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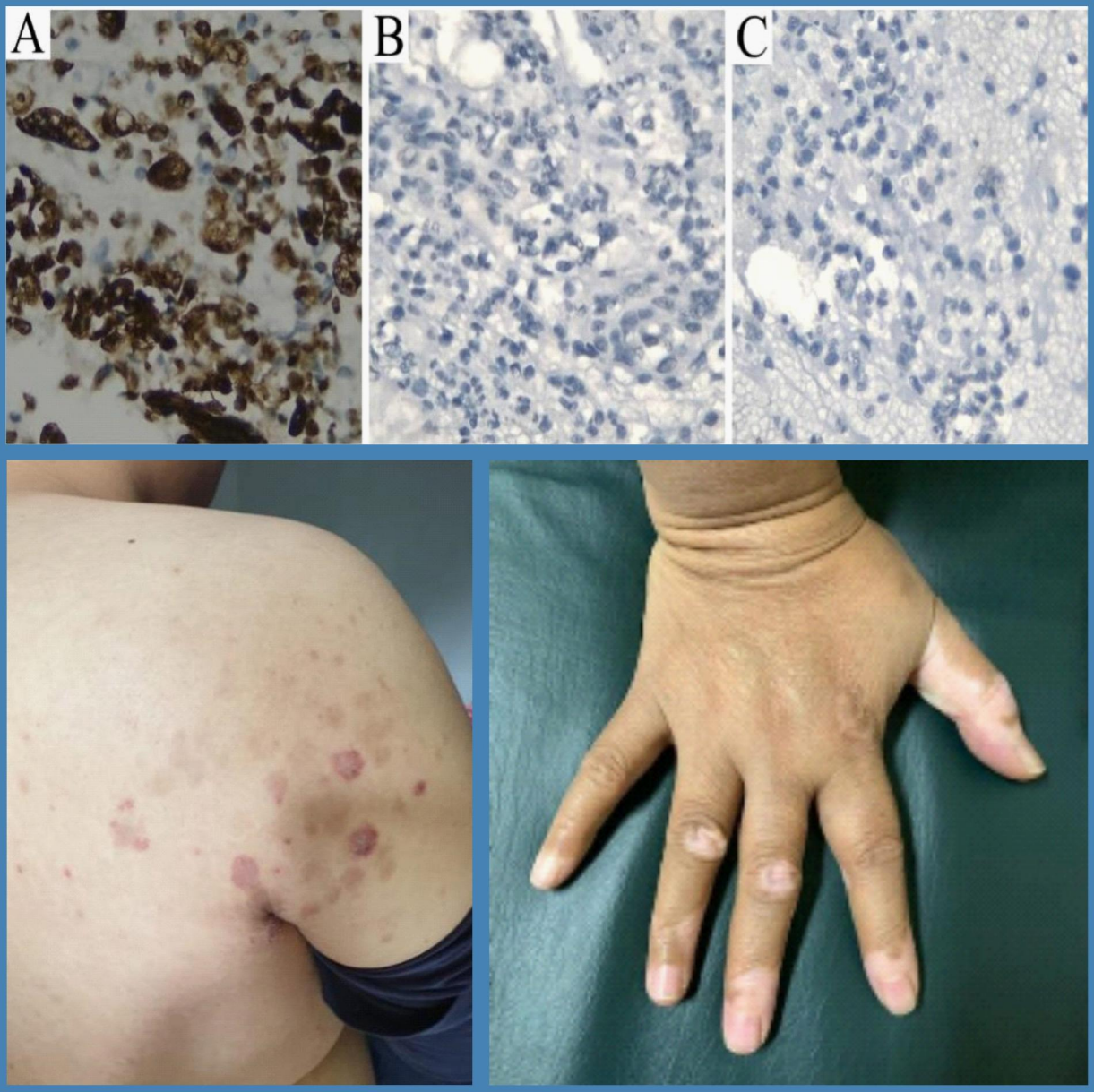

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Table of contents

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Effect of Robusta coffee extract gel on fibroblast and collagen during proliferative phase of IIB degree-burn on Long Evans rats

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ABSTRACT

IIB Degree-burn takes longer to heal because only a small epithelium component remains. It requires a topical agent that can accelerate the wound healing process. One of the wound healing signs is the increased collagen formation as an extracellular matrix produced by fibroblast in the proliferative phase. This study aimed to prove the effect of Robusta coffee extract gel as a topical agent on increasing the number of fibroblasts and collagen density in IIB degree-burn treatment on Long Evans rats. This study used 24 Long Evans rats which were divided into three groups, namely positive control (silver sulfadiazine), negative control (gel base), and treatment group (2.5% of Robusta coffee extract gel). The results showed a higher number of fibroblast ($p<0.05$) and a denser collagen density ($p<0.05$) of the Robusta coffee extract gel compared to the control group on day 8 and day 14. These results indicated that the number of fibroblasts and collagen increases that can scavenge free radicals and stimulate the release of cytokines that play a role in increasing fibroblast proliferation. Robusta coffee affects expanding the number of fibroblasts and collagen density to be an alternative topical agent of second-degree burns treatment.

ABSTRAK

Proses penyembuhan luka bakar derajat IIB membutuhkan waktu yang cukup lama. Hal ini dikarenakan komponen epitel yang tersisa hanya sedikit sehingga memerlukan sediaan topikal yang dapat mempercepat proses penyembuhan luka tersebut. Salah satu tanda proses penyembuhan luka adalah terjadinya peningkatan pembentukan kolagen sebagai matriks ekstraseluler yang dihasilkan oleh fibroblast pada fase proliferasi. Tujuan penelitian ini adalah untuk membuktikan efek gel ekstrak kopi Robusta sebagai agen topikal terhadap peningkatan jumlah fibroblas dan kepadatan kolagen pada perawatan luka bakar derajat IIB tikus Long Evans. Penelitian ini menggunakan 24 ekor tikus yang dibagi menjadi 3 kelompok yaitu kontrol positif (silver sulfadiazine), kontrol negatif (basis gel), dan kelompok perlakuan (gel ekstrak kopi Robusta 2,5%). Hasil penelitian menunjukkan jumlah fibroblas yang lebih tinggi ($p<0.05$) dan kepadatan kolagen yang lebih rapat ($p<0.05$) antara gel ekstrak kopi Robusta dibandingkan dengan kelompok kontrol baik pada hari ke-8 maupun hari ke-14. Hasil ini menunjukkan bahwa ada peningkatan fibroblast dan kolagen yang dapat melindungi dari radikal bebas dan merangsang pelepasan sitokin yang berperan dalam meningkatkan proliferasi fibroblast. Gel kopi Robusta dapat meningkatkan jumlah fibroblast dan kepadatan kolangen sehingga gel ini dapat menjadi alternatif agen topikal dalam perawatan luka bakar derajat IIB.

Keywords:
topical agent;
burn;
deep partial thickness;
gel;
Robusta coffee

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INTRODUCTION

A burn is one of the biggest global health problems. It is estimated around 11 million cases annually worldwide.[1] In Jember, East Java, Indonesia, the burn prevalence reaches 70 cases from January 2014-October 2016, with the most cases severe burn.[2] IIB-Degree burns have a fairly high prevalence in Indonesia, there were 104 of 414 cases during 2013-2015.[3] In the II-degree burns, the damage usually extends to the reticular dermis. After the injury, the burn will cause redness and paleness or splotchy skin followed by the appearance of bullae in the injured area. At this stage, the patients are complaining of pain and decreased capillary refill when pressure is applied to the injury. Patients with IIB-degree burns are able to heal spontaneously without surgery.[1,4]

The proliferative phase plays an important role in restoring tissue integrity by replacing lost tissue and repairing damaged tissue during the wound healing process.[5] Proliferation of fibroblast cells occurs in this phase to synthesize collagen.[6] Collagen is a major protein component in the extracellular matrix to control the inflammatory response to injury.[7] The density of collagen in the wound area determines the strength of the tissue to prevent the risk of wound dehiscence. However, non-optimal wound care will cause a slower wound healing process.[8]

Moist wound treatment promotes the healing process of burn by providing a topical agent, such as silver sulfadiazine.[9] Another way to create a moist environment is to use standard modern dressings.[10] However, the number of modern dressings is very limited, especially in the peripheral area. These problems may cause a physical and psychological burden on the patient because the treatment takes longer.[11] Therefore, natural ingredients that can stimulate the wound healing process and abundant materials can be an alternative therapy with economic value. One of these ingredients is Robusta coffee.

Previous studies showed a positive effect of Robusta coffee extract in accelerating the healing process of incisional wounds in mice, indicating the number of fibroblasts increases.[12] Robusta coffee contains ingredients such as chlorogenic acid, caffeine, flavonoids, tannins, and saponins, which have antioxidant effects so that those ingredients can ward off excess free radicals at the wound base.[13] Flavonoids in coffee have antimicrobial effects that work through the destruction of cell membranes and denaturation of cell proteins. Besides, flavonoids have anti-inflammatory effects.[14] Nowadays, there have been no previous studies that found the use of Robusta coffee extract gel in the healing process of IIB degree-burn. This study was conducted to prove the effect of Robusta coffee extract gel as a topical agent on increasing the number of fibroblasts and collagen density in IIB degree-burn treatment on Long Evans rats.

MATERIALS AND METHODS

Animals

This study used 24 male Long Evans rats aged 2-3 months weighing 150-200 g. Rats were randomly divided into three groups: the 2.5% Robusta coffee extract gel group, the positive control group that was given silver sulfadiazine, and the negative control that was given the gel base. Rats were used in the experiment after one week of acclimatization. The research was conducted after obtaining approval from the Ethics Committee of the Faculty of Medicine, the Universitas Jember, Jember (ref:1508/H25.11/KE/2021).

Extract preparation

Robusta coffee used in this study was obtained from the Coffee and Cacao Research Institute in Jember. Two hundred g of Robusta coffee beans were grinded into powder. The powder was extracted using the ultrasonic method with 1.5 L of ethanol at 40°C for 30 min. The results were filtered using filter paper on a vacuum Buchner funnel and concentrated using a rotary evaporator.

Gel preparation

Methylparaben, nipasol, and propylene glycol were mixed into a glass beaker. Then, the carbopol and distilled water were mixed into the mortar in a ratio of 1:20. 4% triethanolamine was added slowly while stirred. The propylene mixture was put into a mortar containing carbopol and distilled water, then stirred to form a homogeneous gel mass. Robusta coffee extract and remaining distilled water were added to the mortar until homogeneous.

Burn wounds model

Rats were anaesthetized with a mixture of ketamine and midazolam intraperitoneally. Each rat was shaved on its back and cleaned with 70% alcohol. Based on Akhoondinasab *et al.*[15] modifications were carried out under the supervision of a clinically experienced burn and wound consultant plastic surgeon and experimental research related to burns may avoid bias without the need for a blind examiner. The burn was made using an iron plate with 2 x 2.5 cm width, immersed in boiling water at 98°C for 10 min. The hot iron plate was attached to the back of the rats for 20 seconds.

The wound was cleaned with normal saline and Savlon. Then, the gel base, silver sulfadiazine, and coffee extract gel were applied every 2 days according to each group. The wound was covered with a transparent film, sterile gauze, and plaster. Skin tissue was taken by biopsy on day 8 and day 14 to evaluate wound healing microscopically. The tissue was stored in 10% NBF solution for histopathological slides using HE staining.

Observation of histopathological slides

The histopathological observation was carried out using a light microscope with 400x magnification connected to an optical camera. Fibroblasts were identified as spindle-shaped cells or oval, flat, and purple in HE staining. These cells were observed in 6 fields of view, selected zig-zag from top to bottom in each slide, and then the average was calculated.[16] Meanwhile, collagen was identified as long pink fibers, which were interpreted in the scoring system. The scoring was determined based on Rachmanita *et al.*,[17] study in 4 fields of view, selected zig-zag from top to bottom in each slide, and then the average was calculated. The scoring system can be seen in TABLE 1.[17]

TABLE 1. Collagen density scoring

Score	Interpretation
0	No collagen fibers in the wound area
1	Collagen fibers in the low wound area (<25%)
2	Collagen fibers in moderate wound areas (26-50%)
3	Collagen fibers in tight wound areas (51-75%)
4	Collagen fibers in very tight wound areas (76-100%)

Data analysis

Research data analysis used the SPSS program. Data of the study were shown as mean ± standard deviation (SD). The fibroblast's statistical data analysis was carried out using the One Way Anova and continued using the Post Hoc LSD test. Further, the observation results of collagen density were tested using the Kruskal-Wallis' and followed by the Mann-Whitney's. The p <0.05 indicated a significant result.

RESULTS

Number of fibroblasts

Fibroblasts have spindle-shaped cells or oval, flat, and purple colored in HE staining. FIGURE 2 shows the fibroblasts in the burn day 14 of the negative control group (FIGURE 2a), Robusta coffee extract gel (FIGURE 2b), and positive control group (FIGURE 2c). The number of fibroblasts was significantly higher after being given Robusta coffee extract gel on the 8th and 14th day (59.27 ± 7.18 and 64.3 ± 9.93) than in the negative control group (51.07 ± 5 .93 and 53.08 ± 5.33), but it did not differ much compared to the positive control group (59.98 ± 5.27 and 62.71 ± 8.77), as shown in FIGURE 1. Burn treatment with Robusta coffee extract gel showed a significant difference in the number of fibroblasts compared to the negative control group on day 8th and day 14th (p <0.05), but there was no significant difference with the positive control (p>0.05). It was indicated that the Robusta coffee extract gel significantly increased the number of fibroblasts (FIGURE 1).

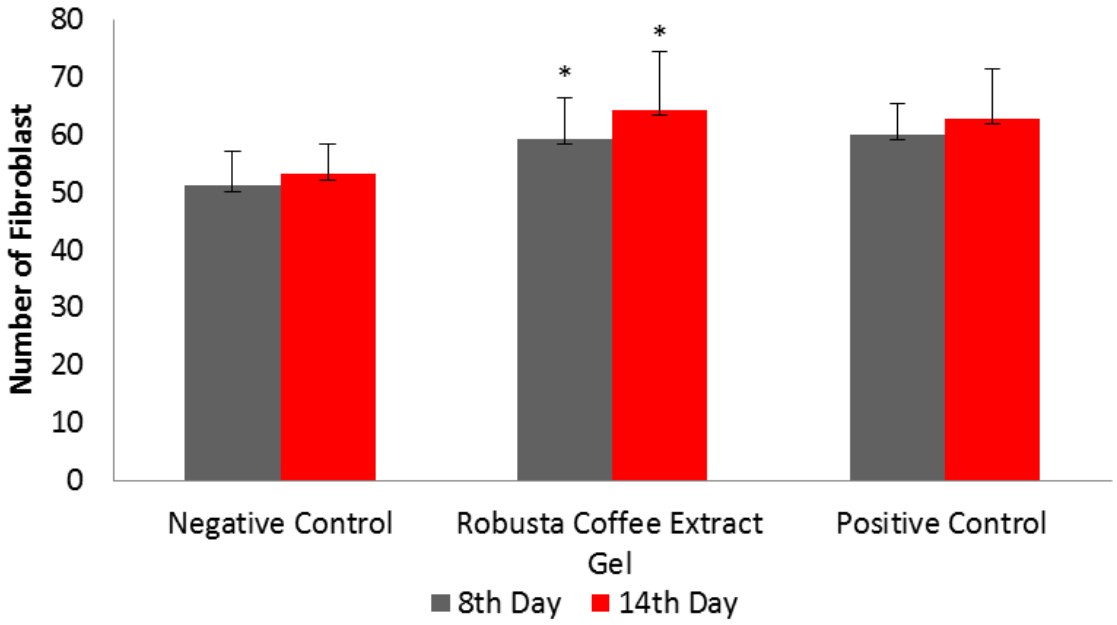

FIGURE 1. Number of fibroblast (mean ± SD) in each group on day 8th and day 14th. Post Hoc LSD Test; *p<0.05

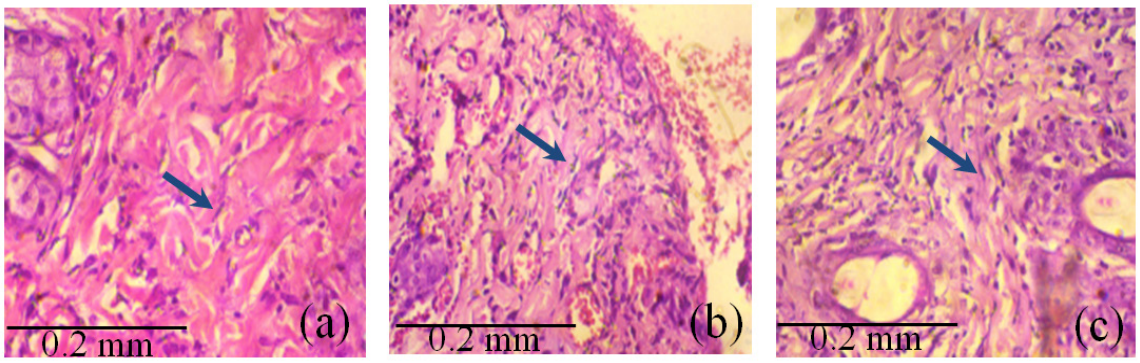

FIGURE 2. Photomicrographs of fibroblast cells in burn day 14th with magnification 400x; (a) negative control; (b) Robusta coffee extract gel; (c) positive control group.

Collagen density

A scoring system was used to measure the collagen density. FIGURE 3 shows that on the 8th and 14th days, the Robusta coffee extract gel has denser average collagen (2.75± 0.33; 3.25± 0.54) compared to the positive control (2.73± 0.44; 3.13± 0.45) and the negative control (2.25± 0.35; 2.65± 0.45). Histopathological description of the collagen density on day 14 of the negative control (FIGURE 4a), Robusta coffee extract gel (FIGURE 4b), and positive control (FIGURE 4c) can be seen in FIGURE 4. Burn treatment with Robusta Coffee extract gel showed a significant difference in collagen density compared to the negative control group on the 8th and 14th days (P<0.05). However, there was no significant difference between the Robusta coffee extract gel group and the positive control group (p>0.05). It was indicated that the Robusta coffee extract gel significantly increases the density of collagen (FIGURE 3).

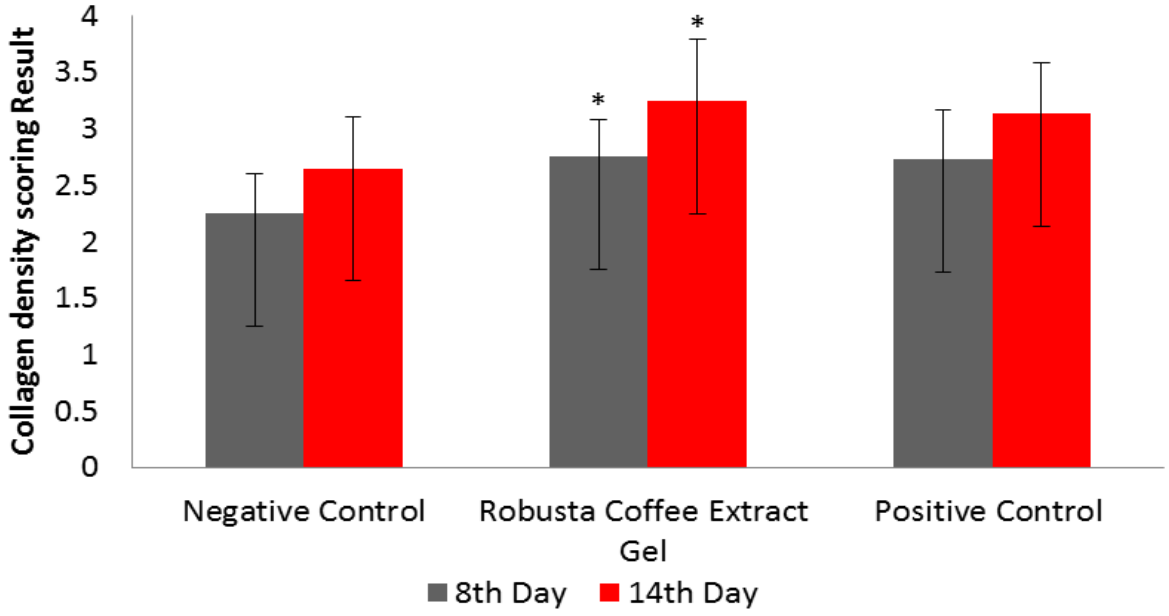

FIGURE 3. Collagen density scoring results (mean ± SD) for each group on 8th and 14th post-burn. Mann-Whitney; *p<0.05

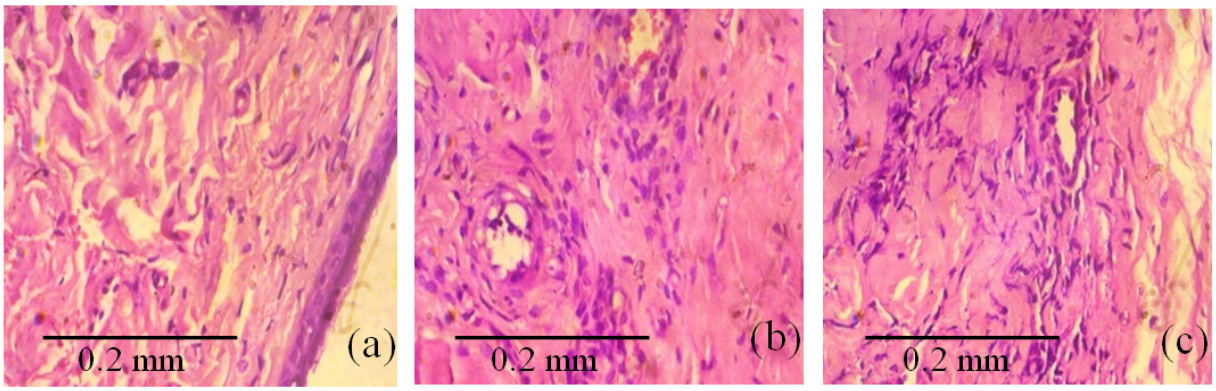

FIGURE 4. Histopathological of collagen density in day 14 burn with magnification 400x; (a) negative control; (b) Robusta coffee extract gel; (c) positive control.

DISCUSSION

In the burn wound healing process, there is a process of restoring tissue integrity by substituting lost tissue and repairing damaged tissue. During the proliferative phase, the process of epithelialization, angiogenesis, and collagen synthesis in the wound area will occur; this leads to the formation of an extracellular matrix to restore vascularity and form granulation tissue.[5] Fibroblasts are cells with a significant role, these cells will proliferate to synthesize collagen.[6] Collagen is a major protein component in the extracellular matrix that can control the inflammatory response to injury through its influence on mitogenesis, differentiation, and cellular migration.[7] The density of collagen can strengthen the tissue. However, insufficient collagen deposition in the wound area can increase the risk of wound dehiscence.[8]

The wound healing process in the negative control group was slower than in the other groups. It is indicated by the lower number of fibroblasts and collagen density. The negative control only contains a gel base. It does not affect the wound healing process because there is no active substance but only as a vehicle. This result is strengthened by Fuadi *et al.*[18] showing that wounds treated without active ingredients had lower fibroblast than others. On the contrary, the number of fibroblasts and collagen density in the positive control is higher due to silver sulfadiazine having antibacterial properties by preventing folic acid synthesis. Silver sulfadiazine plays a role in fibroblast proliferation to produce collagen and fibronectin.[19]

The substance of antioxidant chlorogenic acid, caffeine, and flavonoids in Robusta coffee bean extract caused an increase in the number of fibroblasts and collagen density compared to the negative control group on the 8th and 14th days. Excessive free radicals can disturb the activity of TGF-β in fibroblasts proliferation to synthesize collagen during the proliferative phase.[20,21] Humaryanto also showed that the administration of Robusta coffee extract could assist the wound healing process by increasing the number of inflammatory cells and fibroblast cell proliferation during the proliferative phase.[12] The results of this study were strengthened by Kenisa *et al.*[22] that the administration of Robusta coffee extract was able to significantly increase the number of fibroblasts so that the full thickness healing process was faster than the control group. Shahriari *et al.*[23] reported that the administration of green coffee bean extract improved the healing process of full-thickness wounds in rats. Coffee bean extract and its chemical constituents can promote keratinocytes

and fibroblasts' proliferation and migration, which are highly important in the wound healing process.[23]

Yaqin and Nurmilawati[24], the composition of Robusta coffee bean extract, namely caffeine, flavonoids, and chlorogenic acid, could inhibit the growth of *Staphylococcus aureus* bacteria colonies. The flavonoid contained in Robusta coffee has an antibacterial effect by damaging bacterial cell membranes and denaturing cell proteins to kill the bacteria.[14] Prevention of infection during the wound healing process will accelerate the healing process towards the proliferation phase, so fibroblast cell proliferation occurs faster.[25] It caused the number of fibroblast cells in the Robusta coffee extract group to be higher than in the negative control group on the seventh day. In line with Yaqin and Nurmilawati[24], Yuwono[26] reported in his study that Robusta coffee has a strong zone of inhibition against methicillin-resistant *Staphylococcus aureus* (MRSA).

