	<=0.25
Imipenem	81	29.10%	<=0.25
Linezolid	176	63.30%	<=0.5



Graph 3: Showing sensitivity pattern

Table 4: Resistance pattern of Staph.aureus in different clinical specimen

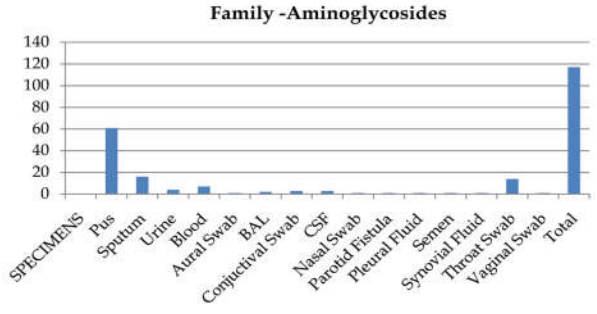
Antibiotic	Total Resistant	MIC Value	Percentage
Vancomycin	148	>=32	53.30%
Tigecycline	23	1	8.30%
Teicoplanin	161	>=32	58%
Tetracycline	94	>=16	33.90%
Trimethoprim/Sulfamethoxazole	175	>=320	63%
Rifampicin	101	>=4	36.40%
P-Benzylpenicillin	24	>=0.5	8.70%
Oxacillin	123	>=4	44.30%
Levofloxacin	96	>=8	34.60%
Gentamicin	53	>=16	19.10%
Erythromycin	203	>=8	73.10%
Daptomycin	138	>=8	49.70%
Clindamycin	190	>=4	68.40%
Ciprofloxacin	213	>=8	76.70%
Amox/clav	191	>=8	68.80%
Ampicillin	272	>=8	97.80%
Amoxycillin	271	>=8	97.50%
Cefazoline	194	>=4	69.80%
Ceftriaxone	198	>=8	71.30%
Cefotaxime	198	>=8	71.30%
Ofloxacin	249	>=16	89.57%
Ampicillin+Sulbactum	199	>=8	71.60%
Imipenem	197	>=8	70.90%
Linezolid	102	>=8	36.70%



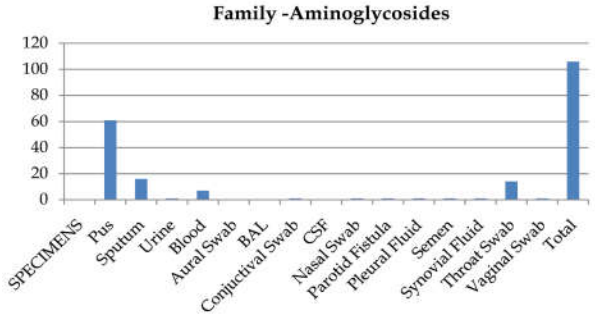
Graph 4: Showing resistance pattern of Staph aureus

Table 5: Showing resistance mechanism of Staph aureus in different clinical specimens

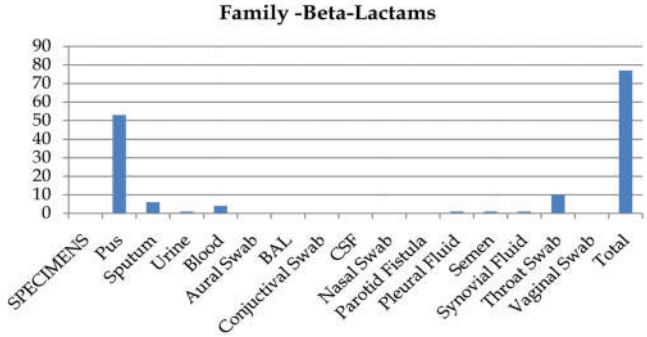
SPECIMENS	Family - AMINOGLYCOSIDES – 18 RESISTANT KAN (APH(3')-III)	Family - AMINOGLYCOSIDES – 19 RESISTANT KAN TOB (ANT(4')(4")	Family - BETA-LACTAMS - 6 ACQUIRED PENICILLINASE	Family - MACROLIDES/LINCOSAMIDES/STREPTOGRAMINS RESISTANT (EFFLUX)	Family - MACROLIDES/LINCOSAMIDES/STREPTOGRAMINS – 8 RESISTANT TO STREPTOGRAMINS (SGA-SGB)	Family - TRIMETHOPRIM/SULFONAMIDES – 1 RESISTANT	Family - TRIMETHOPRIM/SULFONAMIDES – 2 TRIMETHOPRIM RESISTANT	ICR-Inducible Clindamycin Resistance POSITIVE	OXSF-Cefoxitin Screen POSITIVE	Family - BETA-LACTAMS – 21 Modification of PBP (mecA)
Pus	61	61	53	18	30	69	25	18	77	53
Sputum	16	16	6	3	6	9	9	2	36	16
Urine	4	1	1	1	0	3	1	0	4	3
Blood	7	7	4	2	5	3	6	0	3	13
Aural Swab	1	0	0	0	0	0	0	0	1	2
BAL	2	0	0	0	0	0	0	0	2	0
Conjuctival Swab	3	1	0	0	1	0	0	0	3	2
CSF	3	0	0	0	0	0	0	0	3	1
Nasal Swab	1	1	0	0	1	0	1	0	1	1
Parotid Fistula	1	1	0	1	0	0	1	0	1	1
Pleural Fluid	1	1	1	0	1	0	0	0	0	1
Semen	1	1	1	1	0	3	2	1	9	7
Synovial	1	1	1	1	0	0	0	0	1	0



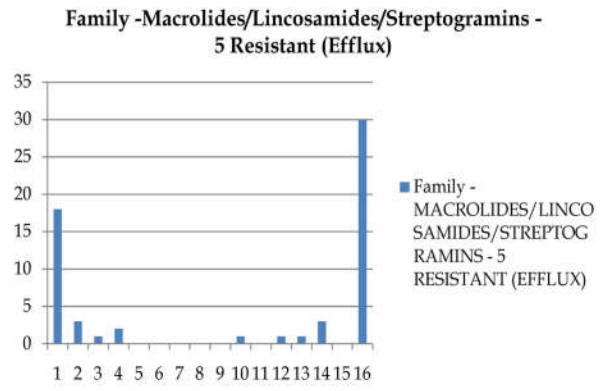
Graph 5: Resistance mechanism RESISTANT KAN (APH(3')-III)



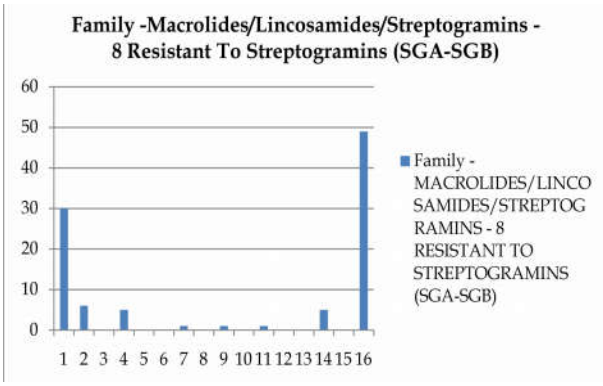
Graph 6: Resistant Kan Tob (ANT(4')(4"))