The gel dosage form in the extract can provide a cold sensation and reduce pain. Acute pain after an injury may cause hemodynamic changes and anxiety in the experimental animals, resulting in slower wound healing. Pain reduces macrophage activity which affects the performance of TGF-β in stimulating fibroblast proliferation.[27] Lack of pain control causes a prolonged catabolic phase, increasing glucagon, corticosteroids, and insulin hormone resistance which can inhibit wound healing.[18]

This study showed that Robusta coffee extract gel had a positive effect on burn healing by increasing the number of fibroblast cells and collagen density. A previous similar study revealed that coffee extract had an impact on healing burns in male rats.[28] The limitation of this study is that the observation of the burn healing process is limited at the end of the proliferative phase (14th day) so the wound that is formed has not healed completely. In addition, Robusta coffee extract is only made in the form of gel preparation. Therefore, further research is needed on the process of burn healing to the remodeling phase or the use of Robusta coffee extract in other preparations such as ointments or creams.

CONCLUSION

The results of this study indicates that the Robusta coffee extract gel can accelerate the healing process of second-degree burns by increasing the number of fibroblasts and collagen density. Therefore, it can be an alternative topical agent in the IIB degree-burn treatment.

ACKNOWLEDGEMENTS

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Antibiotic effectiveness on biofilm - producing *Escherichia coli* isolated from catheterized patients

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ABSTRACT

Biofilm is one of the factors that facilitate the occurrence of catheter-associated urinary tract infection (CAUTI). *Escherichia coli* is reported as one of the most dominant bacteria that have virulence factors including biofilm formation. Uropathogenic *E. coli* (UPEC) shows increasing resistance to several antibiotics. Examination of the antibiotic sensitivity on the biofilm-producing *E. coli* and its activity on biofilm formation are important for selecting high effectiveness antibiotics which is beneficial for the management of CAUTI patients. A total of 35 *E. coli* isolates were recultured in the medium of LB agar and blood agar. The isolates were evaluated the sensitivity based on their MIC value to several antibiotics. In addition, the antibiofilm activity of the antibiotics based on their MBIC value was also evaluated. The data obtained were analyzed both descriptively and analytically. Almost the *E. coli* isolates have good sensitivity to meropenem antibiotics, amoxicillin-clavulanic acid, and Fosfomycin. However, among the evaluated antibiotics, only fosfomycin that showed antibiofilm activity. The different in terms of the resistance phenotype between the urinary isolates and the catheter isolates was observed. However, there were no significantly differences in the MIC value (pMIC=0.522) and the MBIC value (pMBIC = 0.523). In conclusion, the alternatives of antibiotic therapy for the planktonic bacteria are amoxicillin-clavulanic acid and fosfomycin, while for the biofilm bacteria is fosfomycin. A biofilm screening examination on the catheter to improve the effectiveness of therapy management for CAUTI patients is recommended.

ABSTRAK

Biofilm merupakan salah satu faktor yang mempermudah terjadinya infeksi saluran kemih akibat kateter (*catheter-associated urinary tract infection*/ CAUTI). *Escherichia coli* merupakan salah satu bakteri paling dominan yang memiliki faktor virulensi termasuk pembentukan biofilm. Uropathogenic *E. coli* (UPEC) menunjukkan peningkatan resistensi terhadap beberapa antibiotik. Pemeriksaan sensitivitas antibiotik pada *E. coli* penghasil biofilm dan aktivitasnya pada pembentukan biofilm penting untuk memilih antibiotik dengan efektivitas tinggi yang bermanfaat bagi pengelolaan pasien CAUTI. Sebanyak 35 isolat *E. coli* dikultur kembali dalam media agar LB dan agar darah. Isolat dievaluasi sensitivitasnya berdasarkan nilai MIC nya terhadap beberapa antibiotik. Selain itu, aktivitas antibiofilm antibiotik berdasarkan nilai MBIC nya juga dievaluasi. Data yang diperoleh dianalisis secara deskriptif dan analitik. Hampir semua isolat *E. coli* memiliki sensitivitas tinggi terhadap antibiotik meropenem, amoksisilin-asam klavulanat, dan fosfomisin. Namun, di antara antibiotik yang dievaluasi, hanya fosfomisin yang mempunyai aktivitas antibiofilm. Terdapat perbedaan fenotipe resistensi antara isolat urin dan isolat kateter. Namun tidak terdapat perbedaan signifikan pada nilai MIC (pMIC=0,522) dan nilai MBIC (pMBIC=0,523). Kesimpulannya, alternatif terapi antibiotik untuk bakteri planktonik adalah amoksisilin-asam klavulanat dan fosfomisin, sedangkan untuk bakteri biofilm adalah fosfomisin. Disaranakan untuk dilakukan pemeriksaan skrining biofilm pada kateter untuk meningkatkan efektivitas manajemen terapi pada pasien CAUTI.

Keywords:
antibiotics;
biofilm;
CAUTI;
resistance;
planktonic

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INTRODUCTION

Urinary tract infection (UTI) is one of the infections which often occurs most of the time and therefore this infection should be given serious attention.[1-3] Approximately 40% of nosocomial infection cases due to the UTI are related to the use of a catheter exceeding 2 x 24 h. It is well known as catheter-associated urinary tract infection (CAUTI). Around 1-4% of these cases develop into bacteremia that contributes to the rise of mortality rate.[1,2] In previous research, biofilm production was found to take place in the catheter of patients, especially among patients who used the catheter for more than five days. Furthermore, female patients are vulnerable to higher risks compared to male patients.[3]

The CAUTI-causing microorganisms commonly found is uropathogenic *Escherichia coli* (UPEC) with an occurrence percentage of 80%.[4,5] The biofilm-producing *E. coli* is the most dominant bacteria both in the urinary culture and in the catheter culture. In addition, it is also reported that the biofilm-producing *E. coli* has virulence factors that support biofilm production.[3] The presence of the virulence factors within the UPEC facilitates the occurrence of colonization on the surface of the host mucosa, destroys and invades the urinary tract tissue, and causes persistent or recurrent infection, which is in general difficult to be cured through the usual antibiotic therapy.[6,7]

Furthermore, several studies reported that UPEC shows increasing resistance to several antibiotics, especially when *E. coli* has the biofilm-producing capacity.[8-10] In fact, several experts reported that the bacteria within the biofilm have the capacity to form resistance to antibiotics around 100 – 1,000 times stronger in comparison to the free-swimming planktonic bacteria within the urine, known as the free-swimming counterparts. Such higher capacity is caused by the changes within the mutation target, the enzyme modification, and the efflux pump so that the bacteria within the biofilm have higher resistance toward the antibiotics.[11-13] Therefore, data with regards to antibiotic effectiveness on biofilm-producing *E. coli* are needed to identify the antibiotics that have the antibiofilm activity so that the antibiotics can be useful for the therapy management of CAUTI patients.

MATERIALS AND METHODS

Bacteria isolate characteristics

Thirty-five bacteria isolates (30 bacteria isolates from catheter culture and 5 bacteria isolates from urinary culture) of *E. coli* used in the study were the isolation results from the previous study (TABLE 1).[3] Among 35 bacteria isolates, 31 of them were *E. coli* with positive CRA (*E. coli* biofilm-producing), and 4 of them were *E. coli* with negative CRA (non-biofilm-producing *E. coli*).

TABLE 1. Thirty-five *E. coli* isolates used in this study

Isolate number	Origin	CRA test
C23	Urinary catheter	Positive
C26	Urinary catheter	Negative
C27	Urinary catheter	Positive
C34	Urinary catheter	Positive
C44	Urinary catheter	Positive
C48	Urinary catheter	Positive
C57	Urinary catheter	Positive
C74	Urinary catheter	Positive
C103A	Urinary catheter	Negative
C116	Urinary catheter	Negative
C122	Urinary catheter	Positive
C137	Urinary catheter	Positive
C176	Urinary catheter	Positive
C178	Urinary catheter	Positive
C179	Urinary catheter	Positive
C193	Urinary catheter	Positive
C207	Urinary catheter	Positive
C215	Urinary catheter	Positive
C216	Urinary catheter	Positive
C217	Urinary catheter	Positive
C223	Urinary catheter	Positive
C227	Urinary catheter	Positive
C230	Urinary catheter	Negative
C235	Urinary catheter	Positive
C240	Urinary catheter	Positive
C251-2	Urinary catheter	Positive
C256	Urinary catheter	Positive
C255	Urinary catheter	Positive
C275	Urinary catheter	Positive
C279	Urinary catheter	Positive
U57	Urine	Negative
U178	Urine	Positive
U207	Urine	Positive
U227	Urine	Positive
U235	Urine	Positive

Note: Isolate number means C = origin from catheter; U = origin from urine; the code number behind the C/U means patient identity (U57 and C57 are from the same patient).

Isolate preparation

The bacteria culture within the cryotube was recultured on the medium of Luria Bertani Agar and Blood Agar, then incubated at 37^{0}C for 18-20 h.

Isolate identification

A single colony from the Blood Agar Media was picked up and inoculated into a Vitek 2 cartridge (Vitek 2 compact, BioMerieux™, France) according to the manufacturer's instructions to identify the bacterial species.

Antibiotic test

To evaluate the effectiveness of antibiotics in biofilm eradication, the minimum inhibition concentration (MIC) test and minimum biofilm inhibitory concentration (MBIC) were performed.[14] Seven antibiotics used in this study are commonly used for treating UTIs including fosfomycin, ciprofloxacin, cefotaxime, amoxicillin-clavulanic acid, ceftriaxone, meropenem, and amikacin.

Minimum inhibition concentration test

The MIC test was performed by using the microdilution method. The colony of fresh bacteria (18-20 h) that had been cultured on the LB agar was dissolved in the physiological saline with a turbidity level equal to 0.50 according to the McFarland standards. This suspension was inoculated on the microtiter plate well with the antibiotic serial dilution (logarithmic) using the broth Mueller-Hinton medium. The final concentration of the isolates was equal to 5 x 10^5 CFU/mL. After being implanted for 18 h, the MIC assessment was conducted.[14] Then, the assessment of the sensitivity was carried out by using the 2018 CLSI standard.[15] The MIC was defined as the minimum concentration of the extract that did not allow any visible growth or turbidity of the organism in broth. MIC_{90} refers to the concentration of the test compound required to prevent the growth of 90% of organisms tested. The concentration at which all the isolates failed to grow is taken as MIC.

Minimum biofilm inhibition Concentration

The sensitivity was assessed on the polystyrene microtiter plate that had been implanted on the biofilm within the 96 wells in sequence. This initiative was conducted to minimize the occurrence of manipulation of the biofilm. Then, the fresh and sterile Brain Heart Infusion (BHI) was inserted into the plate and incubated at 37^{0}C for the next 24 h.[14] In the MBIC assessment, the biofilm that had grown over the microtiter plate was exposed to different antibiotic concentrations. After the microtiter plate had been exposed to the biofilm and the antibiotic compounds, the plate was washed three times using sterile PBS (pH 7.4).[14] Reading on the thickness was conducted and the results from the biofilm were compared to the control bacteria using the spectrometer with a wavelength of 595 mm. $MBIC_{50}$ was determined as the lowest concentration that causes at least 50% inhibition of the viability of formed biofilm in the presence of a biologically active agent.

Data analysis

The collected thirty-five *E. coli* isolates data were analyzed descriptively based on the MIC mean score and the MBIC mean score of each antibiotic, namely fosfomycin, ciprofloxacin, cefixime, amoxicillin-clavulanic acid, ceftriaxone, meropenem, and amikacin. The author also analyzed five samples of *E. coli* bacteria results from both urine and catheter taken from the same patients to compare the MIC and the MBIC value from each of the samples.

RESULTS

The results of MIC sensitivity on the *E. coli* still showed a high level of sensitivity toward meropenem (100%), amoxicillin-clavulanic acid (97.10%), fosfomycin (80.00%), and amikacin (77.10%). On the contrary, the sensitivity decreased on ciprofloxacin (28.60%), cefixime (17.10%), and ceftriaxone (8.60%) (TABLE 2). In comparison, the sensitivity of biofilm-producing *E. coli* (positive CRA) and the non-biofilm-producing *E. coli* (negative CRA) yields had similar sensitivity on meropenem (100% vs 100%). However, on ceftriaxone, cefixime, ciprofloxacin, and fosfomycin except for amikacin and amoxicillin-clavulanic acid, there was higher sensitivity on *E. coli* positive CRA than *E. coli* negative CRA. Based on the same results, the researchers also found that 60% of all *E. coli* isolates have the nature of extended spectrum beta lactamase (ESBL). This has been identified from the sensitivity pattern inspection using the Vitek 2 Compact (Biomeriux®) tool. Out of 21 ESBL *E. coli* isolates, 17 isolates were found to have positive CRA (81%) while 4 isolates were found to have negative CRA. This nature of ESBL does not confirm the correlation with the biofilm production capacity ($p > 0.05$).

TABLE 2. The pattern of *E. coli* Sensitivity on Numerous Antibiotics

Types of antibiotics	*E. coli* (n=35)				% Total Sensitivity
	Biofilm producing n* = 31[(a)]	% Sensitivity	Non-Biofilm producing n**=4[(b)]	% Sensitivity	
Ceftriaxone	3	9.70	0	0	8.60
Cefixime	6	19.40	0	0	17.10
Ciprofloxacin	10	32.20	0	0	28.60
Amikacin	23	74.20	4	100	77.10
Amoxicillin-clavulanic acid	30	96.80	4	100	97.10
Meropenem	31	100	4	100	100
Fosfomycin	25	80.60	3	75	80.00

[a]) number of drug-sensitive samples from *E. coli* producing biofilm; [b]) number of drug-sensitive samples from *E. coli* not producing biofilm

In addition, to examine the sensitivity, those antibiotics were also evaluated, in terms of biofilm-production inhibiting capacity. The results of the current study showed that the antibiotics with the lowest titer increase on $MBIC_{50}$ in comparison to MIC_{90} are ciprofloxacin and fosfomycin (without titer increase) and meropenem with a 1-fold titer increase (0.032 µg/mL to 0.064 µg/mL). On the contrary, ceftriaxone and cefixime required more than two-fold titer increasing to > 512 µg/mL. Similarly, amikacin required a three-fold titer increase to achieve $MBIC_{50}$ from 4 µg/mL to 32 µg/mL (TABLE 3).

TABLE 3. Comparison of MIC value and MBIC value on biofilm-producing *E. coli* (n = 31)

Antibiotics	MIC_{90} (µg/mL)	Min-Max (µg/mL)	MIC Sensitivity (CLSI)	$MBIC_{50}$ (µg/mL)	Min-Max (µg/mL)
Ceftriaxone	512	0.25–512	≤ 1; ≥ 4	> 512	2– > 512
Cefixime	512	0.50–512	≤ 1; ≥ 4	> 512	1– > 512
Ciprofloxacin	160	0.15–640	≤ 1; ≥ 4	160	0.32– > 640
Amikacin	4	1–16	≤ 16; ≥ 64	32	4– > 512
Amoxicillin-clavulanic acid	6	2–16	≤ 8; ≥ 32	--	--
Meropenem	0.03	0.01–0.05	≤ 1; ≥ 4	0.064	0.02– > 4
Fosfomycin	80	0.63– > 320	≤ 64; ≥ 256	80	2.50– > 320

As previously explained, the *E. coli* isolates used in the study were obtained from different sources, which were urine and catheter. The five samples from both urine and catheter were taken from the same patients to compare the MIC and the MBIC value based on the source of the isolates. Data from the MIC identification show that higher concentrations of all antibiotics were required to inhibit the isolates taken from the catheter (shown as red bars) in comparison to the isolates taken from the urine (shown as green bars) except for ciprofloxacin (FIGURE 1).

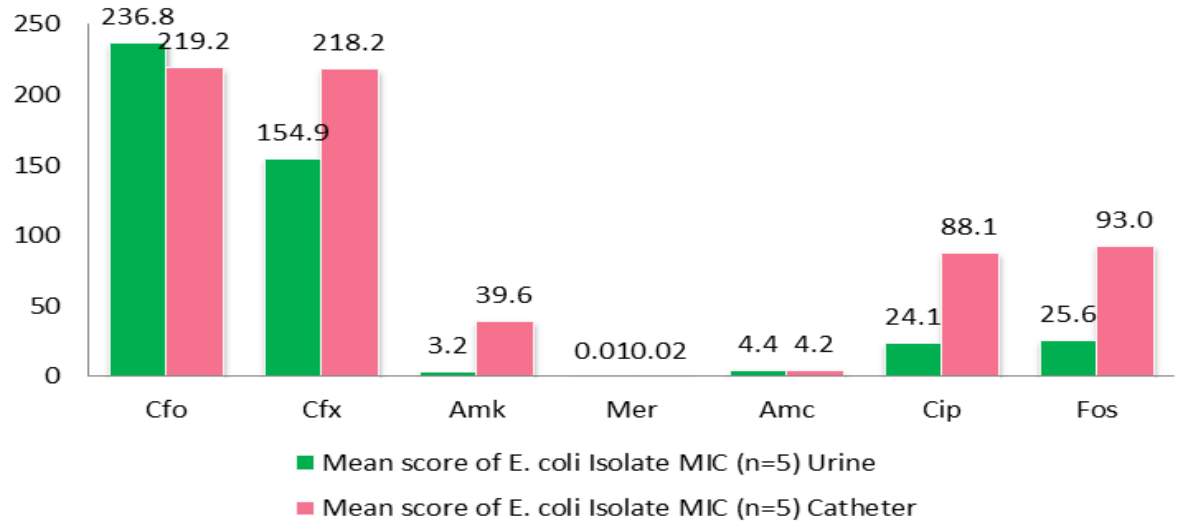

FIGURE 1. Comparison between the MIC mean score (µg/mL) of isolates obtained from the urine (n = 5) (green) and the MIC mean score (µg/mL) of isolates obtained from catheter (n = 5) (pink) CFO = ceftriaxone, CFX = cefixime, AMK = amikacin, MER = meropenem, AMC = Amoxicillin Clavulanic-Acid, CIP = ciprofloxacin, FOS = fosfomycin

On the contrary, the phenotype results of MBIC for the antibiotics on the *E. coli* taken from the urine and the catheter also showed that almost all antibiotics demand higher concentration (2-10 times) on the isolates taken from the catheter (shown in orange) than the isolates taken from the urine (shown as blue bars). These findings described the different phenotype characteristics between the isolates taken from the catheter and the isolates taken from the urine (FIGURE 2).

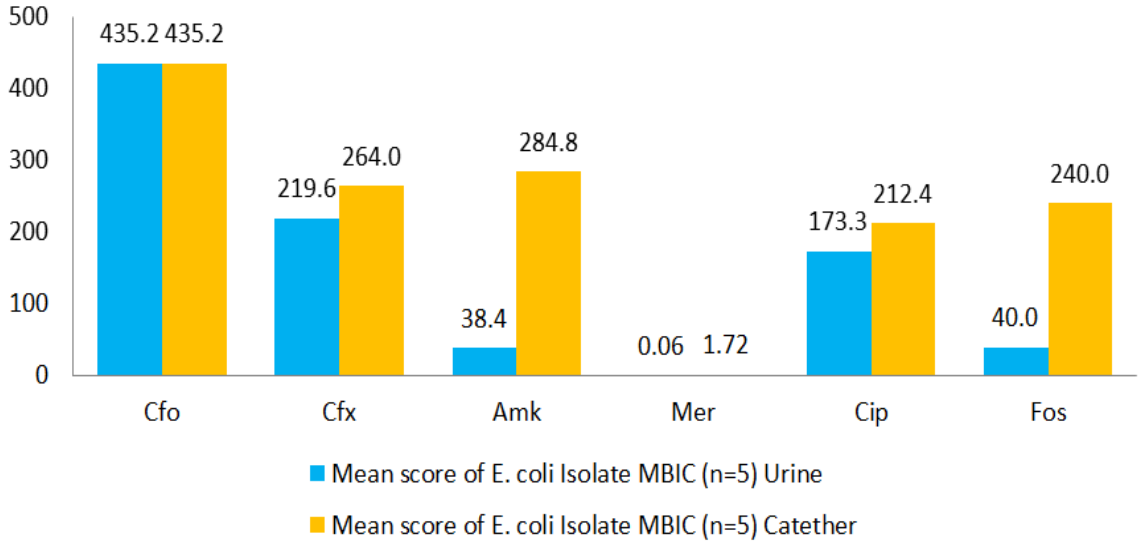

FIGURE 2. Comparison between the MBIC mean score (µg/mL) of isolates obtained from the urine (blue) and the MBIC mean score (µg/mL) of isolates obtained from the catheter (yellow). CFO = ceftriaxone, CFX = cefixime, AMK = amikacin, MER = meropenem, CIP = ciprofloxacin, FOS = fosfomycin

DISCUSSION

The results showed that the bacteria's capacity for forming the biofilm does not influence its sensitivity to antibiotics. In general, the effectiveness of antibiotics was good in inhibiting the development of biofilm-producing *E. coli*, except for several antibiotics such as ciprofloxacin (28.60%), cefixime (17.10%), and ceftriaxone (8.60%) (TABLE 2). The results of this study are similar to those of research conducted in Nepal. This study found that the biofilm-producing *E. coli* shows resistance to the antibiotics like amoxicillin (10.60%), ciprofloxacin (39.40%), and cefixime (24.50%).[16] Resistance to fluoroquinolone (ciprofloxacin) and ceftriaxone was also found in the results of other studies.[17] The results of this study align with those of Neupane *et al.*[16] suggesting that the sensitivity of *E. coli* to amoxicillin is low (10.60%), while the sensitivity of *E. coli* to amoxicillin clavulanic acid is high (97.10%). The difference showed that amoxicillin combined with the lactamase beta inhibitor (amoxicillin-clavulanic acid) has higher effectiveness in inhibiting the development of the biofilm-producing *E. coli* compared to amoxicillin itself.[16,18,19]

Thus, this finding indicated that the effectiveness of the antibiotics used in the present study is not influenced by the capacity of *E. coli* to produce biofilm. On the other hand, the results of a study by Kobir *et al.*[20] showed that the resistant pattern correlates with the biofilm-producing capacity of the uropathogenic *E. coli*. Similarly, the results of a study by Cho *et al.*[21] also showed that the biofilm-producing *Pseudomonas aeruginosa* has a higher resistance level in comparison to the non-biofilm-producing *P. aeruginosa* on amikacin, ceftazidime, and cefepime. Such a difference implies that the antibiotic effectiveness (amikacin) can be different in different species although the bacteria are equally able to produce the biofilm. The different results between this study with other studies might be caused by the small samples of non-biofilm producing *E. coli* (n=4) compared to biofilm producing *E. coli*.

Furthermore, in this study 60% of the *E. coli* taken from the urinary isolates and the biofilm were ESBL.

This figure aligns with the surveillance results in Mexico from 2009 until 2015 for the cases of UTIs, which resulted from nosocomial infection or catheter use.[22] In other words, this finding shows that the presence of bacteria within the biofilm can lead to the occurrence of CAUTI. Based on the high resistance of the biofilm-producing *E. coli* to several antibiotics such as quinolone, amoxicillin, cotrimoxazole, and ceftriaxone in the case of CAUTI, biofilm-screening examination on the catheter should be performed so that the infection management within the CAUTI patients can be conducted effectively.

The effectiveness of antibiotics as antibiofilm has been assessed based on their capacity in inhibiting biofilm production by *E. coli*. To assess this capacity, the MBIC was conducted under the rate $MBIC_{50}$. $MBIC_{50}$ is the lowest antibiotic rate in inhibiting 50% of biofilm production by *E. coli*.[14] At the same time, the results of the current study also show that ciprofloxacin and fosfomycin do not need higher antibiotic concentrations both in the planktonic form (MIC_{90}) and in the biofilm form ($MBIC_{50}$) (TABLE 3). These findings are similar to the results of a study by Gonzales *et al*.[23] which showed that ciprofloxacin can inhibit biofilm production. However, the only difference shown by the results of the current study is the concentration of ciprofloxacin used (160 µg/mL) is very high compared to the range value suggested by CLSI (TABLE 3). This implies that the antibiotic considered the most effective one for inhibiting biofilm production is fosfomycin (80 µg/mL). The results of previous research also showed that fosfomycin can serve as an alternative therapy that can be used as an antibiofilm, especially in combination with gentamicin.[24]

The biofilm resistance to high-dose antibiotics is multifactorial and depends on the class of the antibiotics used, including numerous mechanisms that can be different from one another, such as poor antibiotic diffusion, antibiotic use negligence, and biofilm genetical expression variants.[25] The extracellular matrix of the biofilm is considered responsible for biofilm resistance. Then, consistent with the statement, it had been mentioned previously by Parrino *et al*.[26] that the mechanical and the physiochemical nature of the biofilm matrix can reduce or inhibit several compounds, including antibiotics and antiseptics. The chemical structure of the biofilm matrix is crucial, such as the different types of exopolysaccharide (EPS) and the dependency on the surrounding environment of the biofilm.[10,26] However, the decreasing antibiotic penetration cannot fully explain the biofilm resistance toward antibiotics.[25]

The antibiotics such as fluoroquinolone, rifampin, and ampicillin have quite good penetration toward the matrix although the penetration cannot eradicate 100% biofilm bacteria. For example, in the case of *P. aeruginosa* and *E. coli* during the 24 h *in vitro* experiment, the biofilm bacteria cannot be eradicated by the 24 h therapy using fosfomycin and ciprofloxacin, whereas the two antibiotics have reached 50% maximum concentration within 6 h.[25] In addition, the mean score of the antibiotics' MIC and MBIC is higher on the isolates taken from the catheter in comparison to the isolates taken from the urine except for ceftriaxone (both FIGURE 1 and FIGURE 2 are consistent with the results of other studies, which state that the bacteria in the form of biofilm have 100-1,000 times stronger resistance capacity on the antibiotics compared to bacteria in the form of free-swimming counterparts planktonic).[11-13] The antibiotic effectiveness toward *E. coli* is not influenced by biofilm-producing capacity; instead, since *E. coli* becomes part of the biofilm, the bacteria

will undergo changes in mutation target, enzyme modification, and efflux pump. Consequently, the bacteria will have higher resistance to antibiotics.[11-13]

However, the results of statistical analysis using the Mann-Whitney procedures did not show significant differences between the MIC mean score and the MBIC mean score of the antibiotics on the isolates taken from the catheter compared to the isolates taken from the urine (pMIC = 0.522, pMBIC = 0.523). It showed that the characteristics of *E. coli* found in the catheter are similar to those found in the urine. Consequently, there is a possibility that the bacteria within the urine come from the bacteria colonization found in the catheter and vice versa. This incident further indicated that the presence of colonization or biofilm within the catheter can cause infection in the urinary tract. Such an indication is similar to the results of the previous studies, which stated that the presence of biofilm increases the risk of CAUTI occurrence.[3]

The only limitation found in the study were in the planktonic bacteria sensitivity test (MIC), and the biofilm sensitivity test (MBIC) conducted *in vitro*. In the last several years, the management of biofilm-associated infections has been a challenge because generally the studies are conducted *in vitro*; consequently, the results of these studies are not close to the clinical (*in vivo* studies) manner. Therefore, there should be another approach in addition to relying on the use of antibiotics that have antibiofilm characteristics. The approach that should be developed is the other biofilm therapy target, such as QS inhibition, adhesion-inhibiting-type bacteria, anti-virulence factor, and exopolysaccharide matrix degradation, to overcome antibiotic resistance.[26-28]

CONCLUSION

The alternatives of antibiotic therapy for the biofilm-producing planktonic bacteria are amoxicillin-clavulanic acid and fosfomycin, while the antibiotic that has the antibiofilm characteristics is fosfomycin. There should be a biofilm screening examination on the catheter to improve the effectiveness of therapy management for CAUTI patients.

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A comparison study of GeneXpert and In-House N1N2 CDC Real-Time RT-PCR for detection of SARS-CoV-2 infection

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ABSTRACT

COVID-19 is a disease caused by SARS-CoV-2, a new virus from genus β-coronaviruses. This disease has been declared a pandemic by WHO on 11 March 2020 until now. The nucleic acid tests are the most frequently used assays because of their high sensitivity and specificity. One of the tests is the GeneXpert, a real-time reverse transcription polymerase chain reaction (rRT-PCR)-based assay platform. The use of the GeneXpert shows great public health interest because of the rapid (50 min), the minimum number of trained staff, and less infrastructure and equipment. However, there are limited data on the application of the GeneXpert for the detection of SARS-CoV-2. Therefore, we conducted a comparative study between the GeneXpert and in-house N1N2 CDC rRT-PCR assay. Of 86 samples, 17 were rRT-PCR positive while 13 were GeneXpert positive. Of rRT-PCR positive 17 samples, 7 were GeneXpert negative [58.82% (10/17] sensitivity]. We also found that 3 GeneXpert positive samples showed rRT-PCR negative (95.65% [66/69] specificity). It is concluded that negative results by the GeneXpert can not rule out the possibility of SARS-CoV-2 infection, particularly in close-contact individuals and the interpretation of the positive result should be analyzed carefully, particularly amplification with Ct>40.

ABSTRAK

COVID-19 adalah penyakit yang disebabkan oleh SARS-CoV-2, virus baru dari genus β-coronaviruses. Penyakit ini telah dinyatakan sebagai pandemi oleh WHO pada 11 Maret 2020 hingga sekarang. Tes asam nukleat adalah tes yang paling sering digunakan karena sensitivitas dan spesifisitasnya yang tinggi. Salah satu tesnya adalah GeneXpert, platform pengujian berbasis *real-time reverse transcription polymerase chain reaction* (rRT-PCR). Penggunaan GeneXpert menunjukkan minat kesehatan masyarakat yang besar karena kecepatan (50 menit), jumlah staf terlatih yang minimum, dan infrastruktur dan peralatan yang lebih sedikit. Namun, ada keterbatasan data dalam aplikasi GeneXpert untuk deteksi SARS-CoV-2. Oleh karena itu, kami melakukan studi perbandingan antara GeneXpert dan uji rRT-PCR N1N2 CDC *in-house*. Dari 86 sampel, 17 adalah rRT-PCR positif sementara 13 adalah GeneXpert positif. Dari 17 sampel rRT-PCR positif, 7 adalah GeneXpert negatif (sensitivitas 58,82% [10/17]). Kami juga menemukan bahwa 3 sampel positif GeneXpert menunjukkan rRT-PCR negatif (95,65% [66/69] spesifisitas). Disimpulkan bahwa hasil negatif oleh GeneXpert tidak dapat mengesampingkan kemungkinan infeksi SARS-CoV-2, terutama pada individu yang kontak dekat dan interpretasi hasil positif harus dianalisis dengan cermat, terutama amplifikasi dengan Ct>40.

Keywords:
COVID-19;
SARS-CoV-2;
GeneXpert;
PCR;
nucleic acid tests

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from Family *Coronaviridae*.[1,2] Genetic analysis showed that SARS-CoV-2 has similarities with another coronavirus (SARS-CoV) and is grouped within β-coronavirus genus.[2] SARS-CoV-2 is an enveloped virus and has a positive-sense single-stranded RNA genome enclosed in structural nucleocapsid (N) protein.[2,3] Other structural proteins namely E (envelope), M (membrane), and S (spike) proteins form viral envelope.[2,3] Among these structural proteins, S protein facilitates viral entry to the host cell by binding with the ACE receptor.[3]

This is a new virus discovered in 2019 in Wuhan, China, after some patients showed symptoms of flu-like illness.[4] In March 2020, COVID-19 was declared a pandemic by WHO because of the number of cases and countries with cases increase.[5] Globally, the numbers of COVID-19 patients continuously increased, with 500 million confirmed cases and over 6 million deaths have been reported from the beginning of the pandemic until the third week of April 2022.[6] In Indonesia, COVID-19 cases have been over 6 million and more than 155,746 deaths have been reported.[7]

At the beginning of the pandemic, there is an urgent need for a highly specific and sensitive method to detect the virus.[2,4,8] Currently, many testing methods are available for the detection of SARS-CoV-2.[2] The most common genes for nucleic acid detection of SARS-CoV-2 are orf1a/b, RdRp, S, N, and E.[1] Some diagnostic methods used to detect SARS-CoV-2 are serological (antigen and antibody detection) and nucleic acid tests such as standard real-time reverse transcription-polymerase chain reaction (rRT-PCR) and rapid tests like RT-LAMP, and GeneXpert assays.[2,9] The rRT-PCR is a gold standard for detection of SARS-CoV-2 because its sensitivity and specificity.[1,2,8] On the other hand, the GeneXpert using single cartridge-based assay is a rapid method for detection of COVID-19 compared to the standard rRT-PCR assay.[5,8] The rapid turnover of the GeneExpert result makes it as an increasingly popular choice for the detection of SARS-CoV-2. However, to our knowledge there is limited data on the GeneExpert performance particularly on its sensitivity and specificity. Therefore, in this study we compared standard rRT-PCR based on the N1N2 CDC protocol and GeneXpert assay.

MATERIALS AND METHODS

Clinical specimens

Eighty-six nasopharyngeal/oropharyngeal swabs were obtained from suspected COVID-19 individuals in Jakarta from September-December 2020. The swab samples were collected immediately into 1 mL of the viral transport medium (DMEM containing 1% pen-strep and 5% bovine serum albumin) and stored at 2-4°C for not more than 4 h. The viral transport medium was divided for GenXpert and rRT-PCR tests conducted by two separate teams (blind testing). This study was approved by the Ethics Committee, Faculty of Medicine, Universitas Indonesia (KET-395/UN2.F1/ETIK/PPM.00.02/2020).

Viral RNA extraction

The viral RNA genome was extracted by using QIAmp Viral RNA Mini Kit (Qiagen, Germany) in accordance with the manufacturer's instructions. The final elute was stored at -80°C for not more than 4h.

Real-time RT-PCR (rRT-PCR) assay

The primers and probes for N (N1 and N2) and human RNase P (internal control, IC) genes based on the Centers for Disease Control and Prevention (CDC) were used for the detection of SARS-CoV-2.[10] The rRT-PCR was performed with the following composition (20 µl of total volume): 1x SensiFAST™ Probe No-ROX One-Step mix (Bioline, Cat. No: BIO-76005), 1.5 µL each of primer and probe solution (2019-nCoV RUO Kit, IDT Integrated DNA technologies, Cat. no:10006713), 4U of RNase inhibitor, 2 U of reverse transcriptase enzyme, and 7.9 µL of RNA template. The PCR machine, MA-6000 Real-Time PCR System [Molarray, Suzhou, China]), was used under the following conditions: 50°C for 50 min; 95°C for 50 min; 45 cycles of 95°C for 15 sec and 55°C for 30 sec. The rRT-PCR positive was defined if Ct ≤ 40 for both N1 and N2.[10]

Rapid GeneXpert test

The GeneXpert used Xpert® Xpress SARS-CoV-2 kit based on N (N2 CDC) and E genes for detection of SARS-CoV-2.[11] The procedure and the result interpretation were performed according to the manufacturer's instructions.[11] SARS-CoV-2 positivity was defined if either gene (N2 and E) or only N2 were positive. The presumptive SARS-CoV-2 positive was defined if only the E gene was positive (Ct value ≤ 45).

Statistical analysis

The SPSS 16.0 was used for statistical analysis and a fisher test with a 5% (0.05) level of significance was used for hypothesis testing.

RESULTS

The comparison results between real-time RT-PCR (rRT-PCR) and GeneXpert are shown in TABLE 1. Of 86 samples, 17 were rRT-PCR positive while 13 were GeneXpert positive. Of rRT-PCR positive 17 samples, 7 were GeneXpert negative (41.18% [7/17] discrepancy). The GeneXpert negative samples had Ct values above 34 by rRT-PCR (TABLE 2), indicating that GeneXpert failed to detect SARS-CoV-2 with high Ct values above 34.

TABLE 1. Comparison results between real-time RT-PCR (rRT-PCR) and GeneXpert methods (n=86)

Positive		GeneXpert		p*	OR
		Negative			
rRT-PCR	Positive	10	7	< 0.001	31.429
	Negative	3	66		

TABLE 2. Discrepancy results between real-time RT-PCR (rRT-PCR) and GeneXpert for detection of SARS-CoV-2

Sample ID	Real-time RT-PCR			GeneXpert		
	Region target (Ct value)		Result	Region target (Ct value)		Result
	N1	N2		N2	E	
2809-23	34.22	33.93	+	ND	ND	-
2809-31	37.67	38.15	+	ND	ND	-
2809-20	38.34	37.81	+	ND	ND	-
2809-21	35.91	37.72	+	ND	ND	-
2809-14	ND	ND	-	42.4	ND	+
2909-06	34.75	34.49	+	ND	ND	-
2909-13	36.15	38.28	+	ND	ND	-
0510-05	37.56	36.40	+	ND	ND	-
1008-08	ND	ND	-	44.2	39.2	+
1208-38	ND	ND	-	41.3	ND	+

Note: All tests were valid with internal control Ct of < 30. N1 and N2: Regions of N gene. E: Envelope gene. ND: Not detected. Ct: Cycle threshold. +: Positive. -: Negative.

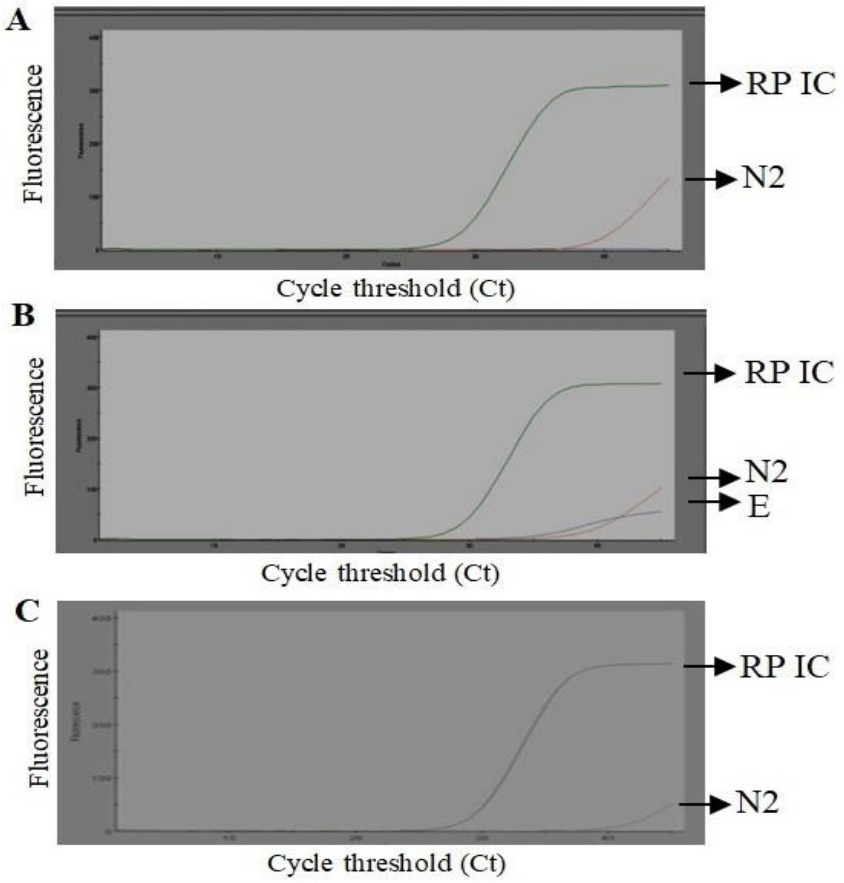

FIGURE 1. Curve Amplification for ID samples 2809-14 (A), 1008-08 (B), and 1208-38 (C). RP IC: Human RNase P gene internal control. N2: Region of N gene. E: E gene.

TABLE 3. Sensitivity, specificity, PPV, and NPV of GeneXpert assay (prior probability of infection 0.05

Test	Value
Sensitivity	0.5882 (58.82 %)
Specificity	0.9565 (95.65 %)
PPV	0.4158
NPV	0.9778

We found that 3 GeneXpert positive samples showed rRT-PCR negative (TABLE 2). Of 3 samples, 2 were detected for N2 (Ct>40) and E (Ct=0), while 1 was detected for N2 (Ct>40) and E (Ct=39.2). Based on manual curve analysis, all samples showed sigmoid curves of RP gene internal controls, while N2 and E curves were not sigmoid (FIGURE 1). Because of the questionable results, another real-time RT-PCR reaction (Detection Kit for 2019-nCoV, Cat. no: #DA-930, Da An Gene Co., Ltd. of Sun Yat Sen University), a kit listed by the WHO Emergency Use for detection of SARS-CoV-2 nucleic acid, was performed for clarification. The results showed that all 3 samples were SARS-CoV-2 negative (Data not shown).

DISCUSSION

The rRT-PCR and GeneXpert compared in this study have the same gene target [nucleocapsid (N)] for SARS-CoV-2; however, the rRT-PCR detects two regions (N1 and N2) of the N gene, while the GeneXpert detects only one region (N2).[10,11] The GeneXpert detects an additional gene, envelope (E) for all coronaviruses.[11] For detection of specific SARS-CoV-2, regions of N gene including N1 and N2 have been reported as rRT-PCR targets with higher sensitivity than other gene targets.[12-14] The high sensitivity might be caused by a high number of subgenomic mRNA of the N gene produced during the replication of coronaviruses.[15] Comparison between N1 and N2 applied for clinical and environmental samples, most of studies reported N1 having higher sensitivity than N2,[12,16-19] and another study reported an otherwise result.[13] Even though N1 was more sensitive than N2, several valid results were N2 positive and N1 negative.[18] Thus, it is suggested that N1 and N2 primer-probe sets should be used for detection of specific SARS-CoV-2.

The GeneXpert failed to detect SARS-CoV-2 in 7 samples that were positive by rRT-PCR (TABLE 2). Procop *et al*. reported that the GeneXpert had afalse-negative rate of 2% compared with N1N2 CDC rRT-PCR.[20] Other studies also reported the false-negative results by the GeneXpert.[21] Based on Ct value, the false-negative occurred in cases with high Ct values above 34 (TABLE 2). The Ct values can be used as surrogate markers for deducing the virus infectivity. For this reason, several studies have reported the association of Ct values with virus infectivity by using cell culture methods. It has been shown that patients with Ct values above 30 or 34 did not excrete infectious viral particles.[22,23]

However, other studies have reported otherwise data.[24-26] Two studies reported that clinical samples with Ct-values above 30 could still be infectious.[24,25] Singanayagam *et al*.[26] reported that 8% of samples with Ct above 35 were still infectious. The different results might be affected by different pre-analytic and post-analytic factors in each laboratory, making Ct values as surrogate markers are unclear and debatable. Thus, Platten

et al.,[27] suggested that the Ct value cut-offs can be defined as acceptable low-risk values; higher Ct values as lower infection risks. Based on the data, it is suggested that SARS-CoV-2 negative by the GeneXpert cannot rule out the possibility of SARS-CoV-2 infection.

On the other side, the GeneXpert showed 3 false-positive results (TABLE 1). Based on Ct values, all 3 samples were detected for N with Ct>40 and only 1 sample was detected for E with Ct= 39.2 (TABLE 2). Moreover, N2 and E amplification curves showed non-sigmoid curves (FIGURE 1). The question results have been clarified by another kit and showed SARS-CoV-2 negative (Data not shown). These GeneXpert false-positive results have been reported by Rakotosamimanana *et al.*,[28] in that they found samples, that were no amplification of E gene (Ct=0) and N2 with Ct>40 by GenXpert, are negative by standard rRT-PCR assay. Other studies also reported the same result patterns.[21,29] Das *et al.* reported 16 (34%) of samples with Ct>35 by GenXpert were only 3 (18.8%) positive by standard rRT-PCR assay.[21] Moreover, Moran *et al.*,[29] reported that the GeneXpert results with E gene (Ct=0) and N2 with Ct>40 were SARS-CoV-2 negative when performing the repeated GeneXpert testing. Therefore, we suggested the repeated GeneXpert testing for clarification when the results were N2 with Ct>40.

CONCLUSION

The negative results by GeneXpert cannot rule out the possibility of SARS-CoV-2 infection, particularly for close-contact individuals. Due to automatic interpretation by the GeneXPert software, the interpretation of the positive result should be analyzed carefully, particularly Ct>40. The sensitivity and specificity of the GeneXpert were 58.82% and 95.65% respectively. However, it is important to know that there is a limitation to this study, namely the small number of the samples used.

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Prediction score for post-stroke cognitive impairment (PSCI) after acute ischemic stroke

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ABSTRACT

It was estimated that patients with ischemic stroke and post-stroke cognitive impairment (PSCI) have been increasing. In addition, this PSCI is often late diagnosed when it has already developed into post-stroke dementia. Only a few studies have developed a scoring system of predictor factors cognitive impairment (CI) for post-acute ischemic stroke in Indonesia. This study aimed **t**o develop a scoring system of predictor factors of CI for post-stroke ischemic patients. The patients included were >18 years old diagnosed with acute ischemic stroke who underwent mini-mental state examination (MMSE) and clock drawing test (CDT) examination on day-30 at Bethesda Hospital Yogyakarta. It was retrospective cohort study design and samples were obtained from the stroke registry and medical records. Patients who had a history of CI and incomplete medical records were excluded. The results of MSSE and CDT at day-30 were the outcomes of this study. To evaluate the relationship between the independent variable and the dependent variable, chi-squared tests were perforemd followed by multivariate logistic regression analysis with Hosmer-Lemeshow tests with backward likelihood-ratio (LR) method and by assessing the final area under the curve (AUC) model. The final model was transformed into a scoring system to determine the value of probability prediction of PSCI, the optimal cut-off point, the sensitivity value and specificity value of the cognitive impairment scoring system at day-30 after acute ischemic stroke. A total of 140 subjects were included in the study with an average age of 62.8 years, 86 (61.4%) males and 54 (38.6%) females. Ninety-one subjects (65%) experienced post-stroke CI. The multivariate analysis showed age >70 years, education level ≤6 years, modified ranking score (mRS) >3 at diagnosis, Barthel index score ≤4 at diagnosis, the number of multiple lesions and the location of lesion in the cortex were independent predictor factors affecting CI 30 days after acute ischemic stroke. The developed predictor score obtained AUC discrimination value of 82.6% (95%CI:0.757-0.896) and calibration value of p>0.366. The scoring system had a value range of 0-7, and with a cut-off ≥1, it had a sensitivity value of 86.8% and a specificity value of 59.2%. It can be concluded that the predictor score has a good performance in predicting the occurrence of PSCI at day-30 after acute ischemic stroke.

Keywords:
Prediction score;
acute ischemic stroke;
cognitive impairment;
post-stroke cognitive impairment;
PSCI score

ABSTRAK

Penderita stroke iskemik dan gangguan kognitif pasca stroke (*post-stroke cognitive impairment*/PSCI) diperkirakan terus meningkat. Selain itu, PSCI ini sering terlambat didiagnsosis ketika sudah berkembang menjadi demensia pasca stroke. Hanya sedikit penelitian yang mengembangkan sistem penilaian faktor predictor gangguan kognitif (*cognitive impairment*/CI) untuk pasca stroke iskemik akut di Indonesia. Penelitian ini bertujuan untuk mengembangkan sistem penilaian faktor prediktor CI pada pasien iskemik pasca stroke. Pasien yang termasuk berusia >18 tahun didiagnosis stroke iskemik akut yang menjalani pemeriksaan *mini-mental state examination* (MMSE) dan *clock drawing test* (CDT) pada hari ke 30 di RS Bethesda Yogyakarta. Penelitian ini menggunakan rancangan studi kohort retrospektif dan sampel diperoleh dari catatan stroke dan rekam medis. Pasien yang memiliki riwayat CI dan catatan medis yang tidak lengkap dikeluarkan. Hasil MSSE dan CDT pada hari

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ke 30 merupakan hasil dari penelitian ini. Untuk mengetahui hubungan antara variabel bebas dan variabel terikat dilakukan uji chi-kuadrat dilanjutkan dengan analisis regresi logistik multivariat dengan model uji Hosmer-Lemeshow dengan metode *backward likelihood-ratio* (LR) dan dengan menilai model akhir *area under the curve* (AUC). Model akhir ditransformasikan menjadi sistem penilaian untuk menentukan nilai prediksi probabilitas PSCI, titik potong optimal, nilai sensitivitas, dan nilai spesifisitas sistem penilaian gangguan kognitif pada hari ke-30 pasca stroke iskemik akut. Sebanyak 140 subjek dilibatkan dalam penelitian dengan usia rata-rata 62,8 tahun, 86 (61,4%) laki-laki dan 54 (38,6%) perempuan. Sembilan puluh satu subjek (65%) mengalami CI pasca stroke. Analisis multivariat menunjukkan usia >70 tahun, tingkat pendidikan 6 tahun, skor rankin termodifikasi (*modified ranking score*/mRS) >3 saat diagnosis, skor indeks Barthel 4 saat diagnosis, jumlah lesi multipel, dan lokasi lesi di korteks merupakan faktor prediktor independent yang mempengaruhi CI 30 hari setelah stroke iskemik akut. Skor prediktor yang dikembangkan diperoleh nilai diskriminasi AUC sebesar 82,6% (95%CI:0,757-0,896) dan nilai kalibrasi p>0,366. Sistem penilaian memiliki rentang nilai 0-7, dan dengan *cut-off* 1, memiliki nilai sensitivitas 86,8% dan nilai spesifisitas 59,2%. Dapat disimpulkan bahwa skor prediktor memiliki kinerja yang baik dalam memprediksi terjadinya PSCI pada hari ke 30 pasca stroke iskemik akut.