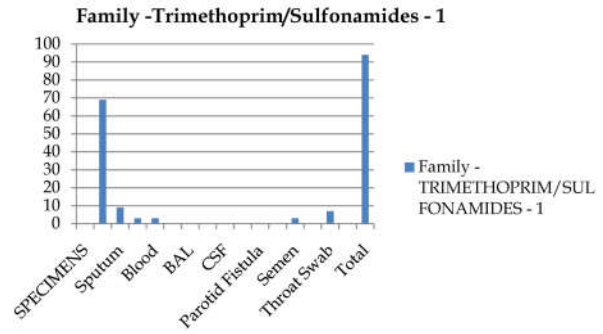
Graph 7: Acquired penicillinase



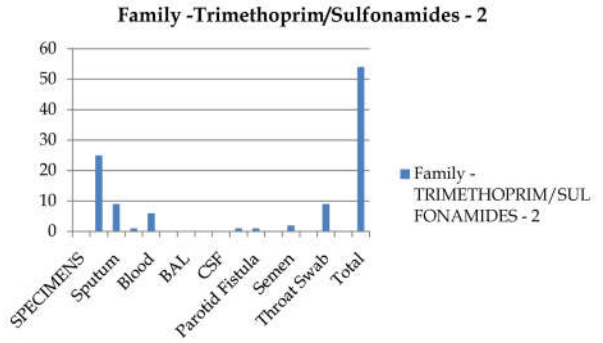
Graph 8: Resistant (efflux



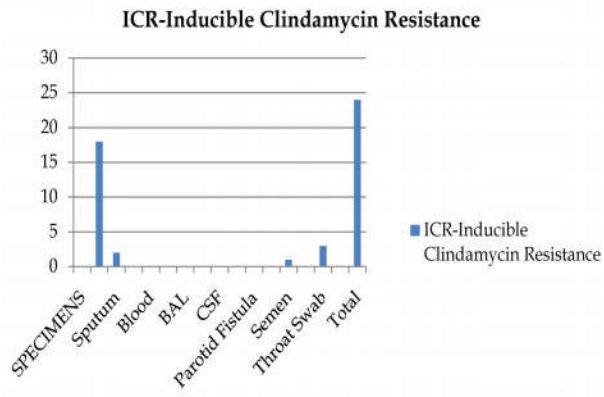
Graph 9: Resistant to streptogramins (SGA-SGB)



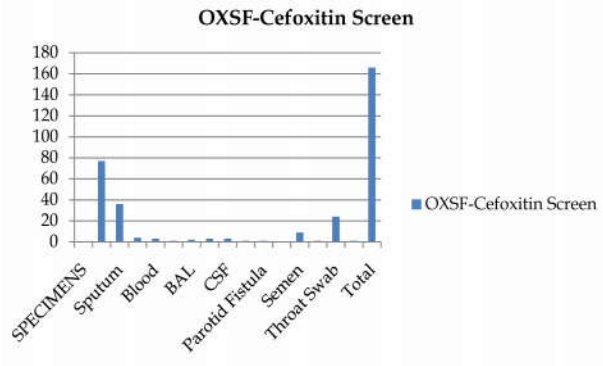
Graph 10: Family-Trimethoprim/Sulfonamodes-1



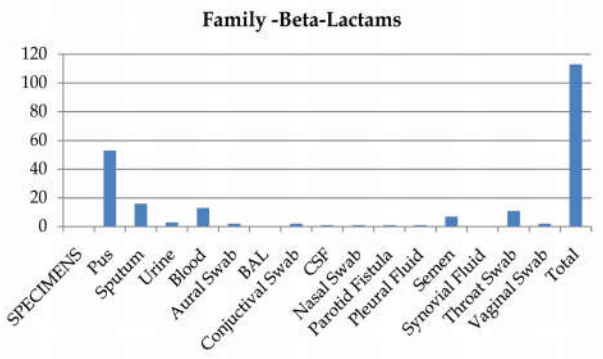
Graph 11: Family-Trimethoprim/Sulfonamodes-2



Graph 12: ICR-Inducible Clindamycin Resistance



Graph 13: Cefoxitin screen positive



Graph 14: Modification of PBP (mecA)

Discussion

Drug resistance in Staph aureus is a major global health problem.It increases the morbidity and mortality among the patients and reduces the chances of using sensitive drugs for future generations.It also adds to the economic burden for healthcare systems.

The rate and magnitude of drug resistance in Staph aureus is mediated by a complex interplay of different epidemiological factors and mechanisms. Overuse and misuse of antibiotics is one of the reasons of resistance.

There are several mecjhanisms by which antibiotics act on the microbes e.g. drugs such as Aztreonam, cefalosporins, penicillins, Vancomycin, Imipenem and Methicillin act by inhibiting the cell wall synthesis of bacteria.Some antibiotics act by inhibiting enzymes involved in DNA synthesis like Quinolones. Drugs like sulphonamides inhibit tetrahydrofolic acid needed for DNA synthesis.Aminoglycosides,Cloramphenicol act by inhibiting protein synthesis [6].

Drug resistance among bacteria develops as a result of mutations in the microorganism's genetic structure or by acquiring extra pieces of genetic material from other bacteria. There are several drug resistance mechanisms like(a) Decreased drug uptake modification of plasma membrane causing reduced permeability, (b)increased drug export caused by increased activity of efflux pumps, (c)inactivation or modification mutations in ribosomal proteins, penicillin binding proteins(PBP), (d) Introduction of new drug-insertion of methicillin resistance gene(MecA), (e)increased production of Beta lactamase gene [7]. Cross resistance develops between members of a class of antibiotics because they are chemically related and have the same target of action in bacterial cells .The The drug efflux mechanism confers resistance to betalactams,aminoglycosides, tetracyclines, macrolides, streptogramins etc.The intracellular antibiotic concentration is reduced by the efflux mechanism thereby delaying the death of bacterium.Absence of or alteration in aminoglycoside transport system, inadequate membrane potential, modification in lipopoly saccheride (LPS) phenotype can result in a cross resistance to all aminoglycosides. The enzymes causing inactivation of aminoglycosides are classified according to the type of modification AAC (acetyltransferases), ANT (nucleotidyl transfereases) or adenyltransferases, APH (phosphotransferases) (Shaw et.al 1993) [8].

Penicillin was introduced in early 1940s and soon developed resistance due to the ability of Staph aureus to produce Beta lactamase enzyme i.e. penicillinase.Penicillin converts the beta lactam nucleus into harmless peniciloic acid. MRSA worsened this situation.Methicillin was introduced in 1961 as it was penicillinase stable beta lactam antibiotic., but since then, MRSA strains have become endemic [9]. MRSA contains MecA gene which is responsible for the production of penicillin binding protein(PBP 2a) [10].

Staph aureus also develops resistance due to NorA multidrug resistance efflux pump resulting in low level quinolone resistance [11].

In the study conducted by Alain C et al. in 2014, 40.6% cases were identified as MRSA and 39.4% were inducible Clindamycin resistance. The found 100% sensitivity for Linezolids followed by tetracycline(95%),while Penicillin G had 0% sensitivity [12]. Our study does not correlate with this study. Our study showed 91.7% sensitivity for Tetracycline, 91.3% for Penicillin, Linezolid(63.3%) and inducible clindamycin resistance in only 24 cases.

In the study of Uwaezuoke et al. ,high sensitivity was found to Gentamycin(91.7%), Cloxacillin (85.4%), Erythromycin(66.7%), Streptomycin (66.7%) [13]. Our study does not correlate with this study as well. Emmanuel et. al. found highest sensitivity to Levofloxacin(100%), followed by Ciprofloxacin (78.9%) and least to Penicillin (7.1%) [14]. Najim Abdulla et al. found Amikacin, Gentamycin And Doxycycline to be highly susceptible [15]. Lowest rates were seen with Amoxycillin, Amoxyclav,Erythromycin, Cotrim-oxazole and Cefuroxime. Our study partly correlates with this study. Mazhar Salim et al. found highest sensitivity to cloramphenicol, Linezolid, Nitrofurantoin, Rifampicin and Teicoplanin but high resistance to Erythromycin and Penicillin. All isolates were sensitive to Vancomycin [16].