TRODUCTION

Stroke is the second leading cause of death and the third-ranking cause of disability in the world.[1,2] The prevalence of stroke in Indonesia has increased from 7 to 10.9% and it is the highest cause of disability among people aged ≥60 years old.[3] Post-stroke disability is not only a physical disability (motoric) but also involves cognitive impairment (CI). Post-stroke cognitive impairment (PSCI) is part of vascular cognitive impairment (VCI) which includes all CI (vascular dementia and CI no dementia) caused by or associated with vascular factors (cerebrovascular).[4]

The prevalence of PSCI varies from 20 to 80% among countries depending on population, race, and diagnostic criteria.[5,6] As many as 61.7% of post-stroke patients in Indonesia experienced CI based on data from the Basic Health Research (*Riset Kesehatan Dasar*/Riskesdas) in 2013.[7] A research conducted at Dr. Sardjito General Hospital, Yogyakarta in 2000 showed that acute ischemic stroke plays a role in CI with the result that 80.6% of patients with acute ischemic stroke aged ≥65 years old have decreased cognitive function.[8]

Patients with PSCI have reportedly returned to normal or progressively worsened into dementia.[9] A study conducted by Suda *et al*.[10] in Japan in 2020 showed that CI can appear immediately after minor ischemic stroke on the fifth day with a prevalence of 63.3% and is associated with decreased function of daily activities. Post-stroke cognitive impairment is an important problem for stroke patients, but not many are aware of it so it is often discovered late when it has developed into post-stroke dementia (PSD).[11] Cognitive impairment after acute ischemic stroke have not been widely reported in Indonesia. Accordingly, a multidomain screening tool is needed to predict PSCI earlier in order to prevent the development of PSD and hopefully improve the recovery of stroke patients. This study aimed to develop a CI predictor scoring system in acute ischemic stroke patients and estimate the magnitude of risk factors to predict CI after acute ischemic stroke.

MATERIALS AND METHODS

Design of study

This an observational study used a retrospective cohort design involving patients aged >18 years with the clinical diagnosis of acute ischemic stroke and radiological head CT-scan at Bethesda Yogyakarta Hospital from December 30th, 2019 to November 14th, 2020.

Procedure

The inclusion criteria for study subjects were patients aged >18 years diagnosed with acute ischemic stroke with the first attack and onset <24 h at Bethesda Yogyakarta Hospital. The exclusion criteria were diagnosed with transient ischemic attack (TIA), had a history of previous cognitive and psychiatric disorders, and incomplete medical records, which did not include complete data on all variables of this study. The calculation of the minimum sample size in this study used the hypothesis testing research formula with descriptive sensitivity, namely 140 subjects. The results of the mini-mental state examination (MMSE) and the clock drawing test (CDT) on day-30 were the outcomes of this study.

This study has received ethical approval from the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada/Dr. Sardjito General Hospital, Yogyakarta with number: KE/FK/1005/EC/2020 and licensing of research institutions from RS Bethesda Hospital Yogyakarta with No.118/KEPK-RSB/IX/20.

Statistical analysis

Statistical analysis was performed using the SPSS version 26 (IBM Corp., Chicago) data package program. Predictor factor analysis used bivariate analysis with the chi-squared method. Variables with a p <0.25 were followed by multivariate logistic regression analysis with Hosmer and Lemeshow tests and backward likelihood ratio (LR) until the variables with each p <0.2 became the final model. The Hosmer-Lemeshow tests used a p >0.05 as statistically significant (calibration) and an area under the curve (AUC) value >0.80 was considered strong or equal to the expected value (discrimination). Furthermore, the final model was transformed into a scoring system, to assess the PSCI probability prediction, and optimal cutoff point, while the sensitivity and specificity scores for acute ischemic PSCI scores were determined.

RESULTS

A total 163 subjects who met the inclusion criteria during the period of 30 December 2019 to 14 November 2020 were recruited in this study. Total sampling was conducted and 23 subjects were excluded because the medical record contained incomplete data and had a previous history of CI. The basic characteristics of the subjects are detailed in TABLE 1. The results of the CI assessment of acute ischemic stroke in this study are shown in TABLE 2. Study subjects on day-30 were assessed by MMSE and CDT. The results of the MMSE assessment showed that 58.6% of subjects experienced acute ischemic PSCI and CDT results showed 45.7% experienced acute ischemic PSCI. In this study, those who were declared to have CI after acute ischemic stroke were those with MMSE values ≤25 and or CDT ≤2. After the assessment, it was found that there were 91 subjects (65%) who experienced PSCI incidents and 49 subjects (35%) who did not experience PSCI events. The basic characteristics of the subjects were obtained through descriptive analysis. In this study, the incidence of PSCI occurred in study subjects who had an average age of 64.8 ± 10.16 years with the proportion predominantly women (68.5%) and educational level of ≤6 years (90.3%).

The final results of the multivariate analysis obtained 6 variables, namely age >70 years, education level <6 years, mRS score >3, BI score ≤ 4, the number of multiple lesions, and the presence of lesions in the cortex location which were statistically selected to be developed into a scoring system with the AUC discrimination value of 0.826 (95%CI: 0.757-0.896; p=0.000) and the Hosmer and Lemeshow test calibration value

of p = 0.366. In the multivariate logistic regression analysis with the backward LR method, the results of the final stage used p <0.20 which was concluded as statistically significant. The results of the bivariate analysis and the final results of the backward LR multivariate logistic regression analysis are shown in TABLE 3.

TABLE 1. Basic characteristics of subjects

Basic characteristics	Total (n=140)	PSCI		p
		Presence n=91 (65%)	Absent n= 49 (35%)	
Age (mean ± SD years)	62.8±10.4	64.8±10.16	59.1±10.03	0.001[a]
Gender [n (%)]				
• Female	54 (38.6)	37 (68.5)	17 (31.5)	0.489[b]
• Male	86 (61.4)	54 (62.8)	32 (37.2)	
Education [n (%)]				
• ≤6 years	31 (22.1)	28 (90.3)	3 (9.7)	0.001[b]
• >6 years	109 (77.9)	63 (57.8)	46(42.2)	
Hypertension [n (%)]				
• Yes	74 (52.9)	46 (62.5)	28 (37.8)	0.456[b]
• No	66 (47.1)	45 (68.2)	21 (31.8)	
Diabetes mellitus [n (%)]				
• Yes	43 (30.7)	29 (67.4)	14 (32.6)	0.687[b]
• No	97 (69.3)	62 (63.9)	35 (36.1)	
Hyperlipidemia [n (%)]				
• Yes	99 (70.7)	64 (64.6)	35 (35.4)	0.892[b]
• No	41(29.3)	27 (65.9)	14 (34.1)	
Smoking [n (%)]				
• Yes	59 (42.1)	39 (66.1)	20 (33.9)	0.816[b]
• No	81 (57.9)	52 (64.2)	29 (35.8)	
At day on admission				
• NIHSS score (median IQR)	6(4–8)	6(5–9)	5(4–6)	0.001[c]
• mRS score (median IQR)	3(3–4)	4(3–4)	3(2–3)	0.000[c]
• Barthel index score (median IQR)	4(2–8)	3(1–6)	7(4–10)	0.000[c]
Number of lesions [n (%)]				
• Multiple	56 (40)	48 (85.7)	8 (14.3)	0.000[b]
• Single	84 (60)	43 (51.2)	41 (48.8)	
Location of lesions [n (%)]				
• Cortex	56 (40)	43 (76.8)	13 (23.2)	0.017[b]
• Subcortex	84 (60.0)	48 (57.1)	36 (42.9)	
Cerebral atrophy [n (%)]				
• Yes	41(29.7)	33 (80.5)	81 (19.5)	0.013[b]
• No	99 (70.3)	58 (58.6)	41 (41.4)	
At 30 day after admission				
• MMSE score (median IQR)	24(19-27)	21(17-23)	27(26-28)	0.000[c]
• CDT score (median IQR)	3 (1-4)	1 (0-3)	4 (3-4)	

[a]: t-test independent, [b]: Chi-squared test, [c]: Mann-Whitney test

TABLE 2. The outcome of patients with CI 30 days after acute ischemic stroke

PSCI day-30	n = 140 (%)
MMSE [n (%)]	
• ≤25	82 (58.6)
• >25	58 (41.4)
CDT [n (%)]	
• ≤2	64 (45.7)
• >2	76 (54.3)
CI [n (%)]	
• MMSE ≤25 and/or CDT ≤2	91 (65)
• MMSE >25 and CDT >2	49 (35)

Note: CI: cognitive impairment; CDT: clock drawing test; MMSE: mini-mental state examination; PSCI: post-stroke cognitive impairment.

TABLE 3. Results of bivariate and multivariate analyses of CI after acute ischemic stroke

Variable	Bivariate Analysis			Multivariate Analysis			
	p	OR	95%CI	p	OR	95%CI	B coefficient/ Standard Error
Age (years)							
• >70	0.003	3.75	1.51–9.26	0.046	2.93	1.01–8.47	1.08/0.54
Gender							
• Female	0.489	1.29	0.62–2.65				
Education							
• ≤6 years	0.001	6.81	1.95–23.78	0.016	5.48	1.38–21.81	1.70/0.70
Hypertension							
• Yes	0.456	0.76	0.38–1.54				
Diabetes mellitus							
• Yes	0.687	1.16	0.54–2.50				
Hyperlipidemia							
• Yes	0.892	0.94	0.44–2.03				
Smoking							
• Yes	0.816	1.08	0.53–2.20				
NIHSS score on admission							
• ≥7	0.004	3.09	1.40–6.80				
mRS score on admission							
• >3	0.000	4.40	2.00–9.69	0.170	2.17	0.71–6.60	0.77/0.56
Barthel Index score on admission							
• ≤4	0.000	5.62	2.62–12.06	0.058	2.82	0.96–8.24	1.03/0.54
Number of lesion							
• Multiple	0.000	5.72	2.41–13.54	0.053	2.68	0.98–7.30	0.98/0.51
Location of lesion							
• Cortex	0.017	2.48	1.16–5.28	0.132	2.06	0.80–5.32	0.72/0.48
Cerebral atrophy							
• Yes	0.013	2.91	1.22–6.95				

After the final model was transformed into a scoring system by utilizing the B and SE values in the multivariate TABLE, a cognitive impairment score system was formed after acute ischemic stroke with a score range of 0–7 where the optimal cutoff point was ≥1 with a sensitivity value of 86.8%, specificity of 59.2% and the predicted PSCI event probability value of 40.49%. The scoring system and the probability value for the occurrence of PSCI are shown in TABLE 4, 5 and FIGURE 1.

TABLE 4. Acute ischemic PSCI scoring system.

Variable	Score
Age	
• ≤ 70 years	+0
• > years	+1
Level of education	
• > 6 years	+0
• ≤ 6 years	+2
Score of mRS	
• ≤ 3	+0
• > 3	+1
Score of Barthel index	
• > 44	+0
• ≤ 4	+1
Number of lesions	
• Single	+0
• Multiple	+1
Location of lesions	
• Subcortex	+0
• Cortex	+1

The maximum score is 7. A score of 1 or higher is at risk of developing PSCI significantly

TABLE 5. The sensitivity, specificity, NPV and PPV of PSCI scoring system.

Cut-point	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
≥0	95.6	40.8	75.0	83.3
≥1	86.8	59.2	79.5	69.0
≥2	60.4	87.8	90.1	54.4
≥3	40.7	91.8	90.4	45.4
≥4	26.4	100	100	42.2
≥5	12.1	100	100	37.9
≥6	3.3	100	100	35.7
≥7	0.0	100	100	35.0

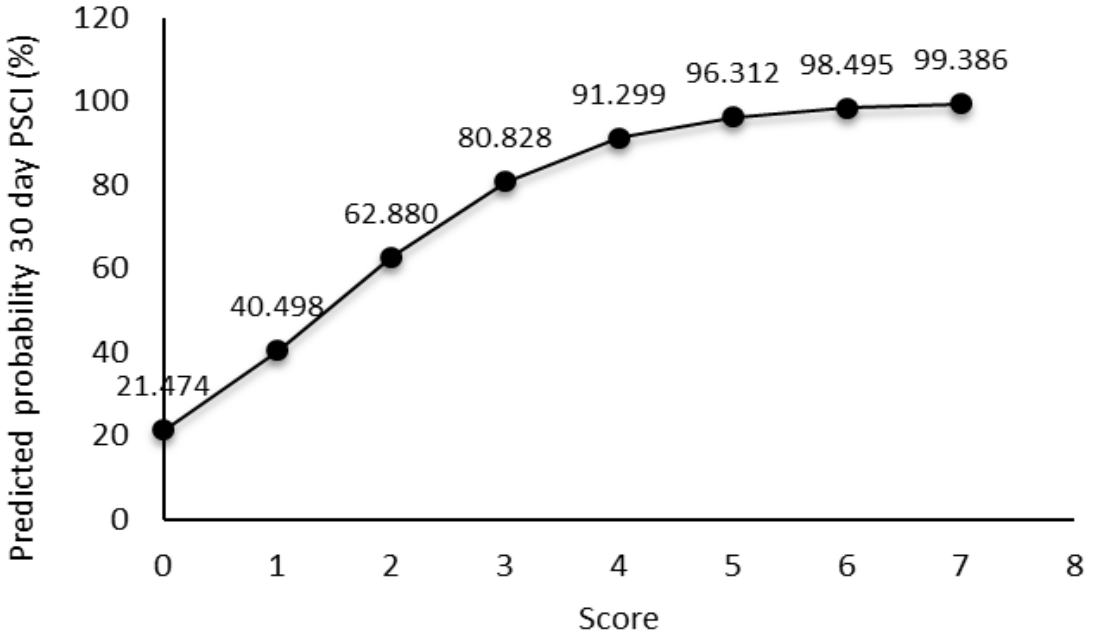

FIGURE 1. PSCI probability prediction graph at day-30.

DISCUSSION

Acute ischemic PSCI is increasing but is often identified late when it has progressed to post-stroke dementia. In this study, it was found that 65% of patients with acute ischemic stroke experienced CI on the 30th day after acute ischemic stroke. Most of those who experienced acute ischemic PSCI at age >70 years, education level ≤6 years, had a BI score ≤4 and mRS score >3 at diagnosis, had multiple lesions, and the presence of lesions in the cortex on CT-scan result of the head which are predictors of CI after acute ischemic stroke. In addition, the main result of this study is the developed predictor score has a good performance in predicting the occurrence of CI at day-30 after acute ischemic stroke as indicated by the AUC discrimination value of 0.826 (95% CI: 0.75–0.89) and the Hosmer-Lemeshow test calibration value of p=0.366. This scoring system has a value range of 0–7, with a cutoff ≥1 having sensitivity value of 86.8%, specificity of 59.2%, and predictive value of the PSCI event probability of 40.49%.

The output observed in this study was the PSCI incident. The incidence of PSCI ranges between 20%–80%, which varies between countries depending on population, time of observation, and outcome criteria.[6] The incidence of PSCI in this study was 65%. These results are similar to those of the study conducted by Yang *et al.*[14] in 2020 in China which reported the results of the PSCI incidence rate at day-30, specifically 62.5%. In addition, the results of previous studies that have been done at RS Bethesda Yogyakarta by Pinzon *et al.*[15] in 2018 obtained the PSCI incidence rate of 68.2%. Different results were seen in study conducted by Chander *et al.*[16] in 2017 in Singapore who reported the PSCI incidence rate was lower at 37.32%. The different results in this study were likely due to several differences, namely, the study used a mild ischemic stroke type, the time was observed at the 3rd month and the observed outcome criteria were only using MMSE.

The results of the analysis of this study showed that acute ischemic stroke patients aged > 70 years were

significantly associated with the incidence of PSCI having an OR value of 2.9 times higher to experience PSCI events. These results are similar to studies conducted by Godefroy *et al.*[17] in 2018 which showed stroke patients aged >70 years have an OR value of 2.5 times higher incidence of PSCI. This condition indicates that as age increases, changes in the structure of the brain tissue can result in decreased cognitive function and the ischemic stroke incidence causes hypoperfusion of brain tissue, thereby accelerating the process of cognitive decline.[18]

The results of this study show there were as many as 90.3% of patients with acute ischemic stroke with an education level of ≤6 years who experienced CI with OR value of 5.4 times higher to experience PSCI. This is similar to the results of research conducted by Chander *et al.*[16] in 2017, where 85.9% of ischemic stroke patients with an education level of ≤6 years experienced CI with OR value of 1.76 times higher to experience PSCI. In addition, research in Korea in 2019 showed that low levels of education were associated with an increased risk of PSCI and this condition was related to the cognitive reverse (CR) theory. The CR is the ability of the brain's endurance (capacity) to slow down or minimize damage to brain tissue. Although the exact mechanism is not clearly known, it is believed that the level of education and work affects the resilience of the neuropathological process where each individual has a different number of synapses and volume of brain tissue in maintaining the neuropathological process. Patients who had an ischemic stroke with an education level of ≤6 years have fewer synapses and a smaller volume of brain tissue, making it less effective at resisting tissue damage.[19]

One of the tools that can be used to assess the functional level of stroke patients is the BI score and the mRS score. The BI score of ≤4 (total dependence) at diagnosis in this study was shown to be a significant predictor of CI after acute ischemic stroke with a value of p:0.05. In addition, the mRS score >3 at diagnosis (moderate to severe disability) for those who cannot meet basic life needs without the help of others has also been shown to be associated with the incidence of PSCI. Research conducted by Monfort *et al.*[20] in 2008 reported that decreased cognitive function affects the independence of stroke patients in their daily activities and increases the risk of post-stroke disability. Another study conducted by Khedr *et al.* [21] in 2009 showed that a low BI score was significant as a predictor of PSCI. This condition showed that the BI score ≤4 and the mRS score >3, when diagnosed with cognitive performance disorders as a whole, resulted in subjects being unable to carry out daily activities which required higher cognitive function for motor control, organization, problem-solving, and memory.[22]

The number of multiple stroke lesions is significant as a predictor factor and can increase the risk of PSCI by 3.06 times higher than with a single lesion. These findings are consistent with research conducted by Godefroy *et al.*[17] in 2018 in Paris which showed that the number of multiple stroke lesions was significant as a predictor factor and could increase the risk of PSCI by 3.78 times higher (95% CI:1.6–8.9, p=0.002) compared to the single lesion. This finding suggests that the number of multiple stroke lesions at multiple locations can lead to a more progressive deficit in cognitive function than a single lesion. Compensation of the brain in developing plasticity and repairing the infarcted brain tissue becomes inefficient.[23]

The acute ischemic stroke patients with the location of the lesion in the cortex experienced the majority of PSCI events (76.8%) and increased the risk of PSCI 2.06 times higher than subcortical lesions. This result does not differ from the results of the study conducted by

Zhang *et al.*[24] in 2012 in China where stroke patients with a location of cortical lesions had a 1.5 times higher risk of PSCI incidence than subcortical lesions and it was reported that cortical location was a predictor of PSCI. This condition is because the location of the lesion in the cortex (cortex-subcortex) is more at risk of causing damage to neural networks such as frontal-subcortical circuits which play an important role in cognitive function in the three domains, namely memory, speed of processing information, and executive function.[23] In this study, the majority of acute ischemic stroke patients were located in the subcortex of 84 (60%) and those who had PSCI were 48 (57.1%). This finding suggests that acute ischemic stroke patients who have lesions located in the subcortex also need more attention because the frequency of cases is large and few of them experienced PSCI events. This finding is supported by a study conducted by Grau-Olivares *et al.*[25] in 2009 that showed lesions in the subcortex that were considered mild can cause MCI in 55% and dementia in 33%–67%.

There are several scores that have been developed in other countries as a comparison in this study, namely the SIGNAL2, CHANGE, and GRECogVASC. The SIGNAL2 score was developed and validated in Singapore by Chander *et al.*[26] in 2015 where the SIGNAL2 score had an AUC value of 0.829 (95% CI: 0.77–0.88) and was effective in identifying patients at risk for PSCI at 3–6 months after stroke. Chander *et al.*[16] in 2017 further developed and validated the CHANGE score which had an AUC value of 0.82 (95% CI: 0.76–0.88) and was effective in screening ischemic stroke patients who were at risk for PSCI up to 18 months after stroke. Another study conducted in Paris by Godefroy *et al.*[17] in 2018 developed and validated the GRECogVASC score which had an AUC value of 0.793 (95% CI: 0.745–0.842) and was effective in screening ischemic stroke patients who were at risk for PSCI at the 6th month after stroke.

The difference between this study and the comparative studies above is that the subjects of this study are patients with acute ischemic stroke for the first time with mild to moderate severity, including variables from clinical factors (NIHSS score, BI score and mRS score at diagnosis), and neuroradiological variables which were assessed. Based on the head CT scan, the observation time for PSCI events was 30 days and the outcome criteria were assessed using MMSE and CDT. The parallel equations of this study with the comparative research above are the place of research in the hospital. In the development of the model, the value of $p \leq 0.20$ was concluded as statistically significant, and the score developed had a good performance with a value of AUC ≥ 0.8.

There are several factors that cause the incidence of PSCI to increase and develop into PSD, namely delays in recognizing CI in post-stroke patients, high stroke severity, and post-stroke patients' non-compliance for rehabilitation. Accordingly, the importance of the predictor score developed by the researcher can be used by clinicians as a basis for knowing stroke patients who are at high risk of CI at day-30 after acute ischemic stroke. At this time, the best course of action in preventing the incidence of PSCI is lifestyle modification by increasing physical exercise, a healthy diet and smoking cessation to reduce stroke severity, prevent stroke complications and prevent recurrent stroke events.[27] In addition, citicoline oral therapy can be given as an effort to prevent PSCI from getting worse or developing into PSD. A study conducted by Cotroneo *et al.*[28] in 2013 stated that after giving citicoline orally at a dose of 500 mg 2 times a day for 9 months, there was an increase in the MMSE score by 0.5 points compared to those who did not

receive therapy. Evaluation at 9 months showed a decrease in MMSE score in the group that did not receive citicoline therapy. From this study, it is known that citicoline administration is effective and safe for elderly patients with mild vascular CI.

This study some weaknesses including (1) there was no adjustment of the MMSE and CDT score assessments with the patient's education level, and (2) there was a possibility of selection bias in the subjects included in the study because the previous history of CI was only based on the nurse's alloanamnesis of the patient's family, (3) the research subjects obtained were ischemic stroke patients with mild to moderate severity so that the NIHSS score variable at diagnosis and cerebral atrophy could not represent the ischemic stroke patient population, (4) the concluded values were statistically significant in developing the predictor model using $p<0.2$, and (5) external validity had not been carried out in other hospitals.

CONCLUSIONS

The developed predictor score had a good performance in predicting the occurrence of cognitive impairment on the day-30 after acute ischemic stroke. Results in this study suggest further research should be conducted with external validity in another hospital with a prospective design and a longer monitoring time, specifically, 1, 3, and 6 months after acute ischemic stroke.

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Therapeutic options for extended-spectrum β-lactamases (ESBLs), AmpC β-lactamases producing *Escherichia coli* and *Klebsiella* sp. isolated from various clinical samples

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ABSTRACT

Escherichia coli and *Klebsiella* sp. are the predominant species isolated from clinical samples. Recent and proper understanding of the antibiotic susceptibility pattern of extended-spectrum β-lactamases (ESBL) and AmpC β-lactamases (AmpC) producing *E. coli* and *Klebsiella* sp. will prevent the distribution and future incidence of ESBL and AmpC. We designed this study to understand antibiotic susceptibility patterns of ESBL and AmpC producing *E. coli* and *Klebsiella* sp. isolated from a tertiary care hospital in North India. A cross-sectional study was conducted from March 2021 to February 2022. During this period, various clinical samples were collected and further tested for ESBL producing *E. coli* and *Klebsiella* sp. by using the Double disc Synergy test, whereas AmpC was detected by the Boronic acid disk potentiation method. Their antibiotic susceptibility patterns were noted. Various clinical specimens were collected, in which 37.95% were shown growth of bacteria. Among them, 46.67% of *E. coli* and 25.21% of *Klebsiella* sp. were identified by standard laboratory protocol. ESBL producing isolates were 44.37% and 34.20% in *E. coli* and *Klebsiella* sp., respectively. Whereas AmpC production was detected in 18.27% of *E. coli* and 29.36% of *Klebsiella* sp. ESBL and AmpC producing *E. coli* and *Klebsiella* sp. isolated from pus, blood, and sputum samples showed the highest sensitivity towards colistin, tigecycline, and imipenem while in urine samples imipenem, meropenem showed the highest sensitivity. Susceptibility patterns of ESBL and AmpC producing *E. coli* and *Klebsiella* sp. from various clinical specimens enhance hospital infection management and help clinicians to prescribe the appropriate antibiotics. The carbapenem, nitrofurantoin, colistin and tigecycline were showed highest susceptible against ESBL and AmpC producing *E. coli* and *Klebsiella* sp.

ABSTRAK

Escherichia coli dan *Klebsiella* sp. adalah spesies utama yang diisolasi dari sampel klinis. Pemahaman tentang pola kerentanan terhadap antibiotik dari *E. coli* dan *Klebsiella* sp. penghasil β-laktamase spektrum luas (*extended-spectrum β-lactamases*/ESBL) dan β-laktamase AmpC (AmpC *β-lactamases*/AmpC) akan mencegah distribusi dan munculnya ESBL dan AmpC ke depan. Penelitian ini dirancang untuk mengkaji pola kerentanan terhadap antibiotik dari *E. coli* dan *Klebsiella* sp. penghasil ESBL dan AmpC yang diisolasi dari rumah sakit perawatan tersier di India Utara. Penelitian potong lintang ini dilakukan dari Maret 2021 hingga Februari 2022. Selama periode ini, berbagai sampel klinis dikumpulkan dan diuji lebih lanjut untuk *E. coli* dan *Klebsiella* sp. penghasil ESBL menggunakan uji sinergi cakram ganda, sedangkan penghasil AmpC dideteksi dengan metode potensiasi cakram asam boronat. Pola kerentanan isolate terhadap antibiotik selanjutnya dicatat. Dari berbagai spesimen klinis yang dikumpulkan, sebanyak 37,95% menunjukkan pertumbuhan bakteri, dengan di antaranya, 46,67% *E. coli* dan 25,21% *Klebsiella* sp. diidentifikasi oleh protokol laboratorium standar. Isolat penghasil ESBL berturut-turut sebesar 44,37% dan 34,20% pada *E. coli* dan *Klebsiella* sp. Sedangkan produksi AmpC terdeteksi pada 18,27% *E. coli* dan 29,36% *Klebsiella* sp. *Escherichia coli* dan *Klebsiella* sp. penghasil ESBL dan AmC diisolasi dari sampel pus, darah,

Keywords:
ESBL;
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E. coli;
Klebsiella sp.

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dan sputum menunjukkan sensitivitas tertinggi terhadap colistin, tigesiklin, dan imipenem sedangkan pada sampel urin imipenem, meropenem menunjukkan sensitivitas tertinggi. Informasi tentang pola kerentanan *E. coli* dan *Klebsiella* sp. penghasil ESBL dan AmpC dari berbagai spesimen klinis dapat meningkatkan manajemen infeksi rumah sakit dan membantu dokter meresepkan antibiotik yang tepat. Karbapenem, nitrofurantoin, colistin dan tigesiklin menunjukkan kerentanan tertinggi terhadap *E. coli* dan *Klebsiella* sp. penghasil ESBL dan AmpC.

INTRODUCTION

The threat of antimicrobial resistance in humans is not new; around 700,000 people death annually around the world due to drug resistance.[1] Lack of accurate detection of bacterial resistance may increase mortality and morbidity, whereas knowledge of current trends of antibiotic sensitivity decreases the risk of bacterial resistance.[2] Since 1970, there has been a growing recognition and medical concern towards extended-spectrum β-lactamases (ESBL) and AmpC β-lactamases (AmpC) producing *E. coli* and *Klebsiella* sp. due to the overproduction of newer β-lactamase enzymes.[3] These enzymes are plasmid-mediated and can be transmitted from one bacterium to another. The ESBLs are enzymes that cause resistance to extended-spectrum cephalosporins (ESCs) such as cefotaxime, ceftriaxone, and ceftazidime, as well as the monobactam aztreonam.[2,3] The AmpC are enzymes that are responsible to cause bacterial resistance to penicillin, second and third generation cephalosporins, and cephamycins. According to Ambler's structural classification, AmpC belongs to the molecular C class while by the scheme of Bush they belong to group-1.[3] *Escherichia coli* and *Klebsiella* sp. are the major bacteria isolated from various community and hospital-acquired infections such as bloodstream infection, urinary tract infection, and meningitis.[4]

Various methods are available for the detection of ESBL and AmpC. Common methods available to detect ESBL are the double disc synergy test.[5] Other are combination disc method (the phenotypic confirmatory disc diffusion test).[6] Three dimensional test[7] such as broth dilution test,[6] ESBL E-Test[8] and VITEK-2.[9]

Escherichia coli and *Klebsiella species* produce ESBL and AmpC enhancing therapeutic problems and treatment failure. As a result detection of ESBL and AmpC producing *E.coli* and *Klebsiella* sp. is important for successful therapy as well as prevention of these resistant bacteria. Furthermore, the proper understanding of susceptible antibiotics in ESBLs and AmpC *E. coli* and *Klebsiella* sp. will prevent the distribution and future incidence of ESBLs and AmpC. The study provides the data on recent resistance patterns of ESBL and AmpC producing *E. coli* and *Klebsiella* sp. as well as their current treatment options. This study focused on the antibiotic sensitivity pattern of ESBL, AmpC producing *E. coli* and *Klebsiella* sp. isolated from various clinical specimens at a North Indian tertiary care hospital.

MATERIALS AND METHODS

Design of study

The study was conducted after the approval of the Ethical Committee with ethical letter no- IEC-2100. In this cross-sectional study, 5214 samples were collected from various clinical sites from blood, urine, pus, sputum, and swabs from the patients, who had septicemia, UTI, wounds infections, lower respiratory infections, and local infections respectively, after consent of the patient by nurses during January 2021 to February 2022. Then specimens were processed in the Microbiology Department of the Maharishi

Markandeshwar Institute of Medical Sciences & Research, Mullana, Ambala, India. All the specimens were inoculated on Blood agar and MacConkey Agar. Gram-positive bacteria were excluded and *E. coli and Klebsiella* sp. were only included in further processing identification and antibiotic sensitivity testing was done by Vitek-2 and confirmed by Kirby Bauer disc diffusion method as per CLSI 2021.[6,9] The bacteria control used in the study were *E. coli* ATCC 25922, and *K. pneumonia*e ATCC 700603.

Detection of ESBL

ESBL screening test

Disc Diffusion Test-Mueller Hinton Agar (MHA) was inoculated with the lawn culture of the test organism (0.5 McFarland`s turbidity). Disc of cefpodoxime (10µg), ceftazidime (30µg), cefotaxime (30µg) was applied on surface of MHA. The zones formed for each drug are as follows; cefpodoxime ≤17mm, ceftazidime ≤22mm, cefotaxime ≤27mm. The zones above indicated ESBL production.[6]

Confirmation of ESBL (combination disc method)

The test inoculum (0.5 MacFarland turbidity) was lawn onto the MHA by using a sterile (cotton swab) a ceftazidime disc (30µg) and a ceftazidime-clavulanic acid disc (20+10 µg) were placed at a distance of 20mm from each other, the plates were inoculated at 37^{0}C for 18 to 24 h and the results was noted, ≥5mm size increased in the zone of inhibition observed in ceftazidime-clavulanic acid than ceftazidime consider as ESBL-producing organism.[6]

Detection of AmpC

Cefoxitin disc test

MHA plate was inoculated with the test organism (0.5 McFarland turbidity). A cefoxitin disc (30 µg) was placed in the center. The plates were incubated at 37^{o}C isolate that yielded a zone diameter of <18 mm and was accepted by AmpC enzyme producers.[10]

Boronic acid disk potentiation method

MHA plate was inoculated with a lawn culture of the test organism (0.5 McFarland turbidity). AmpC production was detected by using a disc of cefoxitin (30 µg) and another disc with boronic acid (20µL) on the culture plate at a distance of 20mm from the center of the disc. The overnight incubation was done at 37^{o}C. The organism was considered an AmpC producer if there was a ≥5mm increase in the zone of cefoxitin plus boronic acid disc as compared to cefoxitin disc.[11]

Preparation of disc containing boronic acid + cefoxitin

As much as 120 mg of phenylboronic acid was dissolved in 3 mL of dimethylsulphoxide and 3 mL of sterile distilled water was added to this solution. As much as 20µL of the stock solution was dispensed onto each disc of cefoxitin (30µg). Discs were allowed to dry for 30-60 minute and used immediately. A ≥ 5 mm increase in the zone diameter around the disc containing cefoxitin + boronic acid than around cefoxitin alone was considered positive for AmpC enzyme production.[11] Data was recorded and interpreted with excel and in the form of charts and TABLEs.

RESULTS

A total of 5214 samples were received in a laboratory during the study period, in which 62.05% samples were sterile and 37.95% were positive for bacterial growth, 41.18% were gram-positive bacteria, 53.91% were gram-negative bacteria, and 4.90% were *Candida* sp. The 59% samples were collected from females and 41% samples were collected

from males and the bacterial positivity rate was 54% in female and 45% in male patients. The age of the patients has also noted classified into different groups in which the highest number of samples i.e. 48% was collected from patients belongs the 21-40 years age group followed by the 41-60 age group (22%), 0-20 age group (19%) and 61-80 years old age group (11%) and the culture positivity was highest in patients belongs to 21-40 age group i.e. 36% followed by 41-60 age group (33%), 61-80 years age group (22%) and lowest in 0-20 age group (5%). The majority of the samples were collected from IPD (71%) and OPD (29%) patients. the culture positivity rate was 58% from IPD patients while 42% from OPD patients.

In total Gram-negative isolates, *E. coli* (46.67%) was the predominant bacteria, followed by *Klebsiella* sp. (25.21)%, *Proteus* sp. (4%), *Pseudomonas* sp. (12.74%), *Acinetobacter* sp. (7.77%), *Providencia* sp. (2.%) and *Sphingomonas* sp. (2%) (FIGURE 1).

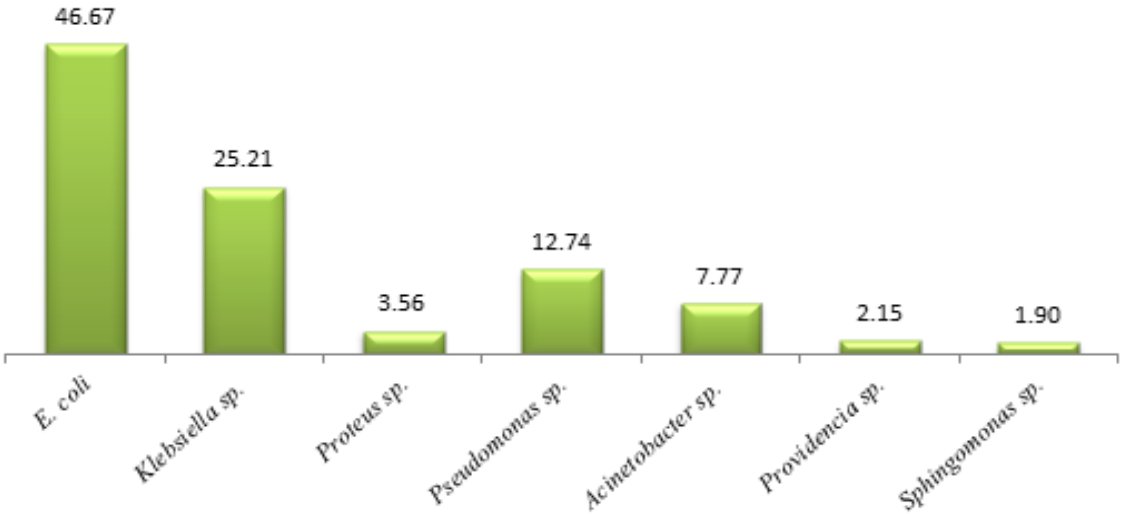


FIGURE 1. Frequency (%) of Gram-negative bacteria isolated in the study

Out of a total of 498 *E. coli*, 44% of *E. coli* were detected as ESBL producers, while 34% of *Klebsiella* sp. were identified as ESBL producers from a total of 269 *Klebsiella* sp. by using CLSI recommended phenotypic confirmatory disc diffusion test (PCDDT). However, 18.27% of *E. coli* and 29.36% of *Klebsiella* sp. isolates were identified as AmpC producers by using a boronic acid test whereas 16% of isolates were found to be co-producers of ESBL and AmpC (TABLE 1).

TABLE 1. ESBL, AmpC and co-producer *E. coli* and *Klebsiella* sp.

Isolates (n=767)	ESBL producers [n (%)]	AmpC producers [n (%)]	Co-porducers [n (%)]
E. coli (n=498)	91 (18.27)	221 (44.37)	58 (11.64)
Klebsiella sp. (n=269)	79 (29.36)	92 (34.20)	39 (14.49)
Total (n=767)	170 (22.16)	313 (40.80)	97 (12.64)

The majority of ESBL producing *E. coli* was isolated from urine specimens i.e. 64.25% followed by pus (17.19%), sputum (2.71%), blood (4.97%), and the majority of ESBL producing *Klebsiella* sp. was isolated from urine specimens i.e. (28.57%), followed by pus (19.78%), sputum (3.29%), and blood (5.49%). Maximum AmpC producing *E. coli* was isolated from (59.34%) urine samples followed by pus (18.68%), sputum (3.29%), blood (5.49)% while in *Klebsiella* sp., maximum isolates were identified from urine samples i.e. 34.17%, followed by pus (18.98%), sputum (5..06%), and blood (15.18%). Whereas co-production of ESBL and AmpC in *E. coli* were maximum in urine samples (55%) followed by pus (22%), sputum (5%), blood (7%) while in *Klebsiella* sp. (23%) co-producers isolated from urine samples followed by pus (18%), sputum (5%), and blood (23%) (TABLE 2).

TABLE 2. Sample wise distribution of ESBL, AmpC, Co-producing *E. coli*, and *Klebsiella* sp. (Others swabs and tissues collected from the various department.)

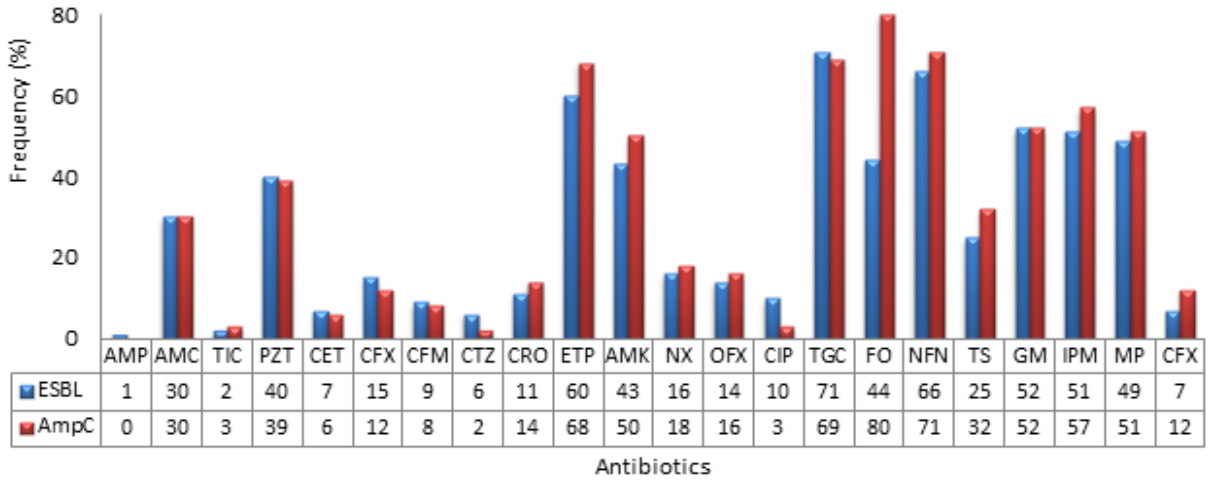
Sample	*E. coli* [n (%)]			*Klebsiella* sp. [n (%)]		
	AmpC producers	ESBL producers	Co-porducers	AmpC producers	ESBL producers	Co-producers
Urine	54 (59.34)	142 (64.25)	32 (55)	27 (34.17)	26 (28.57)	9 (23)
Pus	17 (18.68)	38 (17.19)	13 (22)	15 (18.98)	18 (19.78)	7 (18)
Sputum	3 (3.29)	6 (2.71)	3 (5)	4 (5.06)	11(12.08)	2 (5)
Blood	5 (5.49)	11 (4.97)	4 (7)	12 (15.18)	12 (13.18)	9 (23)
Others	12 (13.18)	24 (10.85)	6 (10)	21(26.58)	24 (26.37)	12 (31)
Total	91 (100)	221 (100)	58 (100)	79 (100)	91 (100%)	39 (100)

The 55% of ESBL producers *E. coli* and *Klebsiella* sp. were isolated from females while 45% from male and AmpC were identified in 52% from females, while 47% from male patients. Co-producers of ESBL and AmpC in females were 53% and in males were 47%, respectively.

The ESBL producing *E. coli* and *Klebsiella* sp. were detected in 42% of IPD patients and 40% in opd patients while AmpC producing *E. coli* and *Klebsiella* sp. were 24% in IPD patients and 19% from OPD patients. Co-producers of ESBL and AmpC in OPD were 13% and IPD were 12%, respectively.

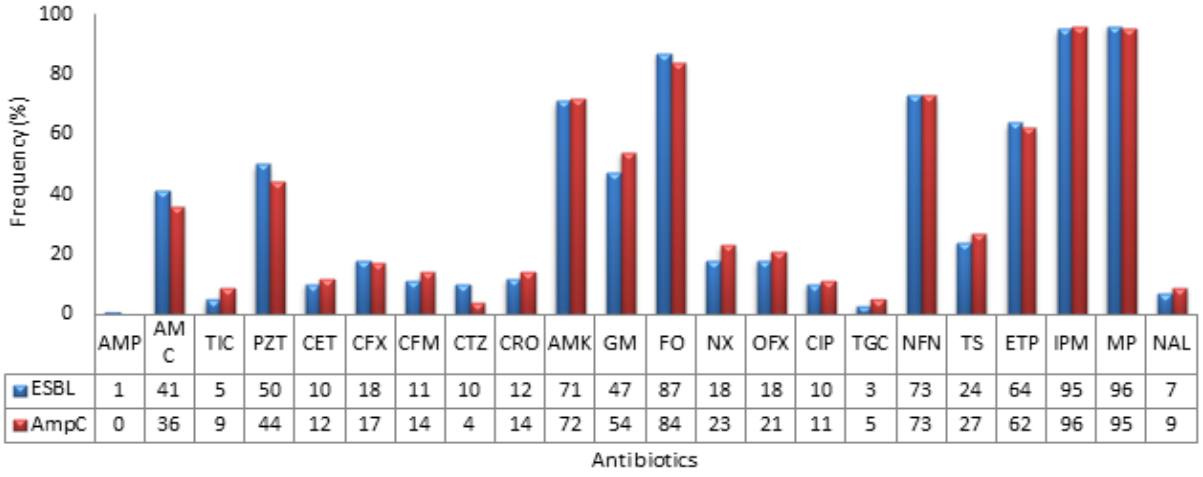
The antibiotic sensitivity pattern of ESBL producing *E. coli* and *Klebsiella* sp. isolated from various clinical samples showed 71.23% strains were susceptible to tigecycline, 66% to nitrofurantoin and 60% to amikacin and AmpC producing *E. coli* and *Klebsiella* sp. showed 80% susceptible to fosfomycin and 71% to nitrofurantoin (FIGURE 2).

The antibiotic sensitivity pattern of ESBL producing *E. coli* and *Klebsiella sp.* isolated from urine samples showed 95% strains were susceptible to imipenem, 96% to meropenem and 87% to fosfomycin and AmpC producing *E. coli* and *Klebsiella* sp. showed 96% susceptible to imipenem, 95% to meropenem and 84% to fosfomycin (FIGURE 3).



	AMP	AMC	TIC	PZT	CET	CFX	CFM	CTZ	CRO	ETP	AMK	NX	OFX	CIP	TGC	FO	NFN	TS	GM	IPM	MP	CFX
ESBL	1	30	2	40	7	15	9	6	11	60	43	16	14	10	71	44	66	25	52	51	49	7
AmpC	0	30	3	39	6	12	8	2	14	68	50	18	16	3	69	80	71	32	52	57	51	12

FIGURE 2. Susceptibility pattern of ESBL and AmpC producing *E. coli* and *Klebsiella* sp. isolated from various sample. Note: AMP (ampicilin); AMC (amoxicillin/clavulanic acid); TIC (ticarcillin); PZT (piperacillin/tazobactam); CET (cefalotin); CFX (cefoxitin); CFM (cefixim); CTZ (ceftazidime); CRO (ceftriaxone); ETP (ertapenem); AMK (amikacin); NX (norfloxacin); OFX (ofloxacin); CIP (ciprofloxacin); TGC (tigecyclin); FO (fosfomycin); NFN (nitrofurantoin); TS (trimehoprim/sulfonamide); GM (gentamicin); IPM (imipenem); MP (meropenem); CFX (cefuroxime).



	AMP	AMC	TIC	PZT	CET	CFX	CFM	CTZ	CRO	AMK	GM	FO	NX	OFX	CIP	TGC	NFN	TS	ETP	IPM	MP	NAL
ESBL	1	41	5	50	10	18	11	10	12	71	47	87	18	18	10	3	73	24	64	95	96	7
AmpC	0	36	9	44	12	17	14	4	14	72	54	84	23	21	11	5	73	27	62	96	95	9

FIGURE 3. Susceptibility pattern of ESBL and AmpC producing *E. coli* and *Klebsiella* sp. isolated from urine specimens. Note: AMP (ampicilin); AMC (amoxicillin/clavulanic acid); TIC (ticarcillin); PZT (piperacillin/tazobactam); CET (cefalotin); CFX (cefoxitin); CFM (cefixim); CRO (ceftriaxone); AMK (amikacin); GM (gentamicin); FO (fosfomycin); NX (norfloxacin); OFX (ofloxacin); CIP (ciprofloxacin); TGC (tigecyclin); NFN (nitrofurantoin); TS (trimehoprim/sulfonamide); ETP (ertapenem); IPM (imipenem); MP (meropenem); NAL (nalidixic acid).

The antibiotic sensitivity pattern of ESBL producing E. coli and Klebsiella sp. isolated from pus samples showed 100% strains were susceptible to colistin, 80% to tigecycline and The antibiotic sensitivity pattern of ESBL producing E. coli and Klebsiella sp. isolated from blood samples showed 100% strains were susceptible to colistin, 57% to tigecycline and 58% to amikacin change into The antibiotic sensitivity pattern of ESBL producing E. coli and Klebsiella

sp. isolated from pus samples showed 100% strains were susceptible to colistin, 80% to tigecycline and 58% amikacin. The antibiotic sensitivity pattern of ESBL producing E. coli and Klebsiella sp. isolated from blood samples showed 100% strains were susceptible to colistin, 57% to tigecycline and 52% to amikacin (FIGURE 4).