Conclusion

A continuous surveillance of antibiotic sensitivity pattern and resistance mechanism is needed for selecting appropriate antibiotic therapy for Staph aureus in different clinical specimens. According to our study Tigecycline, followed by Penicillin, Gentamycin, Tetracycline, Levofloxacin and Linezolid are the antibiotics of choice for treating Staph aureus infections in the present scenario.Ampicillin and Amoxycillin have caesed to be the first line drugs for treating Staphylococcal infections. Multidrug resistance IN Staph aureus is an alarming sign. Newer approaches to therapy and prevention are required to combat this problem. With ithe introduction of automated equipments like Vitek II our understanding of the resistance mechanism is increasing. A larger study population would be required for a better understanding of resistance mechanisms.

Conflict of Interest

none

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Original Article

Journal of Microbiology and Related Research
Volume 2 Number 2, July - December 2016
DOI: http://dx.doi.org/10.21088/jmrr.2395.6623.2216.6

Factors Responsible for Likelihood of Invasive Burn Wound Infections with Their Bacteriological Profile and Antibiotic Suspitibility Pattern

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Received on 12.11.2016, **Accepted on** 17.11.2016

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Abstract

Background: Burn injuries constitute a major health concern with respect to morbidity and mortality. Burns are a very common injury, serious burn injuries are ex-cruciatingly painful and require special care to prevent infection and reduce the severity of scarring. *Objectives:* To determine the predominant bacterial pathogens in the burn wound infection with age, sex and cause of injury, and to know the antibiotics sensitivity profiles of the isolates obtained. *Material & Method:* Burn Patients admitted within 24 hours were included. Whereas Patients admitted after 24 hours were excluded. Two burn swabs were aseptically collected on admission before the start of antibiotics. One swab was subjected for gram staining and the other for culture. All specimens were inoculated on 5% blood agar and MacConkey agar and incubated. Isolated organisms were subjected for Antibiotic Susceptibility. *Result:* Maximum patients (50%) belonged to 21- 40 years age group. and males 39 (65%) were predominant over Female as 21 (35%), Cause of burn in Maximum number of patients was fire burn 25 (41.6 %) Among single isolates *Pseudomonas aeruginosa* was leading (20.8%) followed by *Acinetobacter baumannii* (15.4%), *staphylococcus aureus* (14.1%) and *Klebsiella pneumoniae* (7.5%). Antibiotics susceptibility pattern of gram positive bacteria showed good sensitivity to Gentamycin, Linezolid, Vancomycin, Ciprofloxacin and Clindamycin. Antibiotics susceptibility pattern of gram Negative bacteria showed good sensitivity to Imipenem, Meropenem, Tobramycin, Amikacin, Kenamycin, Cefoperazone, and Tetracycline. *Conclusion:* Microbial colonization was present right from the time of admission in the majority of swabs. The commonest organism isolated was *Pseudomonas Aeruginosa* followed by *Acinetobacter baumannii, staphylococcus aureus, Klebsiella pneumoniae, Enterobacter, Enterococcus species Escherichia coli, proteus mirabilis, CONS* and *Citrobacter species.*

Keywords: Burn wounds, Invasive Infection**,** Pseudomonas Aeruginosa, Antibiotic Susceptibility.

Introduction

The skin forms a defensive barrier against incursion by bacteria, fungi and viruses; any injury to the skin therefore, causes a break in the protective layer surrounding the body [1]. Thermal burns to the skin are caused by any outer heat source. This

may be in the form of an exposed fire from an open fireplace or house fire, a scald from steam, hot or molten liquid, or by direct contact with a burning object such as a hot oven rack or hot cooking pan. Other types of burns consist of radiation burns (from the sun's ultraviolet rays), chemical burns and electrical burns [2]. Burn causes immune suppression.

The burn wound has a much higher incidence of infections compared with other forms of trauma because of extensive skin barrier obliteration as well as alteration of the cellular and humoral immune responses. The dysfunction of the immune system, large cutaneous bacterial load, the possibility of gastrointestinal bacterial translocation, extended hospitalization, and persistent diagnostic and therapeutic procedures all contribute to sepsis [3].

Microorganisms colonizing the burn wound originate from the patient's remote skin and gastrointestinal and respiratory flora. Microorganisms may also be transferred to a patient's skin surface via contact with contaminated external ecological surfaces, water, fomites, air, and the soiled hands of health care workers. Immediately following injury, gram-positive bacteria from the patient's internal skin flora or the external environment predominantly colonize the burn wound. Endogenous gram-negative bacteria from the patient's gastrointestinal flora also rapidly colonize the burn wound surface in the first few days after injury [2,3].

Wound colonization by yeasts and fungi usually occurs later due to the use of broad-spectrum antibiotic therapy. Microorganisms transmitted from the hospital environment tend to be more resistant to antimicrobial agents than those originating from the patient's normal flora [4].

The increase rate of burn wound infection and sepsis is due to overcrowding (such as in developing countries), inadequate sterilization and disinfection practices, gross contamination of environment, and lack of isolation facilities, inadequate hand washing and absence of barrier nursing. Patients have to stay for long period in the hospital and many intravascular and other devices are put in them. Hence they are at greater risk of acquiring hospital-acquired infection [5].

The majority organisms which remain as causative agents of burn wound infection in any burn treatment facility change over time. Gram positive organisms are initially prevalent during hospital stay of patients; then gradually become super quantity unit by gram negative opportunists that appear to have a greater susceptibility to invade [6]. This would allow early management of looming septic episodes with empirical systemic antibiotic before the results of microbiologic culture becomes available thus improving overall infection related morbidity and mortality [7].

In addition to loss of the likely cutaneous barrier to infection, coagulated protein and other microbial nutrients in the burn wound, there is loss of vascularity of the wound leading to microbial colonization. In some patients, colonization is followed by invasion of microorganisms, giving rise to burn wound infections. After the development of effective therapy for fluid and electrolyte abnormalities caused by severe burns, infection and septicemia became the most important causes of death [8].

There should be continuous observation of burn infections and increase strategies for antimicrobial resistance control and treatment of infectious complications. Hence, the present study was under taken to establish the bacteriological profile of the burn wound infection and to formulate empirical treatment guidelines for these patients, so that mortality can be prevented.

Material and Methods

A cross sectional study in which a total of 60 burn patients admitted in burn unit was conducted in the Department of Microbiology of Maharishi Markandeshwar Institute of Medical Science and Research (MMIMSR) Mullana, Ambala Haryana.

Inclusion Criteria

Burn patients admitted within 24 hours were included.

Exclusion Criteria

Burn patients admitted after 24 hours were excluded.

Specimen Processing

Two burn wound swab were collected aseptically on admission before the start of antibiotics and there after weekly for a maximum period of four weeks or till the patient were discharged or expired. Two Swabs were collected from the burn area and immediately transported in a sterile test tube to the microbiology laboratory; one swab was used for the

gram staining and the other for culture. Wound swabs were then subjected to microbiological analysis by Gram's staining and culture. All specimens were inoculated on 5% blood agar and Mac Conkey agar plates and incubated over night at 37^0C. The isolates were identified by standard microbiological techniques. All the organisms isolated were subjected for antimicrobial susceptibility testing by modified Kirby Bauer method according to CLSI guide lines.

Results

Total no of 60 patients were taken for the study and following results were observed.

Table 1: Shows age wise distribution**.** Out of 60 patients that were studied, the commonest age group was 21-40 years with 30 patients (50%). The second commonest and the least common age group was 0-20 years (20%) and more than 60 years (13.3%) respectively, the youngest patient being 2 month old and the oldest being 76 years old.

Total No. of Patients	Age	No. of Patients	Percentage (%)
	0 – 20	12	20
60	21 – 40	30	50
	41-60	10	16.16
	>60	08	13.3
Total		60	100

Table 2: Shows sex wise distribution of Female patients were lesser than male patients; female being 21 (35%) and males 39 (65%).

Total No. of Patients	Sex	No. of Patients	Percentage
N=60	Male	39	65
	Female	21	35
Total		60	100

Table 3: Illustrates cause of burn of Maximum number of patients 25 (41.6%) suffered from fire burn. Followed by 18 (30%) were due to Electric burn and the remaining 11 (18.3%) were due to thermal burn. Fourth burn chemical 6 (10 %)

Cause	No. of Patients		Percentage (%)
	Stove	Other	
Fire	25	-	41.6
Electric	18	-	30
Chemical	06	-	10
Thermal	11	-	18.3

Table 4: Depicts organism isolated from 240 samples from 60 burn patients. Among the total 240 swabs, single organisms were isolated in 161 samples. Mixed growth was seen in 22 samples and no growth in 57 samples. Among single isolates *Pseudomonas aeruginosa* was leading (20.8%) followed by *Acinetobacter baumannii (15.4%), Staphylococcus aureus (14.1%), Klebsiella pneumoniae (7.5%), Enterobacter (2.5%), Enterococcus (1.6%), Escherichia Coli (1.2%), Proteus (1.2%), Coagulase Negative Staphylococcus (1.2%) and citrobacter species (1.2%).*

Isolated Organism	Pure Growth		Mixed Growth		Total	
	No	%	No	%	No	%
Pseudomonas Aeruginosa	50	20.8	14	30.4	64	26.7
Acinetobacter Baumanni	37	15.4	11	23.9	48	20.0
Staphylococcus Aureus	34	14.1	05	10.8	39	16.2
Klebsiella Pneumonia	18	7.5	08	17.4	26	10.8
Enterobacter Species	06	2.5	02	4.3	08	3.3
Enterococcus Species	04	1.6	02	4.3	06	2.5
Escherichia Coli	03	1.2	01	2.1	04	1.6
Proteus Species	03	1.2	0	0	03	1.2
CONS	03	1.2	0	0	03	1.2
Citrobacter Species	03	1.2	03	6.5	03	1.2
No growth	57	23.7	-	-	57	23.7
Mixed	-	-	22		22	9.1

Table 5: Percentage (%) sensitivity of gram positive bacteria

Antibiotics	Staph Aureus	Enterococcus Species	CONS
Vancomycin	90.9%	100%	66.6%
Azithromycin	45.5%	20%	66.6%
Clindamycin	27.5%	20%	100%
Ciprofloxacin	81.8%	100%	33.3%
Gentamycin	100%	100%	66.6%
Tetracycline	63.6%	60%	33.3%
Gatifloxacin	45.5%	40%	0%
Levofloxacin	36.6%	60%	0%
Penicillin	27.2%	20%	66.6%
Rifampicin	9%	0%	0%
Linezolid	100%	100%	90%

Table 6: Shows the Antibiotics susceptibility pattern of gram negative bacteria a total 44 samples isolates that were 100% sensitive to Gentamycin and Linezolid, Vancomycin, Ciprofloxacin and clindamycin

Antibiotics	Pseudomonas Aeruginosa	Acinetobacter Baumanni	Klebsiella Pneumoniae	Enterobacter Species	Escherichia Coli	Proteus Species	Citrobacter Species
Gentamicin	72.7%	27.7%	33.3%	66.6%	66.6%	100%	100%
Ampicillin	80%	38.8%	11.1%	33.3%	33.3%	100%	50%
Amikacin	90%	90%	100%	83.3%	100%	100%	100%
Cefoperazone	70%	16.6%	16.6%	50%	33.3%	100%	50%
Ciprofloxacin	76.8%	11.1%	22.2%	66.6%	66.6%	100%	50%
Levofloxacin	45%	11%	5.5%	16.6%	66.6%	100%	50%
Imipenem	95%	33.3%	27.7%	66.6%	100%	100%	100%
Meropenem	90%	27.7%	33.3%	83.3%	100%	100%	100%
Chloramphenicol	81.8%	22.2%	11.1%	66.6%	66.6%	100%	50%
Kanamycin	90%	33.3%	11.1%	33.3%	100%	100%	50%
Tetracycline	90%	95%	16.6%	66.6%	66.6%	0%	50%
Tobramycin	90%	66.6%	44.4%	50%	66.6%	0%	50%

Discussion

Burn injuries constitute a major health concern with respect to morbidity and mortality as well as cost of management particularly in developing countries. Thermal injuries impairs the skin & normal barrier function, thus there is microbial colonization in burn wound, because of which contamination is almost unavoidable [9]. Burn wound infection is one of the frequent and severe complications in patients who have sustained burns [10]. Because of the variability of both local and systemic clinical manifestation of invasive burn wound infection, great emphasis is given on the proper identification of burn wound microbial flora by clinician treating burn wound sepsis [11].

In this study from 60 patients included, a total of 240 swabs were taken. Out of 60 Patients, majority of the patients were in age group of 21-40 years (Table 1).

Similar findings were seen by Jyotindra et al (2000), and Leila Azimi et al (2011). It was seen that males were affected more than females (Table 2).

The mode of burn injury in our study was fire injury and electric (Table 3). This result is in accordance with the study done by Leila Azimi et al (2011).

Burn research in India started to blossom during 1970s primarily with epidemiological studies. The nature and extent of the problem of thermal injuries in the vastly populated subcontinent of India was almost unknown before early 1970s.

Pseudomonas aeruginosa isolation was maximum in our study in both single (20.8%) and mixed (30.4 %) growth. *Acinetobacter baumanni* was the second most common isolate in both single (15.4%) and

mixed (23.9%) growth. Growth of Staphylococcus was 14.1% in single isolates and 10.8% in mixed growth (Table 4). This finding is in accordance with other studies by Chaya a Kumar et al who reported *Pseudomonas aeruginosa* as the commonest isolates (34.9%) in their study and Shweta et al who reported *Pseudomonas aeruginosa* as the commonest isolates (47 %) in their study.

The high frequency of *Pseudomonas aeruginosa* might be because it is found frequently in hospital environments and burn wound are an ideal medium for their survival. *Pseudomonas aeruginosa* are inherently resistant to commonly used antibiotics and can even survive in common antiseptics (Oncul E. Ulrur A et al 2009) [12].

Regarding growth of *Acinetobacter baumanni*, it was similar to other studies who also reported higher frequency of *Acinetobacter* infections in their studies. In the study conducted by Ýlyas Yolbaþ et al (2013) Acinetobacter *baumanni* (62.3%) was the most common isolated pathogen. Similarly Harvey Chim et al (2007) also reported similar findings in their study.

The persistence of *Staphylococcus* throughout our study could be due to cross infection of micro-organisms in ICU. It may also be due to the fact that proper infection control practices were not followed by relatives of patients and by health care workers.

The antibiotic sensitivity test was performed by Kirby-Bauer disc diffusion method using commercially available discs (Hi-media). The results were interpreted as per CLSI guideline.