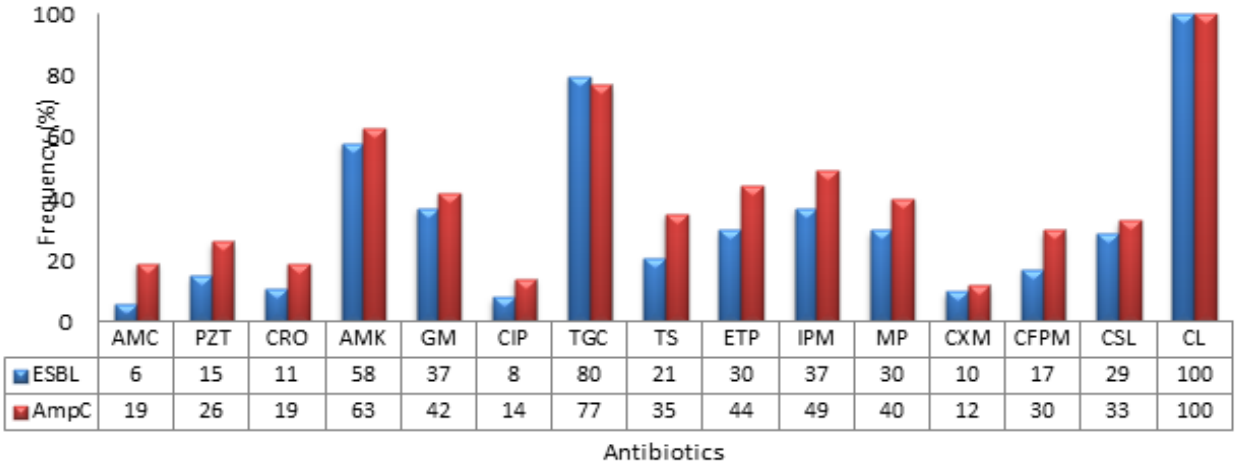


FIGURE 4. Susceptibility pattern of ESBL and AmpC-producing *E. coli* and *Klebsiella* sp. isolated from pus specimens. Note: AMC (amoxicillin/clavulanic acid); PZT (piperacillin/tazobactam); CRO (ceftriaxone); AMK (amikacin); GM (gentamicin); CIP (ciprofloxacin); TGC (tigecyclin); ETP (ertapenem); IPM (imipenem); MP (meropenem); CXM (cefuroxim); CFPM (cefepime); CSL (cefoperazone/sulbactam); CL (colistin).

52% to amikacin and AmpC producing E. coli and Klebsiella sp. showed 100% susceptible to colistin, 59% to tigecycline and 53% to amikacin change into AmpC producing E. coli and Klebsiella sp. showed 100% susceptible to colistin, 59% to tigecycline and 53% to amikacin (FIGURE 5).

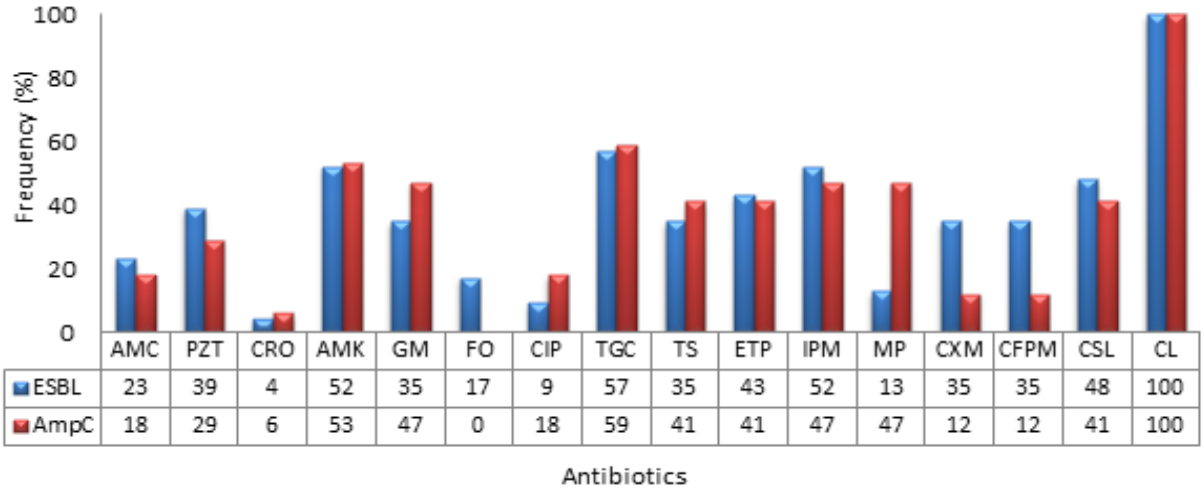
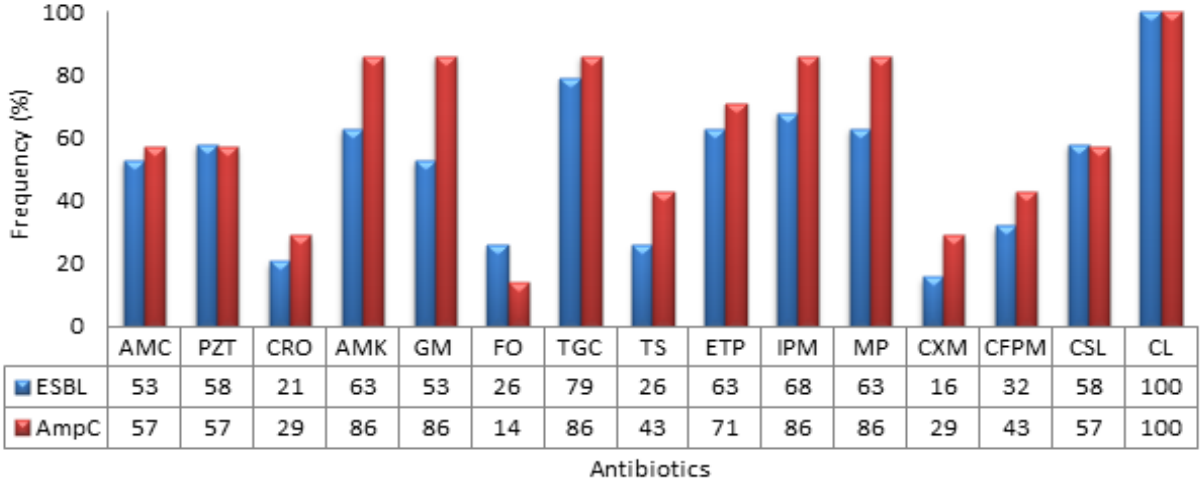


FIGURE 5. Susceptibility pattern of ESBL and AmpC producing *E. coli* and *Klebsiella species* isolated from blood samples. Noted: AMC (amoxicillin/clavulanic acid); PZT (piperacillin/tazobactam); CRO (ceftriaxone); AMK (amikacin); GM (gentamicin); FO (fosfomycin); CIP (ciprofloxacin); TGC (tigecyclin); TS (trimehoprim/sulfonamide); ETP (ertapenem); IPM (imipenem); MP (meropenem); CXM (cefuroxim); CSL (cefoperazone/sulbactam); CL (colistin).

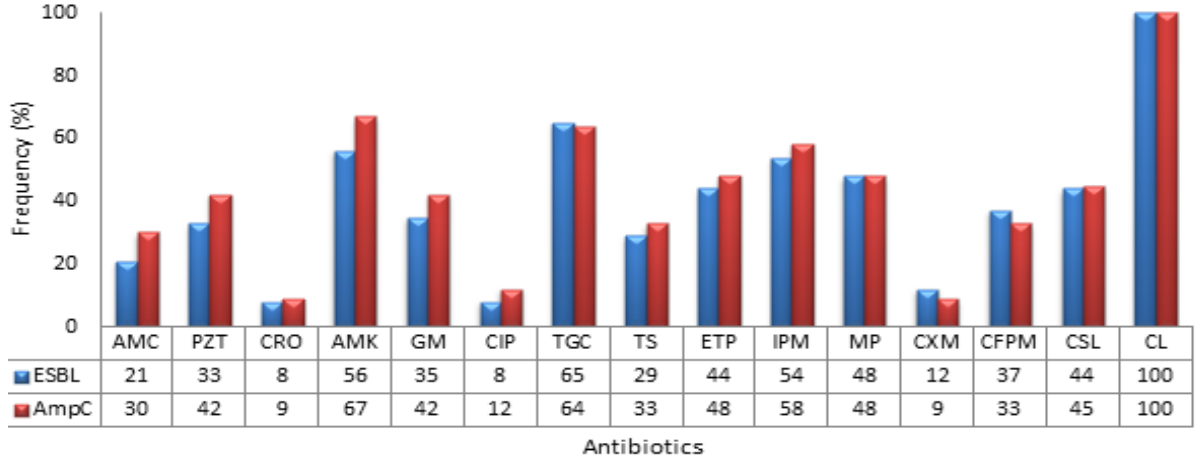
The antibiotic sensitivity pattern of ESBL producing *E. coli* and *Klebsiella* sp. isolated from sputum samples showed 100% strains were susceptible to colistin, 79% to tigecycline, 68% to imipenem, and AmpC producing *E. coli* and *Klebsiella* sp. showed 100% susceptible to colistin, 86% to tigecycline, and 86% to imipenem (FIGURE 6).



	AMC	PZT	CRO	AMK	GM	FO	TGC	TS	ETP	IPM	MP	CXM	CFPM	CSL	CL
ESBL	53	58	21	63	53	26	79	26	63	68	63	16	32	58	100
AmpC	57	57	29	86	86	14	86	43	71	86	86	29	43	57	100

TABLE 6. Susceptibility pattern of ESBL and AmpC producing *E. coli* and *Klebsiella* sp. isolated from sputum samples. Note: AMC (amoxicillin/clavulanic acid); PZT (piperacillin/tazobactam); CRO (ceftriaxone); AMK (amikacin); GM (gentamicin); FO (fosfomycin); TGC (tigecyclin); TS (trimehoprim/sulfonamide); ETP (ertapenem); IPM (imipenem); MP (meropenem); CXM (cefuroxim); CFPM (cefepime); CSL (cefoperazone/sulbactam); CL (colistin).

The antibiotic sensitivity pattern of ESBL producing *E. coli* and *Klebsiella* sp. isolated from other samples showed that 100% of strains were susceptible to colistin, 65% to tigecycline, 56% to amikacin, and AmpC producing *E. coli* and *Klebsiella* sp. showed 100% susceptible to colistin, 64% to tigecycline, and 67% to amikacin (FIGURE 7).



	AMC	PZT	CRO	AMK	GM	CIP	TGC	TS	ETP	IPM	MP	CXM	CFPM	CSL	CL
ESBL	21	33	8	56	35	8	65	29	44	54	48	12	37	44	100
AmpC	30	42	9	67	42	12	64	33	48	58	48	9	33	45	100

FIGURE 7. Susceptibility pattern of ESBL and AmpC producing *E. coli* and *Klebsiella* sp. isolated from other samples (i.e. swabs and tissues). Noted: AMC (amoxicillin/clavulanic acid); PZT (piperacillin/tazobactam); CRO (ceftriaxone); AMK (amikacin); GM (gentamicin); CIP (ciprofloxacin); TGC (tigecyclin); TS (trimehoprim/sulfonamide); ETP (ertapenem); IPM (imipenem); MP (meropenem); CXM (cefuroxim); CFPM (cefepime); CSL (cefoperazone/sulbactam); CL (colistin).

DISCUSSION

Escherichia coli and *Klebsiella* sp. are the predominant bacteria isolated from various community and hospital-acquired infections. *Escherichia coli* and *Klebsiella* sp. that produce ESBLs and AmpC enzymes will cause therapeutic problems and failure, including illness and death. As a result detection of ESBLs and AmpC is important for successful therapy as well as prevention of those resistant bacteria.[8] Precise detection of ESBL and AmpC producing *E. coli* and *Klebsiella* sp. would decrease the mortality and multidrug-resistant organisms. Proper understanding of susceptible antibiotics against ESBLs and AmpC producing *E. coli* and *Klebsiella* sp. can help in the treatment of these organisms. There is a need for susceptibility pattern for ESBLs and AmpC producing *E. coli* and *Klebsiella* sp. from various samples.

In the present study *E. coli* was showed 46.67% growth Rate While *Klebsiella* sp. showed 25.21% followed by *Proteus* sp. 3.56%, *Pseudomonas* sp. 12.74%, *Acinetobacter* sp. 7.77%, *Provedencia* sp. 2.15% and *Sphingomonas* sp. 1.90% (FIGURE 1). As reported by study of Sah *et al.*,[13] among 109 Gram negative bacteria isolates, 40.3% were *E. coli, 30% Klebsiella* sp. and 11% were *Acinetobacter* sp. As the similar study conducted by Nepal *et al.*,[14] stated that *E. coli and* Klebsiella sp. were 51.5% and 14.6%, respectively. As per the both studies *E. coli and Klebsiella* sp. were the predominenet gram negative bacteria isolates. The number of the bacteria might be different because of the locations of the study.

In this present study ESBL producer *E. couple* was 44.17%, whereas *Klebsiella* sp. were 18.27% and AmpC producers *E. coli* were 34.20%, *Klebsiella* sp. 29.36%. Co-producers of ESBL+AmpC also observed in 11.64% in *E. coli* and 14.49% in Klebsiella sp. (TABLE 1). Similar study conducted by Vijaya *et al.*,[15] found about 16% *E. coli*, 6% *Klebsiella* sp. were ESBL positive, 9% *E. coli,* and 3% *Klebsiella* sp. were AmpC producers. Co-production of ESBL+AmpC seen in approximately 15% of total isolates. A study conducted by Nasir *et al.*[16] showed that ESBL production was slightly higher as compared to AmpC in both *E. coli* and *Klebsiella* sp. 12 % *E. coli* and 10% *Klebsiella* sp. were Co-producers for ESBL + AmpC. The increasing number of ESBL and AmpC are the matter of concern that can be resolved by the proper understanding of the antibiotic sensitivity pattern of the samples before starting the empirical treatment or at least send the sample for antibiotic sensitivity testing before starting the empirical treatment.

ESBL-producing *E. coli* and *Klebsiella* sp. from urine samples were 64.25% and 28.57% respectively, followed by pus 17.19% and 19.78%, sputum 2.71% and 12.08%, blood 4.97% and 13.18% respectively. Out of 170 AmpC, 59.34% *E. coli* and 34.17% *Klebsiella* sp. were isolated from urine samples followed by 18.68% and 18.98% from pus, 5.49% and 15.18% in blood samples positively (TABLE 2). A study conducted by Yusuf *et al.*[17] reported that higher ESBL producers detection in *E. coli* and *Klebsiella* sp. was 22.2% in blood samples, followed by 17.6% in urine, 14.5% in urogenital swabs, and 13.6% in wound swab samples whereas AmpC detection in *E. coli* and *Klebsiella* sp. were 50% in urine specimens followed by 20% in catheter tip, 10% in ear swab and10% in wound swab. Saffar *et al.*[18] reported that a higher prevalence rate of ESBL and AmpC producing *E. coli* and *Klebsiella* sp. were seen in the wound and pus samples followed by respiratory samples, body fluid, and blood whereas in AmpC-producing *E. coli* and *Klebsiella* sp. higher rate came from body fluids 63% followed by blood 57%, urine 56%, and respiratory samples 28%. These studies showed that blood and body

fluid was the major source of ESBL and AmpC producing *E. coli* and *Klebsiella* sp. whereas we identified urine as a major source of ESBL and AmpC producing *E. coli* and *Klebsiella* sp. UTI patients should be assessed for antibiotic sensitivity testing as early possible as.

In the present study, 55% of ESBL producers *E. coli* and *Klebsiella* sp. were isolated from females while 45% were from males, and AmpC was identified in 52% from females, while 47% were from male patients. Co-producers of ESBL+AmpC in females were 53% and in males were 47% respectively. The ESBL-producing *E. coli* and *Klebsiella* sp. was detected in 42% IPD patients and 40% in opd patients while AmpC producing *E. coli* and *Klebsiella* sp. were 24% in IPD patients and 19% from opd patients. Co-producers of ESBL+AmpC in OPD were 13% and ipd were 12% respectively. Another study conducted by Yusuf *et al.*[17] showed that 52% of ESBL producers in *E. coli* and *Klebsiella* sp. were isolated from males while 48% from females and AmpC were identified in 60% from male, while 40% from female patients. Somily *et al.*[19] reported that the prevalence rate of ESBL and AmpC producers i.e. 32% of patients were female and 27% patients were male. The ESBL and AmpC producing *E. coli* and *Klebsiella* sp. were detected higher percentage in ipd patients as compared to opd patients. So as per our study females had a higher chance of getting infected with ESBL and AmpC producers *E. coli* and *Klebsiella* sp. We identifed more patients with UTIs and females had more chance of getting UTIs than males. Co-producers are a matter of concern in UTIs of females.

The treatment options against ESBL and AmpC producing *E. coli* and *Klebsiella* sp. depend on the antibiotic sensitivity pattern of the samples. It is very important that every hospital should have their local antibiotic surveillance systems or the hospital may use studies that provide the antibiotic sensitivity pattern on current basics. The sensitive antibiotics can be utilized for the treatment of ESBL and AmpC producing *E. coli* and *Klebsiella* sp.

Antibiotics susceptibility patterns of ESBL and AmpC producing *E. coli* and *Klebsiella* sp. were found to be variable. Most of the ESBL producing isolates were susceptible to tigecycline (71.23%), followed by nitrofurantoin (66%) and (60%) amikacin. Highest sensitivity in AmpC producing isolates was susceptible to fosfomycin (80%), nitrofurantoin (71%), tigecycline (69%), and amikacin (68%) (FIGURE 2). A similar study conducted by Nepal *et al.*[14] showed most of the ESBL producing bacteria were sensitive to imipenem followed by piperacillin/tazobactam, amikacin, and cefoperazone/sulbactam. This is in accordance with the study conducted by Sasirekha *et al.*[20] that reported the highest susceptibility seen in AmpC-producing isolates were imipenem (100%), and amikacin (93%). As per, Nepal *et al.*[14] and Sasirekha *et al.*[20], imipenem and meropenem were the highest sensitive antibiotics in our study imipenem and meropenem were lower than 50% resistant in case of ESBL and AmpC. Fosfomycin, nitrofurantoin, and tigecycline were the highest sensitive antibiotics.

Urine and their antibiotic susceptibility pattern of AmpC and ESBL producing *E. coli* and *Klebsiella* sp. showed high-level susceptibility to imipenem and meropenem 96% and 95% respectively, followed by fosfomycin (81%), nitrofurantoin (71%), and amikacin (70%) (FIGURE 3). Cho *et al.*[21] also showed the susceptibility against ESBL and AmpC producers where highly sensitive antibiotics were imipenem and meropenem (100%) respectively followed by Fosfomycin (96%), amikacin(91%), and nitrofurantoin (90%). A similar study conducted by Halabi *et al.*[22] showed that maximum sensitivity was observed in ertapenem and imipenem followed by

fosfomycin, and amikacin. The resistance towards imipenem, meropenem, and nitrofurantoin have been increased over time. Even though their resistance to carbapenem was also noted and 30% resistance was seen in nitrofurantoin.

Pus samples and their Antibiotic sensitive pattern of ESBL and AmpC producing *E. coli* and *Klebsiella* sp. the highest susceptibility found in colistin (100%) followed by tigecycline (80%), amikacin (75%), and carbapenems (75%) (FIGURE 4). A similar study conducted by Hedaoo *et al.*[23] showed maximum susceptibility to ciprofloxacin followed by amikacin, and cefotaxime. In the pus samples, the colistin and tigecycline was the only option left for the treatment of wound infections, even imipenem was 30 % resistant.

The sensitive pattern of ESBL and AmpC producing *E. coli* and *Klebsiella* sp. from blood samples, the highest susceptibility seen in colistin (100%), tigecycline (58%), followed by amikacin (53%), and imipenem (50%) (FIGURE 5). This is in accordance with the study performed by Saikumar *et al.*[24] that reported colistin, polymyxin-B and carbapenems (100%) sensitive against ESBL producing Gram negative bacteria. The resistance was very high in blood samples. It was 50% for imipenem and colistin was the highest sensitive antibiotic. Susceptibility pattern of ESBL and AmpC producing *E. coli* and *Klebsiella* sp. from sputum showed colistin 100% sensitivity, followed by tigecycline (85%), and carbapenems (80%) (FIGURE 6). However, a similar study was conducted by Malik *et al.*[25] that reported amikacin (80%) and gentamycin (80%) were highly susceptible and followed by cotrimaxazole (69%) and imipenem (55%). In sputum samples, colistin, tigecycline, and carbapenems were the highest sensitive antibiotics. These are all costly antibiotics that increase the cost of treatment so early detection of antibiotic sensitivity may prevent the incident of ESBL and AmpC in patients.

The antibiotic sensitive pattern of ESBL producing *E. coli* and *Klebsiella* sp. from other samples (i.e. swabs and tissues) showed that colistin was higher sensitive (100%), followed by tigecycline (65%), and amikacin was (56%) (FIGURE 7). A similar study conducted by Tekele *et al.*[26] showed that ESBL and AmpC producing *E. coli* and *Klebsiella* sp. were highly sensitive to amikacin (100%), followed by imipenem (98%), and meropenem (96%). Other samples included swabs such as ear swabs, eye swabs, body fluids, and tissues imipenem and meropenem showed resistance in these samples. The condition of humanity is worrisome, especially in the case of antibiotics. Every patient should be assessed for ESBL and AmpC.

Some limitation of the study was observed. No molecular testing of antibiotic resistance in isolates was done, even though it's possible that isolates possess resistant genes but do not exhibit them phenotypically. As a result, they may be able to pass on their resistance to other bacteria. Our findings are highly exciting and therapeutically important since we evaluated the susceptibility pattern of ESBL and AmpC producing *E. coli* and *Klebsiella* sp. isolated from various sources. More in depth surveys with a larger sample size, as well as collaboration with other hospitals, might result in more enticing offers.

CONCLUSION

Multidrug-resistant Gram-negative bacteria (MDR-GNB) isolated from clinical samples are increasing day by day. Extended-extended-spectrum β-lactamase and AmpC enzymes play a major role to refurnish susceptible bacteria into MDR-GNB. The easy spread of these pathogens in hospitals is becoming a major public health issue. As a result, continual screening for resistance mechanisms in nosocomial

infections is essential. Susceptibility patterns of ESBL and AmpC-producing *E. coli* and *Klebsiella* sp. from various specimens enhance hospital infection management and help doctors prescribe the most sensitive antibiotic.

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The influence of tympanic membrane perforation site on the hearing level of conductive hearing loss in chronic suppurative otitis media

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ABSTRACT

Chronic suppurative otitis media (CSOM) is an infection of the middle ear cavity both partially and totally. It is characterized by ear discharge through a tympanic membrane perforation for over a period of 2 to 6 weeks. Hearing loss is the most common complication of CSOM. One of the degrees of hearing loss in tympanic membrane perforation is depending on the site of perforation, but this premise is still debatable because of pros and contras by some researchers. This study aimed to assess the degree of hearing loss in relation to the site of tympanic membrane perforation. A cross-sectional prospective study design was performed involving 43 patients of safe type CSOM who came to the Department of Otolaryngology Head and Neck Surgery from the period January 2016 to November 2018. All subjects were divided into 4 groups based on the site of perforation. There was a perforation in the posteroinferior, the posterosuperior, the anteroinferior, and the anterosuperior. A statistical analysis using Anova along with multivariate analysis was conducted. Our result showed that the most common site of tympanic membrane perforation was at the anteroinferior (30 samples, 59.8%). The highest hearing threshold was seen at posteroinferior with a mean hearing level of 37.7±2.0 dB, anteroinferior with a mean hearing level of 31.7±0.7 dB, anterosuperior with a mean hearing level 30.7±1.4 dB, and posterosuperior mean hearing level 28.9±1.5 dB. The difference was found significant with p=0.004. Posteroinferior tympanic membrane perforation had a higher number of hearing loss compared to the other sites. In conclusion, the tympanic membrane perforation site has an important role in the hearing level of conductive hearing loss in CSOM.

ABSTRAK

Otitis media supuratif kronis (OMSK) adalah infeksi pada rongga telinga tengah baik sebagian maupun seluruhnya. OMSK ditandai dengan keluarnya cairan dari telinga melalui perforasi membran timpani selama lebih dari 2 sampai 6 minggu. Gangguan pendengaran adalah komplikasi OMSK yang paling umum. Derajat gangguan pendengaran tergantung pada lokasi perforasi membran timpani, tetapi dugaan ini masih diperdebatkan karena adanya pro dan kontra dari para peneliti. Penelitian ini bertujuan menilai derajat gangguan pendengaran dalam kaitannya dengan lokasi perforasi membran timpani. Rancangan penelitian prospektif potong lintang terhadap 43 pasien OMSK tipe aman yang datang ke poli THT-KL rawat jalan periode Januari 2016 sampai November 2018. Subjek penelitian dibagi menjadi empat kelompok berdasarkan letak perforasi yaitu posteroinferior, posterosuperior, anteroinferior, dan anterosuperior. Analisis statistik dilakukan menggunakan Anava disertai analisis perbedaan multivariat. Hasil penelitian menunjukkan bahwa tempat perforasi membran timpani yang paling umum adalah di anteroinferior (30 sampel, 59,8%). Ambang pendengaran tertinggi terlihat pada posteroinferior dengan tingkat pendengaran rata-rata 37,7±2,0 dB, anteroinferior dengan tingkat pendengaran rata-rata 31,7±0,7 dB, anterosuperior dengan tingkat pendengaran rata-rata 30,7±1,4 dB, dan tingkat pendengaran rata-rata posterosuperior 28,9±1,5 dB. Perbedaan ditemukan signifikan dengan p=0,004. Perforasi membran timpani posteroinferior memiliki jumlah gangguan pendengaran yang lebih tinggi dibandingkan dengan tempat lain. Dapat disimpulkan letak perforasi membran timpani memiliki peran penting terhadap derajat pendengaran tuli konduktif pada OMSK.

Keywords:
chronic suppurative otitis media;
tympanic membrane perforation;
site of perforation;
hearing threshold level;
hearing loss

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INTRODUCTION

Chronic suppurative otitis media (CSOM) is an infection of the middle ear cavity (eustachian tube, tympanic cavity, and mastoid air cell) characterized by ear discharge through a tympanic membrane perforation for over a period of two to six weeks.[1] It is classified into safe type (benign) and unsafe type (malignant) depending on the likelihood of coexisting cholesteatoma.[2]

In Yemen, Muftah *et al*.[3] reported that the prevalence of CSOM in school children from April 2011 to June 2011 was 51 cases with a total of 686 children. This CSOM is significantly related to hearing loss. Anggaraini *et al*.[4] investigated children whose age is 6 to 15 years old suffering CSOM in Indonesia. There are 116 children of the 7005 children studied who suffered CSOM, 30 children sustained acute otitis media, and 26 children sustained otitis media with effusion. In this study, the prevalence of CSOM was 26.4 per 1000 children in the rustic area, and in an urban area, the prevalence was 7 per 1000 children. Data from medical records at Otolaryngology Head and Neck Surgery Department Dr. Sardjito General Hospital, Yogyakarta, Indonesia between 1998-1999, there were 40 patients with CSOM malignant type, and 62.5% of them underwent a mastoidectomy procedure.[5] Another study in the Chikhwawa District in Southern Malawi, CSOM was diagnosed in 15 of 281 (5.3%) cases in children between 4 to 6 years old.[6]

Hearing loss is the most common complication of CSOM. The effect on hearing is variable. It is often mild even though both ears can be affected.[7,8] The degree of hearing loss depends on the site of perforation, however, the mechanism of sound wave transmission through tympanic membrane perforation has not yet been understood. The hearing level can be defined as a degree of hearing status measured by an audiometer that is described in decibels and is expressed by dB HL. The hearing level can be classified into seven degrees. There is normal hearing as -10 to 15 dB HL, slight hearing loss as 16 to 25 dB HL, mild hearing loss as 26 to 40 dB HL, moderate hearing loss 41 to 55 dB HL, moderately severe hearing loss as 56 to 70 dB HL, severe hearing loss as 71 to 90 dB HL, and Profound hearing loss as >90 dB HL.[9,10]

Studies on the effect of the site perforation on the hearing loss had been undertaken several times. Most authors had generally stated that the hearing loss depended on the site of perforation, but the results were found to be conflicting and inconclusive.[11] It was observed that the site of tympanic membrane perforation was influencing the degree of hearing loss. The worst hearing loss of the tympanic membrane perforation sites was at the posteroinferior quadrant site, but another study found that the degree of hearing loss did not relate to the site of perforation.[12-14]

The study of the effects of tympanic membrane perforation on the sound transmission of middle ears was required for an audiologist to determine the frequency and level of hearing loss.[15] Information obtained from the audiometry test could estimate the difference in hearing threshold values with the site of the tympanic membrane perforation, especially in CSOM patients. This study aimed to assess the degree of hearing loss in relation to the site of tympanic membrane perforation.

MATERIALS AND METHODS

Design and subjects

A cross-sectional study was conducted and the data were gathered by investigation of medical records at the medical record installation of Dr. Sardjito General Hospital, Yogyakarta, Indonesia from January 2016 to November 2018. During the period of study 43 patients (43 ears) were selected according to the inclusion criteria. The inclusion

criteria include 1) Safe type CSOM, 2) Tympanic membrane perforation was not exceeding one quadrant, and 3) audiometric examination revealed conductive hearing loss <45 dB. The exclusion criteria were 1) traumatic tympanic membrane perforation, 2) previous history of ear surgery, and 3) sensorineural hearing loss and mixed hearing loss. All patients who visited the Otolaryngology Head and Neck Surgery Department were assessed by collecting the detailed history and general ENT examination. The previous hearing condition was assumed as a normal hearing level if the patient did not feel deafness beforehand.

Procedure

The tympanic membrane was examined using rigid endoscopy with 4 mm in diameter, 4.5 cm in length, and 0^{o} angle. The patient's hearing level in decibel were determined using pure tone audiometry (Interacoustic AD226) at frequencies of 500 Hz, 1000 Hz, 2000 Hz respectively. All subjects were divided into 4 groups based on the site of perforation. The site of perforation was classified according the quadrant involved, anterosuperior (AS), anteroinferior (AI), posteroinferior (PI), and posterosuperior (PS).

Statistical analysis

Statistical analysis was performed using Anova. The results were considered to be statistically significant if the p value < 0.05. After the Anova test was carried out and the results were significant, the Post Hoc Tukey test was enforced to determine which groups had significant or insignificant differences. Ethical committee approval from the Medical and Health Research Committee (MHREC) Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada/Dr. Sardjito General Hospital, Yogyakarta was obtained with reference number: KE/FK/297/EC/2018.

RESULTS

This study involved 43 patients (43 ears) consisting 24 females (55.8%) and 19 males (44.2%). The age group of patients in this study ranged from 10 to 52 years old with a mean age of 33 years old. Unilateral involvement was more common than bilateral. Hearing loss was the most common presenting complaint. TABLE 1 describes the demographic profile of the patients.

TABLE 1. Demographic profile of the subjects.

Variable	Mean (min-max)	n (%)
Age (years)	33 (10-52)	43 (100)
Gender		
• Male		24 (55.8)
• Female		19 (44.2)
Ear perforation		
• Right side		18 (40.5)
• Left Side		24 (57.1)
• Bilateral		1 (2.4)
Presenting complaint		
• Hearing loss and tinnitus		11 (25.6)
• Hearing loss		30 (69.8)
• Tinnitus		2 (4.6)

In our study, there were 30 ears (69.8%) in the AI quadrant, 5 ears (11.6%) in the AS quadrant, 5 ears (11.6%) in the PI quadrant, and 3 ears (10%) in the PS quadrant. The distribution of the site perforation was shown in TABLE 2. It showed that the hearing loss found in the PI quadrant was more severe than in the other quadrants. Mean hearing loss in the PI group is 37.7±2.0 dB followed by the AI quadrant is 31.7±0.7 dB, AS quadrant is 30.7±1.4 dB, and PS quadrant is 28.9±1.5 dB. In the patient with bilateral tympanic membrane perforation, we just measured the right side because the hearing level of the conductive hearing loss on the contralateral side was more than 45 dB.

TABLE3 showed the significant difference between the PI quadrant versus the AI quadrant, PI quadrant, and also AS quadrant (p = 0.004) with the Post Hoc Tukey test. It was found that there was a statistically significant difference in the hearing threshold value in the PI quadrant compared to the AI quadrant (p = 0.007). The PI quadrant compared with the PS quadrant was statistically significant (p = 0.009). The PI quadrant compared with the AS quadrant was statistically significant (p = 0.019) as well. Then it was a significant difference (p = 0.004) in the degree of hearing loss comparison in each quadrant with the Anova.

TABLE 2. The site of tympanic membrane perforation and hearing loss in relation to the site of perforation

Sites of perforation	n (%)	Hearing loss level (dB)
AS	5 (11.6)	30.7 ± 1.4
PS	3 (10.0)	28.9 ± 1.5
AI	30 (69.8)	31.7 ± 0.7
PI	5 (11.6)	37.7 ± 2.0
Total	43 (100)	

TABLE 3. Hearing loss related with the site in Post Hoc Tukey test and hearing loss related with the site in Anova.

Comparison of the site of perforation	p	95% CI	
		Lower bound	Upper bound
PI – AI	0.007[a,c]	0.91	13.1
PI – PS	0.009[a,c]	1.70	15.8
PI – AS	0.019[a,c]	1.30	10.7
PI – AI – PS-AS	0.004[b,c]		

[a]: Post Hoc Tuckey test; [b]: Anova; [c]: significant difference

DISCUSSION

Based on TABLE 2, there were 30 ears (69.8%) in the AI quadrant, 5 ears (11.6%) in the AS quadrant, 5 ears (11.6%) in the PI quadrant, and 3 ears (10%) in the PS quadrant. Similar to the study conducted by Patel-Chudasama,[16] it was reported that the most common site of tympanic membrane perforation (81.4%) was the pars tensa (including AI quadrant and PI quadrant). The average hearing loss level in the PI group is 37.7±2.0 dB followed by the AI quadrant is 31.7±0.7 dB, AS quadrant is 30.7±1.4 dB, and the PS quadrant is 28.9±1.5 dB. It means that the PS perforation group has the worst hearing level. This result is also similar to other studies. Pannu *et al.*[17] reported that hearing loss of posterior perforation was worse than anterior perforation at 250 Hz. In another study conducted by Nepal *et al.*[13] also found that PI was the worst hearing loss level among AI, AS, and PS at a frequency less than 2000 Hz and 2000- 6000 Hz. In contrast with study conducted by Virk *el al.*[18] that found the average air-bone gap of PI, AI, and AS were resemblant, that was 13 dB. The average intensity of the PS group was the lowest hearing loss that was 11 dB. This study is in line with the clinical study conducted by Voss *et al.*[11] who showed no difference between perforation locations in the degree of hearing loss.

TABLE 3 showes a significant difference in hearing loss in every quadrant (p = 0,004). There was a statistically significant difference between the PI quadrant versus AI quadrant, PI quadrant, and AS quadrant ($p < 0.05$) with Post Hoc Tukey test. Patel-Chudasama *et al.*[16] reported that there was significantly difference (p=0.0001) in the mean reduction level between the PI quadrant (44.3 dB) and the anterior quadrant (26 dB). A study conducted by Alsarhan *et al.*[19] reported a statistically significant difference between the PI and AI quadrants (p = 0.039), between the PI and AS quadrants (p = 0.031) and between PI to PS quadrants (p = 0.043). It is suitable with our study that it proved the theory of the disappearance of the round window baffle effect.

The function of the tympanic membrane is not only as a conductor of sound waves from the outer ear to the middle ear but also functions as a protector, scilicet protecting the middle ear from infection and the round window from direct sound waves. This function is needed to create a phase difference so that sound waves do not hit oval and round windows at the same time. This will mitigate the flow of sound energy that is transmitted in a unilateral direction from the oval window through the perilymph. The effect of increasing the surface area ratio of the tympanic membrane to the oval window increases sound pressure by about 27 decibels while the movement of the ossicle lever contributes about 3 dB.[14]

Ali *et al.*[20] reported that a conductive hearing impairment in the tympanic membrane perforation could be resulted from two processes. There is ossicular coping that caused a pressure difference between the tympanic membrane surfaces on the inside and outside which would cause a decrease in phase between oval and round windows, and the surface of the tympanic membrane was subjected to interference with the transmission of sound waves from the external auditory canal-ossicles-cochlea. This premise is supported by Ibekwe *et al.*[14] that reported the tympanic membrane perforation would cause the formation of a surface area of the tympanic membrane to transmit sound pressure and cause the disturbance of the sound wave transmissions to the middle ear.

Mehta *et al.*[21] reported that there were several factors that influenced the hearing threshold value of the tympanic membrane perforation, that was the location of the quadrant of the

perforation, the size of the perforation and the mastoid cavity. The impact of the location of the perforation on the hearing threshold by comparing the location of the quadrant of the perforation with almost the same size of the perforation was found that the perforation in the anterior quadrant had a lower air-bone gap (1.8 dB) than the posterior quadrant, although it was not statistically significant.

A perforation in PI had more severe hearing loss than perforation in the anterior central. It is because the position of the round window is parallel to a PI quadrant of the tympanic membrane. When it happens, the sound waves that enter the middle ear will bother the rarefaction effect of the round window by their pressure, and then the hearing loss effect will be present even worse than in other sites. The location of the tympanic membrane perforation also influenced decreasing the hearing threshold. The greater the tympanic membrane perforation the smaller the effect of ocular coupling, so that the sound pressure on the oval window and round window were almost the same. This caused a decrease in the different phases between the two windows also influenced decreasing the hearing threshold.[22,23] Perforation of the tympanic membrane will cause an increase of acoustic coupling from 0-20 dB causing to loss of protective function. The increased acoustic coupling will cause conductive hearing loss of 40-50 dB.[24]

Ravi *et al.*[24] reported that in addition to the location and size of the tympanic membrane perforation, decreased hearing was also influenced by the air resonance of the mastoid bones. The smaller the volume results, the larger the air-bone gap. The sound pressure produced in the ear cavity is inversely proportional to the volume of the middle ear. In the tympanic perforation membrane with a smaller volume of the middle ear air cavity, it will produce a greater hearing threshold value. A study conducted by Voss *et al.*[11] reported that perforations of the same size in two different ears could have different conductive hearing loss thresholds of 20-30 dB when the volume of the middle ear air cavity was different. In normal ears, the volume of the air cavity of the middle ear can vary from 2 cm^3 to 20 cm^3.

CONCLUSION

The tympanic membrane perforation site has an important role in the hearing level of conductive hearing loss in CSOM.

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Characteristics of patients with Hepatitis B and C at Dr. Moewardi General Hospital in Surakarta, Indonesia

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ABSTRACT

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections remain a significant health burden in the world, which is mainly attributed to patients who develop chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). The epidemiology of hepatitis B and C in Surakarta, Central Java Province, Indonesia has never been reported. This study aimed to investigate the demographic, clinical, and laboratory characteristics of patients with hepatitis B and C who were admitted to Dr. Moewardi General Hospital, Surakarta in 2019. The medical records of patients with hepatitis B (n=94) and hepatitis C (n=75) were examined, and the data were analyzed using the chi-square and Mann-Whitney tests. The patients with hepatitis C were generally older, more likely to develop jaundice and ascites, and had higher levels of serum urea, creatinine, AST, and total bilirubin compared to those with hepatitis B. In conclusion, patients with HCV infection had worse clinical presentation and laboratory profiles than those with HBV infection. However, further research is needed on a wider scale to confirm this result.

ABSTRAK

Infeksi virus hepatitis B (HBV) dan C (HCV) masih merupakan beban kesehatan yang signifikan bagi dunia terutama pada pasien yang penyakitnya berkembang menjadi hepatitis kronis, sirosis hati, dan karsinoma hepatoseluler (HCC). Epidemiologi hepatitis B dan C di Surakarta, Provinsi Jawa Tengah, Indonesia belum pernah dilaporkan sebelumnya. Penelitian ini bertujuan untuk menganalisis karakteristik demografrik, klinik, dan laboratorik dari pasien hepatitis B dan C yang dirawat inap di RS Dr. Moewardi pada tahun 2019. Data diperoleh dari rekam medis pasien dengan hepatitis B (n=94) dan hepatitis C (n=75) dan dianalisis menggunakan uji *chi-square* dan uji Mann Whitney. Pada pasien dengan hepatitis C ditemukan rerata usia yang lebih tua, jumlah kasus dengan ikterik dan ascites yang lebih banyak, serta kadar ureum, kreatinin, SGOT dan bilirubin total di serum yang lebih tinggi dibandingkan pada pasien dengan hepatitis B. Berdasarkan hasil penelitian tersebut, dapat disimpulkan bahwa infeksi HCV berhubungan dengan derajat penyakit yang lebih berat dibandingkan infeksi HBV. Namun demikian, perlu penelitian lebih lanjut dalam skala yang lebih luas untuk mengkonfirmasi hasil ini.

Keywords:
hepatitis B;
hepatitis C;
HBV;
HCV;
epidemiology

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INTRODUCTION

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are common in the world. It is estimated that there are 2 billion people have been infected by HBV and currently 257 million people live with chronic HBV infection.[1] Meanwhile, there are 71 million people infected with HCV in the world and 2.3 million people of them are co-infected with HIV.[2] Altogether, hepatitis B and C had caused 1.2 million deaths as a result of acute and chronic infections and their complications.[3]

The prevalence of HBV is classified as moderate to high in Indonesia, ranging from 2.5% to 10% of the general population, depending on which region of the country.[4] In Central Java Province, for instance, the prevalence of HBV infection is moderate; i.e., 6-7% of the population. Fortunately, Indonesia has a fairly low prevalence rate of anti HCV antibodies; i.e., around 0.8%. Thus, it can be estimated that the prevalence of HCV infection is much lower than that of HBV.[5]

Hepatitis B and C are a global health burden since these diseases may develop into more severe liver diseases, such as chronic hepatitis, cirrhosis of the liver, hepatocellular carcinoma (HCC), and liver failure. Each spectrum of the disease has its characteristics that can serve to assess disease progression.[6]

During the development of HBV and HCV infection, many metabolic processes are disturbed and these can be detected in the physical examination as well as the results of laboratory investigation. The clinical manifestations of the disease that are associated with impaired liver function include jaundice of the skin and sclera, dark urine, and ascites. A blood test is often necessary to evaluate the disease progression by monitoring the increased levels of serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT).[7] The differences in clinical manifestations as well as laboratory profiles among patients with hepatitis B and hepatitis C may provide important information for monitoring disease progression as well as predicting the outcomes. Some knowledge of the local disease burden is important to develop an effective strategy for infection control and management of hepatitis B and C. This study aimed to describe the epidemiology of hepatitis B and hepatitis C in Surakarta, Central Java Province, Indonesia. Furthermore, the clinical and laboratory characteristics of the patients would be further evaluated.

MATERIALS AND METHODS

Design and subjects

It was an analytic observational study with a cross-sectional design, conducted from January to March 2020. Data were collected from patients who had the diagnosis of hepatitis B or hepatitis C and were admitted to Dr. Moewardi Hospital, Surakarta in 2019. Dr. Moewardi General Hospital is the main referral hospital in Central Java Province, Indonesia. The hospital is well equipped with a clinic of gastroenterohepatology and endoscopy facilities. The diagnosis of hepatitis B was confirmed by HBsAg serology whereas hepatitis C was confirmed by the presence of anti-HCV antibody and HCV RNA.

Procedure

As the initial search of the records, we employed the international classification of diseases 10 (ICD-10) Verison 2019 and the search codes were B18.1 for “chronic viral hepatitis B without delta-agent” and B18.2 “for chronic viral hepatitis C”. After the medical records were collected, the records of patients who had comorbidities that could affect liver function were excluded; such as those with a history of alcohol consumption and those taking hepatotoxic drugs. Records with incomplete data were also excluded as well as those with

HBV and HCV coinfection. The search enabled researchers to identify patients with HBV or HCV-associated cirrhosis and hepatocellular carcinoma and automatically excluded hepatitis D infection. The data collected include demographic characteristics (i.e., patients' age, gender, and address), the presence of clinical manifestations (i.e., jaundice, nausea, vomiting, abdominal pain, ascites, dark urine, and oedema), and the results of laboratory investigation (i.e., the levels of serum SGPT, SGOT, urea, creatinine, and total bilirubin.

Statistical analysis

Data were analyzed using the IBM SPSS software version 22.0 (IBM Corp., Armonk, NY, USA). The differences between groups were analyzed by using the chi-square test and the Mann-Whitney test and the results were considered statistically significant if the p-value is less than 0.05 ($p<0.05$).

RESULTS

A total of 94 cases of hepatitis B and 75 cases of hepatitis C who were treated at Dr. Moewardi Hospital during the period of 2019 were evaluated. TABLE 1 shows that on average, patients with hepatitis C were older than those with hepatitis B. Patients with hepatitis B were mostly from Karanganyar district whilst hepatitis C cases were more prevalent in Surakarta district. Both hepatitis B and C sufferers were male predominant, accounting for around two-thirds of the total cases. TABLE 2 shows that patients with hepatitis C had a higher incidence of jaundice, dark urine, and ascites compared to those with hepatitis B.

TABLE 1. Demographic characteristics of patients with Hepatitis B (n=94) and C (n=75) at Dr. Moewardi General Hospital

Variable	Hepatitis B [n (%)]	Hepatitis C [n (%)]	p
Age (mean±SD years)	49.79±13.30	58.91±14.05	0.000*
• 21-30	8 (8.5)	2 (2.7)	
• 31-40	12 (12.8)	9 (12.0)	
• 41-50	27 (28.7)	7 (9.3)	
• 51-60	31 (33.0)	22 (29.3)	
• 61-70	8 (8.5)	16 (21.3)	
• >70	8 (8.5)	19 (25.3)	
Address			
• Surakarta	12 (12.8)	22 (29.3)	
• Sragen	19 (20.2)	8 (10.7)	
• Karanganyar	25 (26.6)	12 (16.0)	
• Sukoharjo	13 (13.8)	13 (17.3)	
• Wonogiri	4 (4.3)	6 (8.0)	0.79
• Boyolali	7 (7.4)	6 (8.0)	
• Klaten	1 (1.1)	2 (2.7)	
• Yogyakarta	2 (2.1)	2 (2.7)	
• East Java	11 (11.7)	4 (5.3)	
Sex			
• Male	62 (66.0)	49 (65.3)	0.932
• Female	32 (34.0)	26 (34.7)	

*Statistically significant ($p<0.05$)

TABLE 2. Clinical characteristics of patients with Hepatitis B (n=94) and C (n=75) at Dr. Moewardi General Hospital

Variable	Hepatitis B [n (%)]	Hepatitis C [n (%)]	p
Jaundice	25 (26.6)	34 (45.3)	0.011*
Nausea	33 (35.1)	37 (49.3)	0.062
Vomiting	33 (35.1)	22 (29.3)	0.426
Abdominal pain	54 (57.4)	34 (45.3)	0.117
Ascites	18 (19.1)	26 (34.7)	0.022*
Dark urine	2 (2.1)	10 (13.3)	0.005*
Oedema	4 (4.3)	4 (5.3)	0.743

*Statistically significant (p <0.05).

The results of laboratory investigations were shown in TABLE 3. Patients with hepatitis C had significantly higher levels of serum urea, creatinine, SGOT, and total bilirubin. In contrast, the levels of hemoglobin, hematocrit, thrombocyte, and erythrocyte in hepatitis B cases were significantly higher than that in hepatitis C.

TABLE 3. Laboratory characteristics of patients with Hepatitis B (n=94) and C (n=75) at Dr. Moewardi General Hospital

Variabel	Hepatitis B	Hepatitis C	p
Urea (mg/dL)	40.84±46.41	71.36±74.82	0.000*
Creatinine (U/L)	1.49±3.07	2.34±4.18	0.023*
SGPT (U/L)	64.35±84.43	60.49±59.46	0.499
SGOT (U/L)	86.03±101.55	115.33±118.95	0.036*
De Ritis Ratio	1.67±1.29	1.98±1.21	0.006*
• <1	25	8	0.009*
• >1	69	67	
Total bilirubin (mg/dL)	2.45±4.77	5.53±10.25	0.001*
Hemoglobin (g/dL)	13.47±15.98	10.10±2.51	0.000*
Hematocrit (%)	35.92±8.52	30.47±8.21	0.000*
Leucocyte (10^3/uL)	7.59±5.56	8.69±7.62	0.528
Thrombocyte (10^3/uL)	193.96±99.78	168.99±127.87	0.009*
Erythrocyte (million/uL)	4.10±1.02	3.42±0.86	0.000*

*Statistically significant (p <0.05)

We calculated the De Ritis ratio; i.e., the ratio between the serum levels of SGPT and SGOT (SGPT/SGOT). Most patients in this study had the De Ritis ratio with a value of >1, indicating chronic infection that leads to cirrhosis.[8] We found that hepatitis C patients had a significantly higher mean of De Ritis ratio than those with hepatitis B.

DISCUSSION

HBV and HCV infection may develop into a chronic infection and severe

complication such as liver cirrhosis and HCC.[5,6] The clinical manifestations of hepatitis B and C can be different depending on the phase of the disease and the viral respective characteristics.[7] In this study, we analyzed demographic, clinical, and laboratory characteristics of patients who had been admitted to a tertiary hospital in Indonesia with hepatitis B and C, irrespective of the stage of the disease.