Resistance patterns among nosocomial bacterial pathogens may vary from country to country and also within the same country, over time. In this study antibiotics sensitivity profile of the isolates were also observed. *Pseudomonas aeruginosa* isolates in our study were susceptible to imipenem (95%) and amikacin (90%). This contrasts with the antibiotic sensitivity pattern of study done by Saha et al (2011) and similar to studies done by Vinod Kumar C.S. (2013) and Jyotindra et al (2000).

In the present study, almost all strains of *Staphylococcus aureus* showed good sensitivity to Linezolid and Gentamycin (Table 5). Chaya a Kumar et al (2010) reported 100 % sensitivity to Vancomycin and Linezolid in Staphylococcus aureus.

Similarly Acinetobacter *s*pecies showed higher rate of resistance to ciprofloxacin, amikacin, ceftazidime, and piperacillin in our study. Strains showed good sensitivity to amikacin and tetracycline. Other studies have reported high degree of resistance to almost all the antibiotics. We attribute these differences in the susceptibility of strains to differences in the patient population studied by us. Most of our patients were from surgical wards. Furthermore, our patients came from rural areas without much exposure to antibiotics.

Conclusion

Microbial colonization was present right from the time of admission in the majority of swabs. The rate of colonization progressively increased from second and upto the end of third week. The commonest organism isolated was *Pseudomonas aeruginosa* followed by *Acinetobacter baumannii, staphylococcus aureus, klebsiella pneumoniae, Enterobacter, Enterococcus species Escherichia coli, proteus mirabilis, CONS and Citrobacter* species. All the gram-negative organisms had good sensitivity to imipenem and meropenem and resistant to commonly used antibiotics like gentamycin, and cefoperazone, levofloxacin. And all Gram positive organisms had good sensitivity to Linezolid, Gentamycin, and Vancomycin.

Acknowledgement

We are thankful to Dr.Varsha A. Singh (Prof. & Head, Department of Microbiology), MMU, Mullana, Ambala for standing by us while carrying out this study. We extend our earliest gratitude to each and everyone in the Department of Microbiology, who were very kind to us throughout our research.

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Original Article

Journal of Microbiology and Related Research
Volume 2 Number 2, July - December 2016
DOI: http://dx.doi.org/10.21088/jmrr.2395.6623.2216.7

Truelab Micro PCR In Diagnosis of Extrapulmonary Tuberculosis: Our Experience

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Received on 31.08.2016, **Accepted on** 15.09.2016

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Abstract

Introduction: India carries about 25% burden of tuberculosis. It is estimated that about 15-20% of all TB cases are of extrapulmonary origin. Recently, molecular amplification methods such as PCR and RT PCR have attracted interest of laboratorians as the diagnosis can be made in a short time and treatment initiated at the proper time.The present study was undertaken to evaluate the role of Truelab Micro PCR (Molbio diagnostics) in diagnosis of EPTB. *Materials and Methods:* This was a retrospective study conducted between January 2014 to June 2016.Atotal of 447 patients suspected of having EPTB clinically were included in the study. Specimens were collected from patients, both male and female, from different sites like pus, body fluids, lymph node aspirates, CSF, semen, urine, menstrual blood and endometrial tissue etc.according to standard protocols. All the specimens were run on Truelab MicroPCR system(Molbio diagnostics) according to standard protocols. *Results:* A total of 447 patients data was used for this study. Maximum number of samples were endometrial tissue (59.9%) followed by lymph node aspirates (11.63%), pus (4.92%),urine (4.47%), pleural fluid & menstrual blood (3.57%) CSF (3.13%), Ascitic fluid (2.90%) Synovial fluid (1.1%), vitreous fluid and peritoneal fluid (0.89%) , BAL fluid (0.67%) . Out of these different extra pulmonary samples 19(4.25%) were positive by RT PCR and 95.75% were negative. Highest positivity rate was observed inpus (27.2%), followed by CSF (21.4%), menstrual blood & pleural fluid (6.25%), lymph node aspirates(5.76%), urine(5%) and 1.49% in endometrial tissue. *Conclusion:* Truelab Micro PCR is portable,easy to use and provides accurate diagnosis of disease like EPTB with a rapid turn around time. It does not require skilled manpower and elaborate infrastructure and can be easily installed in resource limited settings. A complete patient centric solution for patients suffering from EPTB is a necessity and rapid, accurate diagnosis with Truelab Micro PCR and prompt therapywill help control TB burden in India.

Keywords: EPTB; Real Time PCR; Molecular Methods; True Lab MicroPCR.

Introduction

Tuberculosis is a major global health problem and it is estimated that approximately 8.8 million new cases were reported in year 2012 out of which 1.3 million succumbed to the disease [1]. India carries about 25% burden of tuberculosis and it is estimated

that about one third of the total TB cases are left undiagnosed and hence untreated.There is no reliable data available from India about the burden of extrapulmonary TB, but it is estimated that about 15-20% of all TB cases are of extrapulmonary origin.

The clinical presentation of EPTB is varied, depending upon the site involved, hence many cases may be missed or there might be delay in diagnosis and treatment due to lack of standardized methods for diagnosing EPTB,unlike pulmonary TB. Moreover, the paucibacillary nature of EPTB hinders the diagnosis. Conventional methods like smear and culture have limited diagnostic value in diagnosis of EPTB as it lacks sensitivity and specificity. Recently, molecular amplification methods such as PCR and RT PCR have attracted interest of laboratorians as the diagnosis can be made in a short time and treatment initiated at the proper time.

The present study was undertaken to evaluate the role of Truelab MicroPCR (Molbio diagnostics) in diagnosis of EPTB.

Materials and Methods

This was a retrospective study conducted between January 2014 to June 2016.Atotal of 447 patients suspected of having EPTB clinically were included in the study. Specimens were collected from patients,both male and female,from different sites like pus, body fluids,lymph node aspirates, CSF, semen, urine, menstrual blood and endometrial tissue according to standard protocols. Specimens were subjected to direct smear microscopy for presence of acid fast bacilli when the quantity and quality of specimen permitted. Provisional diagnosis of EPTB was made on the basis of cytology, radiology and response to anti tubercular treatment when confirmatory histopathological diagnosis was not available. All the specimens were run on Truelab MicroPCR saystem (Molbio diagnostics) according to standard protocols.

Results

A total of 447 patients data was used for this study . The patients were divided into 0–20 years, 21-40, 41–60 , 61–80 and more than 80 years age group in both the sexes.

Maximum patients (76.2%) were in 21-40 years age group, followed by (10.7%) in 0-20 years age group, 9.17% in 41-60 years, 3.57% in 61-80 years and 0.22 % in more than 80 years age group 19.2% were males and 80.8% were females. Male female ratio was 0.23:1 with maximum number of female patients in 21-40 years age group (Table 1).

Maximum number of samples were endometrial tissue (59.9%) followed by lymph node aspirates(11.63%), pus (4.92%), urine (4.47%), pleural fluid & menstrual blood (3.57%) CSF (3.13%), Ascitic fluid (2.90%) Synovial fluid (1.1%), vitreous fluid and peritoneal fluid (0.89%), BAL fluid (0.67%), and other sites like semen,ovarian cyst fluid, fluid from Pouch of Douglas(0.44%)and 0.22%from cervical tissue, tissue from sinus tract and skin lesion (Table 2).

Out of these different extra pulmonary samples 19(4.25%) were positive by RT PCR. and 95.75% were negative. Highest positivity rate was observed in pus (27.2%), followed by CSF (21.4%) menstrual blood & pleural fluid (6.25%), lymph node aspirates(5.76%), urine(5%) and 1.49% in endometrial tissue(Table 3).

No positive result was observed in vitreous fluid BAL fluid, Ascitic, Synovial and peritoneal fluids and other sites like ovarian tissue, fluid from Pouch of Douglas etc (Table 4).

268 patients with endometrial tissue as sample were divided into less than 20, 21-30, 31-40, 41to50 and more than 50 years age group, out of which 63.4% patients where in 21-30 years age group followed by 30.9% in 31-40 years, 3.73% below 20 years, 1.49% in 41-50 years age and 0.37% in more than 50 years age group (Table 5).

Table 1: Showing demographic distribution of patients

Age in years	Male	Female	Total	Percentage
0 To 20	24	24	48	10.70%
21 to 40	31	310	341	76.20%
41 to 60	19	22	41	9.17%
61 to 80	11	5	16	3.57%
>80	1		1	0.22%
TOTAL	86	361	447	
Total Percentage	19.20%	80.80%	100%	

Graph 1

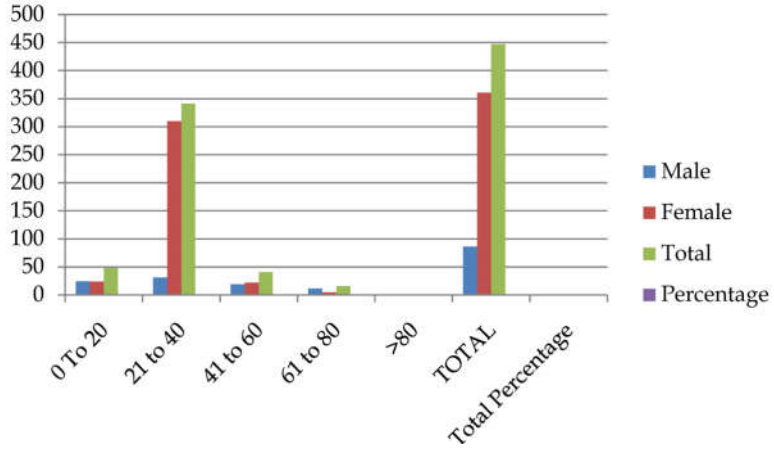


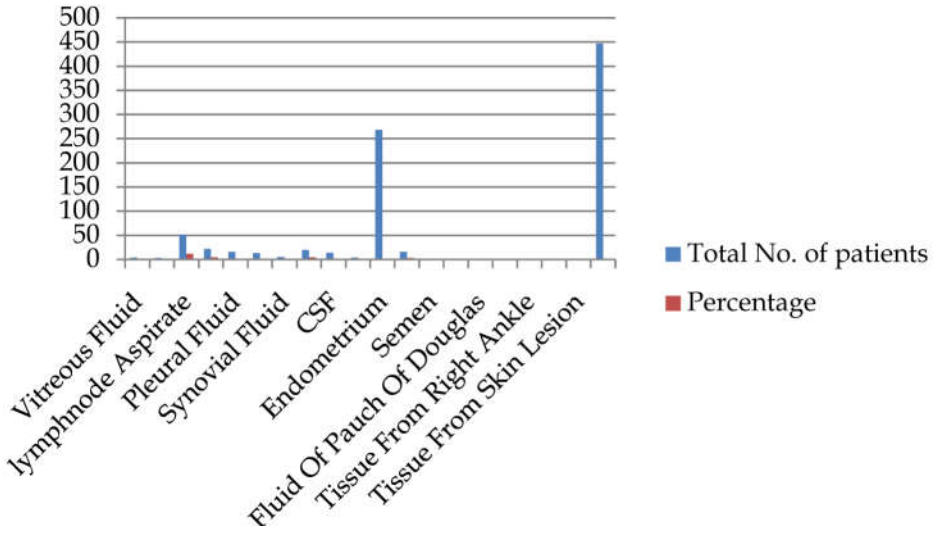
Table 2: Showing number of patients with different clinical specimens

Specimen site	Total No. of patients	Percentage
Vitreous Fluid	4	0.89%
Bal Fluid	3	0.67%
lymphnode Aspirate	52	11.63
Pus	22	4.92
Pleural Fluid	16	3.57%
Ascitic Fluid	13	2.90%
Synovial Fluid	5	1.11
Urine	20	4.47
CSF	14	3.13%
Peritoneal Fluid	4	0.89%
Endometrium	268	59.90%
Menstrual Blood	16	3.57
Semen	2	0.44
Ovarian Cyst Fluid	2	0.44
Fluid Of Pauch Of Douglas	2	0.44
Cervical Tissue	1	0.22
Tissue From Right Ankle	1	0.22
Tissue From Sinus Track	1	0.22
Tissue From Skin Lesion	1	0.22
Total	447	

Table 3: Showing number of positive patients with different clinical specimens

Specimen site	Total Positivepatients	Percentage
Vitreous Fluid	0	0.00%
Bal Fluid	0	0.00%
lymphnode Aspirate	3	5.76
Pus	6	27.2
Pleural Fluid	1	6.25%
Ascitic Fluid	0	0.00%
Synovial Fluid	0	0
Urine	1	5
CSF	3	21.4%
Peritoneal Fluid	0	0.00%
Endometrium	4	1.49%
Menstrual Blood	1	6.25
Semen	0	0.00%
Ovarian Cyst Fluid	0	0.00%
Fluid Of Pauch Of Douglas	0	0.00%
Cervical Tissue	0	0.00%
Tissue From Right Ankle	0	0.00%
Tissue From Sinus Track	0	0.00%
Tissue From Skin Lesion	0	0.00%
Total	447	4.25

Graph 2:



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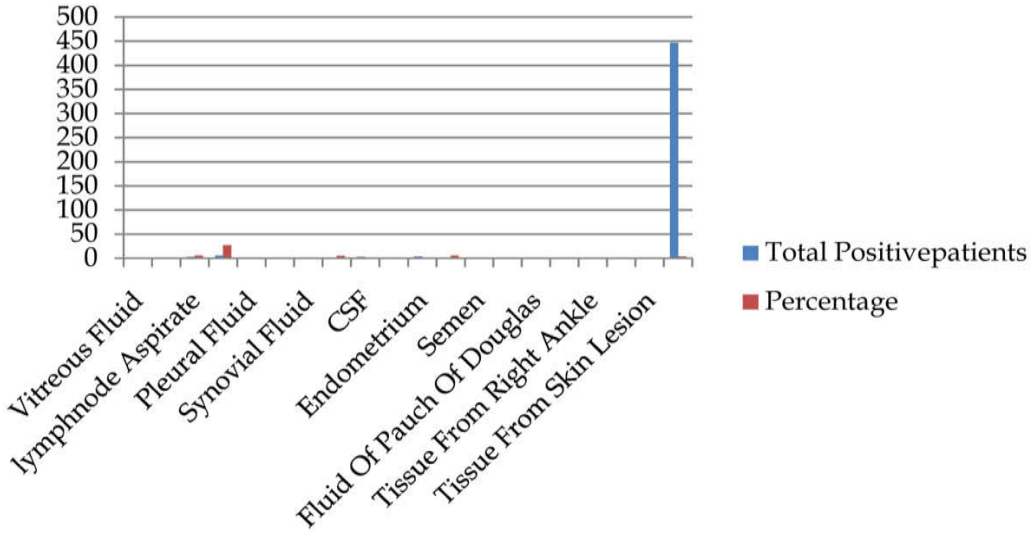


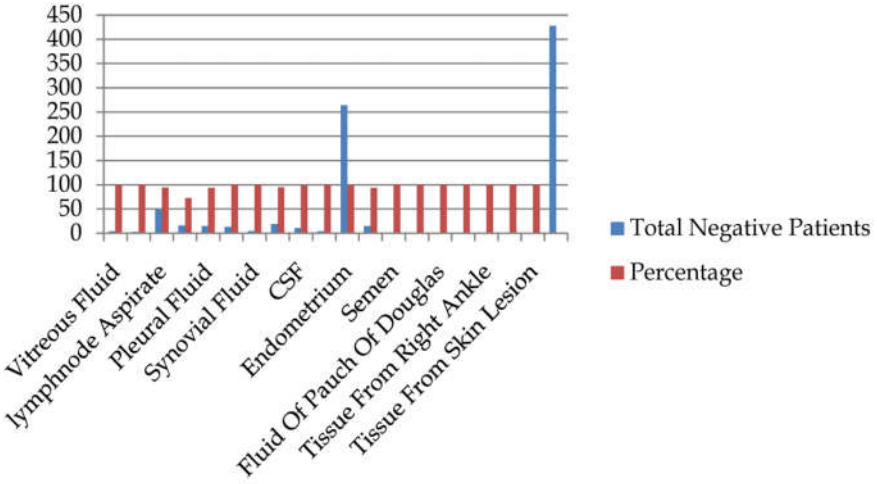
Table 4:

Specimen site	Total Negative Patients	Percentage
Vitreous Fluid	4	100
Bal Fluid	3	100
lymphnode Aspirate	49	94.24
Pus	16	72.8
Pleural Fluid	15	93.75
Ascitic Fluid	13	100
Synovial Fluid	5	100
Urine	19	95
CSF	11	78.6
Peritoneal Fluid	4	100
Endometrium	264	98.5
Menstrual Blood	15	93.75
Semen	2	100
Ovarian Cyst Fluid	2	100
Fluid Of Pauch Of Douglas	2	100
Cervical Tissue	1	100
ssue From Right Ankle	1	100
issue From Sinus Track	1	100
issue From Skin Lesion	1	100
Total	428	

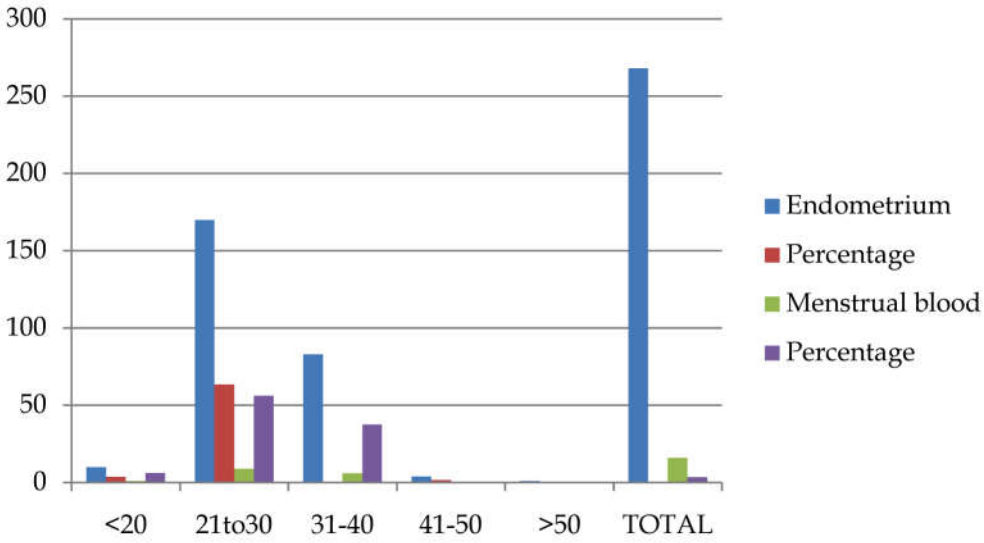
Table 5: Age wise distribution of patients(endometrium & menstrual blood)

Age	Endometrium	Percentage	Menstrual blood	Percentage
<20	10	3.73	1	6.25
21to30	170	63.4	9	56.25
31-40	83	30.90%	6	37.5
41-50	4	1.59	0	0
>50	1	0.37	0	0
TOTAL	268	59.90%	16	3.57

Graph 4:



Graph 5:



Graph 6:

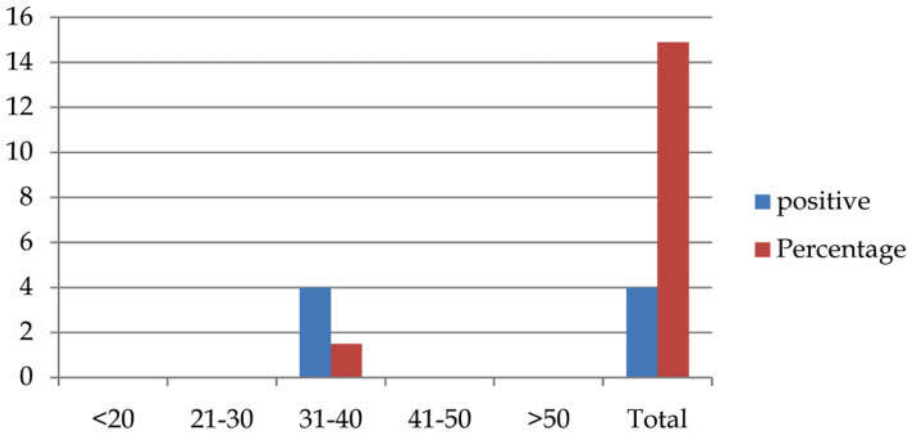


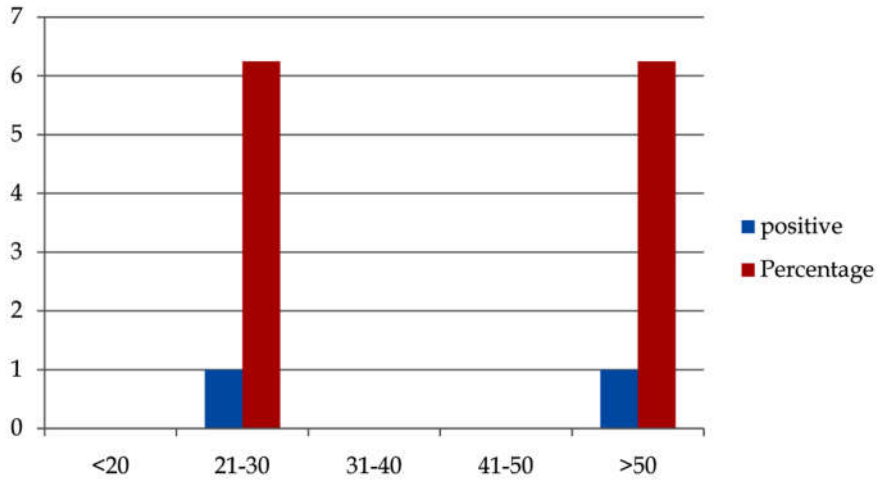
Table 6: Age wise distribution ofnumber of positive patients with endometrium

Age in years	Positive	Percentage
<20	0	0
21-30	0	0
31-40	4	1.49
41-50	0	0
>50	0	0
Total	4	14.9

Table 7: Age wise distribution ofpositive patients with menstrual blood as specimen

Age in years	Positive	Percentage
<20	0	0
21-30	1	6.25
31-40	0	0
41-50	0	0
>50	1	6.25

Graph 7:



Out of these 4.81% were detected to be positive for T.B. in 31-40 years age group and rest were negative, the overall positivity rate was 1.49% (Table 6). All positive results were correlated with histopathology of endometrium. 2 out of 4 positive cases were positive by histopathoolgy also.

The overall positivity rate with menstrual blood as sample was 6.25% (1/16) with one patient being in 21-30 years age group.Rest all were negative (Table 7).

Discussion

TB is a major health problem specially in the Indian subcontinent . The diagnosis of EPTB remains a problem because the diagnostic tools available are not sensitive enough to detect EPTB in early stages specially in paucibacillary cases.

The role of Real time PCR in diagnosis of EPTB has attracted much attention in recent years and some studies have been conducted to evaluate the role of RT PCR and the results have been variable with a sensitivity from 42 % to 100% and specificity between 85% to 100% using different PCR targets [6,7,8]. V. Mahesh Kumar et al in 2014 and M.Singh et al in 2013 observed PCR to be a sensitive and quick method to diagnose EPTB as compared to culture methods [9,10].

Sharma et al found a positivity rate of Zheil Neelson stain, culture and PCR to be 30%, 26.3% and 91.3%respectively [11]. Recently, Xpert MTB RIF assay (Cefeid Sunnyvale CA,USA) has been found to have a sensitivity of 81.2% for lymph nodes, 62.8% for CSF and 21.4% for pleural fluid [12].

Navarro Viasaro et al observed that urogenital TB was the third most frequent EPTB infection ,the first two being pleural and lymph node TB [13].

Sreerama Reddy et al observed 42.6% positive rate for lymph node TB and 14.8%in peritoneum and intestinal TB [14].

Gunal et al observed a positivity rate of 9.7% to 10.7%in TB of joints and bones ,pleura,lymph

nodes,skin and peritoneum [15].

Mazza Stalder et al in 2012 observed that the most frequent EPTB sites were lymph nodes,pleura and osteoarticular TB.They observed that peritoneum, meningeal and urogenital TB were less frequent, may be due to low sensitivity of diagnostic tests including culture and molecular amplification tests [16].

Singh et al observed a 20%-25%positive ratf EPTB with urogenital TB accounting for 4% of the burden [17].

Our findings correlate more or less with these findings in terms of frequency .But the low positivity rate (4.25%) of EPTB in our study as compared to 20% to 25% in other studies needs further analysis. It might be due to paucibacillary nature of the specimens. To overcome the limitations of our study, a larger, multicentric prospective study is required to correctly estimate the positivity rate of EPTB in Indian settings. However, the initial study on Microlab PCR system seems promising**.**

Conclusion

Truelab Micro PCR is portable,easy to use and provides accurate diagnosis of disease like EPTB with a rapid turn around time of about an hour.It is specially useful in rural areas where availability of cost effective and rapid diagnostic tools is a major problem.The diagnosis of EPTB can be made during patient's first visit only and treatment initiated as soon as the diagnosis is made.It does not require skilled manpower and elaborate infrastructure and can be easily installed in resource limited settings. A complete patient centric solution for patients suffering from EPTB is a necessity and rapid ,accurate diagnosis with Truelab MicroPCR and prompt therapywill help control TB burden in India.

Conflict of Interest

none

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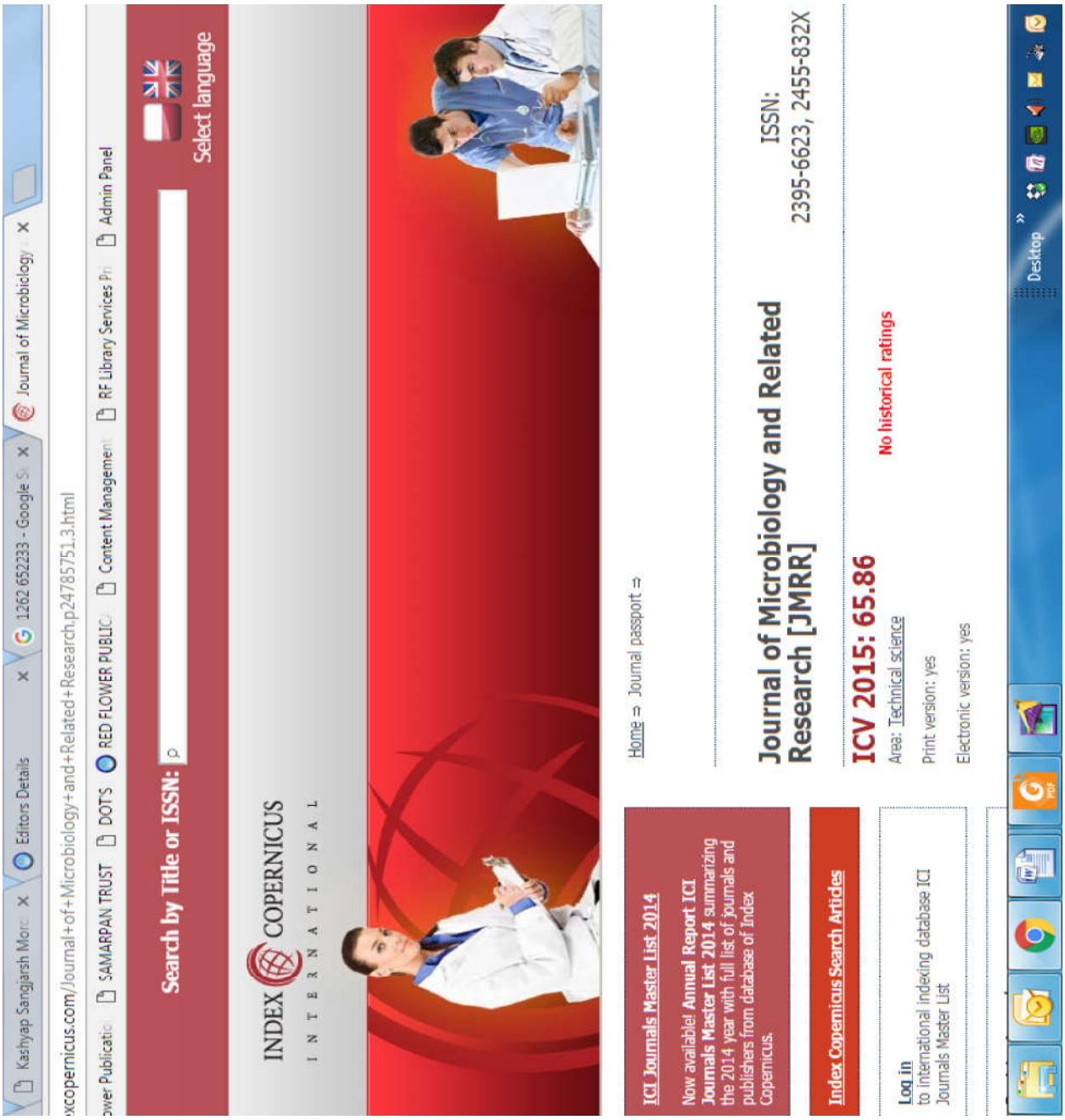

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[1] Flink H, Tegelberg Å, Thörn M, Lagerlöf F. Effect of oral iron supplementation on unstimulated salivary flow rate: A randomized, double-blind, placebo-controlled trial. J Oral Pathol Med 2006; 35: 540-7.

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Article in supplement or special issue

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Corporate (collective) author

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