We found that the sufferers of hepatitis B and hepatitis C were male predominant. This phenomenon is likely due to hormonal differences between males and females. It is important to note that the liver is a sexually dimorphic organ that expresses androgen and estrogen receptors. In hepatitis B, androgen can increase serum HBsAg levels whereas an increase in estrogen will reduce serum HBV DNA levels. Hepatitis B viral protein, the HBx protein, can increase the activity of androgen receptors, causing the development of more progressive HBV in males.[9] In hepatitis C, a previous study has reported that women tend to have a spontaneous clearance of HCV.[10]

Patients with hepatitis C are on average older than those with hepatitis B. This may be attributed to two factors: the virus' mode of transmission and the pathogenesis of the disease. Most HCV infections occur in adults, mainly acquired during blood transfusions or associated with risky lifestyles. In contrast, HBV infection is mostly transmitted vertically so that infection is initiated at an early ages.[11]

Pathogenically, there are differences in how HBV and HCV infection develop into HCC. HBV infection can develop into HCC in three ways: chronic necroinflammation, combining HBV DNA with hepatocyte DNA, and the influence of HBV proteins, such as HBx.[12] Chronic inflammation of hepatitis causes a continuous cycle of necrosis-inflammation-regeneration. This continuous proliferation of hepatocytes is likely to cause epigenetic changes, oncogenic mutations, and telomere shortening. The integration of viral DNA with hepatocyte DNA causes changes in the genetic structure of the host DNA thereby increasing the hepatocarcinogenesis process. HBx protein can alter the expression of growth-stimulating genes. This process causes an acceleration of tumor formation. HBx can accelerate tumor formation by promoting the proliferation of "altered cells". Furthermore, HBx acts as a cofactor in the hepatocarcinogenesis process.[13] In contrast with HBV, the development of HCC in HCV infection is mainly due to chronic inflammation. Moreover, HCV is an RNA virus so there is no merger between the virus and the host genome. This condition causes a longer process in developing HCC in HCV infection.[14,15]

The study of clinical characteristics shows that there are significant differences in the prevalence of ascites, dark urine, and jaundice among hepatitis B and C cases. It has been known that ascites can appear only in chronic hepatitis B and C while dark urine and jaundice usually appear in both acute and chronic hepatitis. Ascites develops as a result of portal hypertension in patients with cirrhosis of the liver whilst dark urine and jaundice are caused by hyperbilirubinemia.[16]

HBV and HCV infection causes damage to the hepatocytes which results in disruption of bilirubin excretion. Disruption excretion of bilirubin leads to retention of bilirubin in the blood resulting in jaundice. The conjugated bilirubin in the blood is finally excreted through urine causing dark-colored urine.[17] Our study found that patients with hepatitis C had a higher level of total bilirubin in their serum compared with those who had hepatitis B (p = 0.017).

Levels of serum urea and creatinine are indicators of kidney health and increased levels of urea and creatinine

are associated with kidney damage.[18] In this study, we found that the levels of serum urea and creatinine in hepatitis C were higher than that in hepatitis B (p <0.05), indicating a worse renal dysfunction in hepatitis C. Liver cirrhosis, which is more common in hepatitis C, can facilitate kidney damage due to portal hypertension.[19] In addition, chronic hepatitis C triggers an immune reaction that attacks the kidneys resulting in glomerulonephritis.[20] Treatment of hepatitis C may also decrease kidney function. Sofosbuvir is thought to have a nephrotoxic effect on the kidneys, although this opinion is still being debated.[21] Nevertheless, patients with kidney disease requiring hemodialysis can be the source of HBV and HCV transmission.[22] A previous study conducted in Yogyakarta, Indonesia found that the prevalence of HBV and HCV in hemodialysis patients was 24.2% and 83.2%, respectively. [23]

The study found that the levels of serum SGPT and SGOT, as well as total bilirubin, were higher in hepatitis C than those in hepatitis B, confirming the results of previous studies.[24,25] In particular, a significant increase in SGOT levels is associated with a higher degree of liver inflammation and damage among patients with hepatitis C. However, the exact mechanism by which HCV causes more damage to the liver is not known with certainty. On average, our study participants had the De Ritis ratio values of >1. An increase in the De Ritis ratio value of >1 is associated with the progression of fibrosis to cirrhosis although this assessment has poor sensitivity.[8] A combination of De Ritis ratio values with the results of a platelet count of <150.000/mm3 and prothrombin time test provides a better sensitivity in predicting the development of chirrosis.[26]

This study implies that public health measures should be taken to reduce the prevalence of hepatitis B and hepatitis C in Surakarta as well as to prevent further transmission of HBV and HCV in the population. In clinical practice, close monitoring should be carefully and regularly performed particularly for patients with HCV infection as it can rapidly deteriorate patients' health due to the disease itself and the medication. The results of liver and renal function tests can serve as key indicators in monitoring disease progression and evaluating therapy. In more-advanced laboratory settings, molecular studies should be performed to identify high-circulating viral genotypes in the community as well as genetic polymorphism of the patients. Such studies are important for portraying HBV and HCV molecular epidemiology as well as analyzing the complex correlation between the agent, host, disease, and therapeutic responses.

CONCLUSION

The patients with HCV infection are more often presenting with worse disease progression than those with HBV infection. Further research is needed on a broader scale to confirm this phenomenon.

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Correlation of neutrophil ratio to lymphocyte levels before therapy with the incidence of metastasis, lymph node involvements, in urothelial type muscle invasive bladder cancer in Indonesia

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ABSTRACT

Bladder cancer is cancer originated from the bladder mucosa or urothelium. Bladder cancer is the 9th most common malignancy worldwide and the most common malignancy of the urinary tract. Studies show that cancer triggers an inflammatory response, which causes changes in circulating inflammatory cells. Examination of neutrophils and lymphocytes is an inexpensive examination, reproducible, and easily obtained. Neutrophil to lymphocyte ratio (NLR) values have been used in several studies to evaluate the inflammatory response that occurs in tumors. In urology, the importance of NLR has been recognized in predicting progression and aggressiveness in urothelial bladder tumors, kidney cancer (RCC/renal cell carcinoma), and upper tract urothelial carcinoma (UTUC). This study was a cross-sectional study obtained retrospectively by evaluating the medical records of patients diagnosed with muscle-invasive bladder cancer (MIBC) at Dr. Sardjito General Hospital, Yogyakarta, Indonesia from January 2017 to December 2019. The NLR data were categorized into NLR < 2.5 and > 2.5. As much as 150 patients with bladder cancer were included in this study, with a mean age of 56.43 ± 13.60 years. In the comparison of NLR values and the incidence of metastasis, there were 15 people (20%) with NLR values < 2.5 who had metastasis while 32 people (42.7%) from the group with NLR > 2.5 had metastasis (p = 0.003). In the comparison of NLR values and nodule involvement, there were 25 (33.3%) patients with NLR < 2.5 and 39 (52%) patients with NLR > 2.5 (p = 0.021). This study showed that patients with metastatic bladder tumors and lymph node involvement had a significantly higher NLR value. It can be concluded the NLR value can be used to predict the metastatic level and lymph node involvement in patients with bladder tumors. Even though it is not a specific marker of inflammation, the NLR examination is simple, affordable, easy to obtain, and widely available.

ABSTRAK

Kanker kandung kemih adalah kanker yang berasal dari mukosa kandung kemih atau urothelium. Kanker kandung kemih adalah jenis keganasan urutan kesembilan yang paling umum terjadi di seluruh dunia dan merupakan jenis keganasan yang paling umum terjadi pada traktus urinarius. Penelitian menunjukkan bahwa kanker memicu proses inflamasi yang menyebabkan perubahan pada sirkulasi sel inflamasi. Pemeriksaan neutrofil dan limfosit merupakan pemeriksaan yang murah, reprodusibel dan mudah dilakukan. Nilai *neutrophil to lymphocyte ratio* (NLR) telah digunakan untuk melihat respon inflamasi yang terjadi pada tumor. Dalam urologi, NLR telah digunakan untuk memprediksi perkembangan dan agresivitas pada tumor kandung kemih urothelial, kanker ginjal (RCC/*renal cell carcinoma*) dan *upper tract urothelial carcinoma* (UTUC). Penelitian ini merupakan penelitian retrospektif menggunakan data rekam medis pasien yang didiagnosis kanker otot kandung kemih invasif (KKKIO) di RSUP Dr. Sarjito, Yogyakarta, Indonesia dari Januari 2017 hingga Desember 2019. Data NLR dikategorikan menjadi NLR < 2,5 dan > 2,5. Populasi pada penelitian ini yaitu 150 pasien dengan kanker kandung kemih, dengan usia rata-rata 56,43 ± 13,60 tahun. Perbandingan nilai NLR dengan kejadian metastasis yaitu terdapat 15 pasien (20%) dengan nilai NLR < 2,5 yang mengalami metastasis, sedangkan sebanyak 32 pasien (42,7%) dengan nilai NLR > 2,5 mengalami metastasis (0,003). Perbandingan nilai NLR dengan keterlibatan nodul, terdapat 25 pasien (33,3%) dengan NLR < 2,5 dan 39 pasien (52%) dengan NLR > 2,5 (0,021). Hasil penelitian menunjukkan bahwa pasien dengan tumor kandung kemih dengan metastasis dan ada keterlibatan kelenjar getah bening secara bermakna memiliki nilai NLR lebih tinggi. Dapat disimpulkan bahwa nilai NLR dapat digunakan untuk memprediksi tingkat metastasis dan keterlibatan kelenjar getah bening pada pasien dengan tumor kandung kemih. Meskipun bukan penanda inflamasi yang spesifik, pemeriksaan NLR merupakan pemeriksaan yang sederhana, terjangkau, mudah diperoleh dan tersedia secara luas.

Keywords:
NLR;
bladder cancer;
metastasis;
marker;
renal cell carcinoma;
inflammatory cells

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INTRODUCTION

Bladder cancer is cancer that originates from the bladder mucosa or urothelium.[1] Bladder cancer is the ninth most common malignancy worldwide and the most common malignancy of the urinary tract.[2] Urothelial bladder cancer has a high aggressiveness. To determine the appropriate management of patients with bladder cancer, accurate perioperative risk stratification is necessary.

Studies showed that cancer triggers an inflammatory response, which causes changes in circulating inflammatory cells. Tumor tissue in addition to inducing a systemic inflammatory response also causes a local inflammatory response due to disruption and destruction of tumors in the surrounding tissue.[3,4] Inflammation that occurs has an important role in the emergence and progression of tumors.

Currently, many systemic inflammatory mediators are investigated to evaluate the inflammatory response on tumors patients. Many inflammatory mediators are used as biomarkers to predict the prognosis of cancer, but most of these biomarkers are relatively expensive.[5,6] Examination of neutrophils and lymphocytes is an examination that is inexpensive, reproducible, and easily obtained examination.[4] Furthermore, meta analytic studies concerning the correlation between the neutrophil to lymphocyte ratio (NLR) and metastatic bladder cancer has been conducted in China, Australia, Korea, Japan and Canada.[7] However, the similar study on Indonesia population has not been conducted, yet. We hope our studies could shine a light to the Indonesian population's characteristic of NLR value to metastatic bladder cancer.

The NLR is obtained by dividing the absolute neutrophil level by the absolute lymphocyte level.[8] The NLR value has been used in several studies to assess the inflammatory response that occurs in tumors. Higher NLR values are associated with a poorer prognosis in bronchoalveolar carcinoma, melanoma, squamous cell carcinoma of the head and neck, as well as in kidney cancer. In urology, the importance of NLR has been recognized in predicting progression and aggressiveness in urothelial bladder tumors, kidney cancer (RCC/renal cell carcinoma), and upper tract urothelial carcinoma (UTUC).[9-12]

Previous studies have shown that NLR can be used as a biomarker for prognostic assessment, also can be used to help clinicians and patients in making decisions regarding their tailored treatment options for muscle invasive bladder cancer (MIBC).[13] Other studies also suggest that the use of NLR in predicting disease aggressiveness, the outcome of the disease oncology, and the response in the treatment of urothelial carcinoma management. Even though there were limitations of the study, such as the inter-study heterogenicity, bias of the publication possibility, the restricted number of studies, also there are no previous randomized controlled studies.[14]

This study aimed to investigate the correlation between NLR levels with the incidence of metastasis, lymph node involvement, in bladder cancer. The result of this study is expected to be a predictor of prognosis in patients with bladder cancer.

MATERIALS AND METHODS

Design of study

It was a cross-sectional study obtained retrospectively by reviewing the medical records of patients diagnosed with MIBC at Dr. Sardjito General

Hospital, Yogyakarta, Indonesia from January 2017 to December 2019. One hundred and fifty patients with MIBC were included in this study.

Procedure

Patients with incomplete medical record data and modalities were excluded. NLR data were categorized into NLR < 2.5 and > 2.5. Each patient and/or relative in charge of the research subject was given an explanation of the objectives, working methods, benefits, and risks of the research. If the prospective research subject or and/or relatives in charge understand and agree to participate in the research, they were asked to sign a written research agreement. NLR, demographic, clinical and pathological data including tumor staging, nodule staging, and metastasis were collected. Protocol of the study has been approve by the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing/Dr. Sardjito General Hospital, Yogyakarta (ref. no. KE/FK/0524/EC/2021).

Statistical analysis

The data obtained were validated, coded, recapitulated, and tabulated.. The existing data were entered into a computer using the SPSS program for further statistical analysis. The relationship between the incidence of metastasis, lymph node involvement, and neutrophil-lymphocyte levels were analyzed by Chi-Square test or Fisher's Test, with p value < 0.05 was considered to be statistically significant.

RESULTS

Among 150 patients involving in this study, 118 were male (78.1%) while 32 were female (21.2%) with the mean age of patients was 56.43 ± 13.60 years. A total of 76 patients (50.7%) had tumors of the T2 category, while 51 (34%) had tumors of the T3 category, and 23 (15.3%) had tumors of the T4 category. Metastatic nodules were found in 64 patients (42.7%), while metastasis were found in 47 patients (31.3%). The mean absolute neutrophil level in the patients was 5.46 ± 6.50 with a range of values from 1.2 to 14.2. The mean absolute lymphocyte level in patients was 2.3 ± 11.50 with a range of values from 0.6 to 23.7. The characteristics of patients are shown in TABLE 1.

In the comparison of NLR values and the incidence of metastasis, there were 15 people (20%) with NLR values less than 2.5 who had metastasis while 32 people (42.7%) from the group with values above 2.5 had metastasis. A statistically significant relationship between NLR values and the incidence of metastasis in bladder cancer patients was observed (p=0.003) as shown in TABLE 2.

In the comparison of NLR values and nodule involvement, 25 (33.3%) patients with NLR < 2.5 had nodule involvement (N+), while 39 (52%) patients with NLR > 2.5 had nodule involvement (N+). A statistically significant relationship between NLR values and nodule involvement in bladder cancer patients was observed (p=0.021) as shown in TABLE 3.

TABLE 1. Sample characteristic

Variable	n (%)	Mean (SD)
Age (years)		56.43 ± 13.60
Gender		
• Male	118 (78.1%)	
• Female	32 (21.2%)	
Tumor staging		
• T2	76 (50.7%)	
• T3	51 (34%)	
• T4	23 (15.3%)	
Nodule staging		
• N1-3	86 (57.3%)	
• N0	64 (42,7%)	
Metastasis		
• M1	47 (31.3%)	
• M0	103 (68.7%)	
Neutrophil		5.46 ± 6.50
Lymphocyte		2.30 ± 11.50

TABLE 2. Univariate analysis of NLR

Variable	NLR		p
	< 2.5	> 2.5	
Metastasis [n (%)]			
• M1	15 (20.0)	32 (42.7)	0.003
• M0	60 (80.0)	43 (57.3)	
Nodule [n (%)]			
• N1-N3	25 (33.3)	39 (52.0)	0.021
• N0	50 (66.7)	36 (48.0)	

TABLE 3. Multivariate analysis logistic regression

Variable	Coefficient	S.E	Wald	df	p	OR	95% CI	
							Lower	Upper
Metastasis	1.051	0.371	8.029	1	0.005	2.86	1.38	5.91
Nodule	-0.076	0.539	0.020	1	0.888	0.92	0.32	2.66

DISCUSSION

Neutrophil to lymphocyte ratio can be used as an independent predictor that provides prognostic value, such as disease-free survival (DFS) and progression-free survival (PFS) in malignant tumors.[15] The NLR can be used as an independent prognostic factor in patients with bladder cancer undergoing

radical cystectomy.[16] The NLR is an important and useful parameter for predicting locally advanced organ-limited stage in MIBC so it needs to be part of clinical staging before radical cystectomy is performed.[17] Several studies reported that preoperative patients with elevated NLR can be up-staged and may benefit from neoadjuvant chemotherapy.[18-20] Recently, there is no standard cut-off value that is used as a standard in assessing NLR. Some studies use the NLR cut-off value ranging from 2-5.[21] In this study we used a cut-off value of 2.5.[4,22-24]

Patients with a higher NLR in bladder cancer are associated with a higher risk of developing bladder cancer with higher rates of cancer aggressiveness and more advanced disease. Higher disease stage, lymph node involvement, more number metastasis were found to have higher NLR.[18,19,21,25-28]

This study showed that there was a statistically significant difference where patients with metastatic bladder tumors (p=0.003), lymph node involvement (p=0.021), had higher NLR values. A previous study reported that patients with NLR 2.38 (p = 0.007) and metastatic lymph nodes (p = 0.030) have a high mortality risk.[16] In addition, a meta-analysis conducted by Gu *et al.*[22] regarding the relationship between NLR values and overall survival (OS) involving 9 studies with 2,300 patients showed that high NLR values are associated with lower OS (p=0.027). In line with the results of this study, which also showed a correlation of NLR values with the incidence of metastasis and lymph node involvement.

The hypothesized mechanism of NLR in relation to cancer is through the increase of growth factors, survival factors, pro-angiogenic factors, enzymes from the extracellular matrix, and induction of signals that cause epithelial to mesenchymal transition.[19,30-32] Patients with high NLR have relatively lower lymphocyte counts and higher neutrophil counts. This shows that the immune response by T lymphocytes against malignancy is not good where there is a decrease in cytotoxicity by T lymphocytes against malignant cells which ultimately causes tumor development. An increase of neutrophil count is associated with an increase in vascular endothelial growth factor (VEGF) which plays a role in tumor progression and angiogenesis.[6,18,22,33-37]

Inflammation has a role at every stage of tumor development starting from the initiation, promotion, malignant transformation, invasion, and metastasis phases.[38] Chemokines and cytokines produced by inflammatory cells as a result of interactions between tumor cells and immune cells have a role in tumor development by a way of regulating the growth, migration, and differentiation of all tumor cell types including neoplasms, fibroblasts, and endothelial cells.[39] Cancer cells will stimulate monocytes and neutrophils through myeloid growth factor and other pro-inflammatory mediators to secrete interleukin-6 (IL-6), VEGF which will stimulate tumor neovascularization, and transforming growth factor beta (TGF-ß) which will cause immunosuppression by inducing lymphocyte apoptosis and causing decreased lymphopoiesis.[4,8,15-17,40]

Some limitations in this study was identified. First, the data collected were obtained from a single clinical source, which is in Dr. Sardjito General Hospital,Yogyakarta. The data were limited from its confounding factors that will affect neutrophil to lymphocyte ratio, and factors that will affect metastasis in MIBC, such as the presence of other illnesses like congestive heart failure (CHF), atrial fibrillation (AF), anemia, or the presence of both high PTH, or low vitamin D.[41] Furthermore, this study also did not consider other confounding factors such as previous chronic bladder irritation and infections, personal history of bladder or urothelial

cancer, bladder birth defects, genetics, and family history, previous history of smoking, workplace exposures, and any history of chemotherapy or radiation therapy.[42,43]

Neutrophils affect the migration of cancer cells which ultimately play a role in the process of metastasis. Tumors induce neutrophil activation to release inflammatory mediators that promote malignant cell metastasis. Neutrophils residing in tumor cells (TANs/tumor-associated neutrophils) release enzymes that degrade the basement membrane and cause invasion of malignant cells across the basement membrane. The tumor cells then circulate. In the circulation, neutrophils play a role in helping tumor cells survive by inducing tumor cell aggregation. Circulating tumor cells adhere to the vascular endothelium and then cause tumor cell extravasation which ultimately plays a role in metastasis.

CONCLUSION

Neutrophil to lymphocyte ratio is a non-specific marker of inflammation that is simple, inexpensive, easy to obtain, and easy to calculate from peripheral blood tests. It can be used to predict the aggressiveness and extent of tumor invasion in patients diagnosed with MIBC.

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Cytologic diagnostic approach of pleuropulmonary blastoma: a case report

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ABSTRACT

Pleuropulmonary blastoma (PPB) is a very rare pediatric lung tumor that arises in the pulmonary parenchyma, mediastinum, and pleura. The tumor has rapid disease progression and therefore the prognosis is remarkably poor. We reported a 4-year-old girl who complained of high fever and shortness of breath for the past 8 weeks. The patient was referred from the previous hospital with a pulmonary mass. CT scan of the chest with contrast showed a solid cystic mass with necrotic areas in the 1st, 2nd, and 3rd segments of the left lung with sized 4.8 x 8.1 x 6.6 cm^3. As the tumor mass was inoperable, an ultrasound-guided fine-needle aspiration biopsy (FNAB) was conducted to diagnose the pulmonary lesion. We concluded that the lung tumor was a PPB based on FNAB cytology and immunocytochemistry staining. The histopathology feature of PPB appeared similar to fetal lung tissue. Cytologic features obtained from fine-needle aspiration cytology smears and cell blocks followed by immunocytochemistry assay could provide a proper and accurate diagnosis in an inoperable surgical pathology case.

ABSTRAK

Pleuropulmonary blastoma (PPB) merupakan jenis tumor paru yang sangat langka pada anak yang berkembang pada parenkim paru, mediastinum, dan pleura. Tumor jenis ini berkembang dengan cepat sehingga prognosisnya sangat buruk. Kami melaporkan pasien anak perempuan berusia 4 tahun yang datang dengan keluhan demam tinggi dan sesak napas selama 8 minggu terakhir. Pasien dirujuk dari rumah sakit sebelumnya dengan adanya suatu massa pada paru. Hasil CT scan dada dengan kontras menunjukkan adanya suatu massa kistik padat dengan area nekrotik pada segmen 1, 2, 3 paru kiri dengan ukuran 4,8 x 8,1 x 6,6 cm^3. Karena massa tumor tidak dapat dioperasi, maka biopsi aspirasi jarum halus (AJH) dengan panduan ultrasonografi dilakukan untuk penegakan diagnosis lesi paru. Dari hasil pemeriksaan sitologi AJH yang dilanjutkan dengan pemeriksaan imunositokimia, kami berkesimpulan bahwa tumor paru tersebut adalah PPB. Gambaran histopatologi PPB memiliki kemiripan dengan histologi jaringan paru janin. Gambaran sitologi yang diperoleh dari apusan sitologi AJH dan blok sel yang diikuti dengan pemeriksaan imunositokimia dapat membantu penegakan diagnosis yang tepat dan akurat pada kasus patologi bedah yang tidak dapat dioperasi.

Keywords:
pleuropulmonary blastoma;
pediatric tumor;
cytology;
pulmonary parenchyma;
mediastinum

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INTRODUCTION

Pleuropulmonary blastoma (PPB) is an infrequent type of pediatric tumor originating in the lungs and usually presenting in early childhood with the majority of cases diagnosed in children less than 6 years of age.[1] There are only about 300 reported cases of PPB worldwide.[2] Confirming the diagnosis of PPB is important not only for the patient but also for close family members since the incidence of hereditary PPB and other malignancy in patients with PPB and their young close relatives are about 25%.[3,4] PPB is commonly associated with cystic malformations of the lung and is classified into three different subtypes, extending from type I (entirely cystic), type II (mixed cystic and solid), and type III (entirely solid), based on the histopathological features.[5] Multimodal therapy has increased the survival rates and approximately >90% of pediatric patients with type I, >70% with type II, and >50% with type III PPB can be fully recovered.[5,6] PPB has been well described in histological studies but only a few literatures describe its cytological features. We described a case report of PPB in a 4-year-old girl in addition to cytological, immunocytochemical, and radiological findings.

CASE

A 4-year-old girl suffered from high-grade fever and dyspnea for the past 8 weeks before hospital admission. The patient was initially diagnosed with an upper respiratory tract infection and treated with antipyretic and antibiotic drugs for 2 weeks at the previous hospital, but the complaints did not improve with the therapy. The patient was referred to our hospital with a pulmonary mass as the diagnosis. When came to our hospital the patient, she was suffering from a fever and shortness of breath. Results of a physical examination showed signs of tachypnea (38 breaths per min), tachycardia (140 beats per min), microcephaly, hypertelorism, bilateral exophthalmos, bilateral proptosis, conjunctival anemia, maxillary hypoplasia, mandibular prognathism, decreased vesicular breath sound on the left lung, and hepatomegaly. Laboratory examination revealed anemia, leukocytosis, neutrophilia, and lymphopenia. CT scan of the chest revealed a solid cystic mass with necrotic areas in the 1st, 2nd, and 3rd segments of the left lung (FIGURE 1). The initial diagnosis of this patient was a left pulmonary mass with suspected Pfeiffer syndrome.

Based on clinical decision-making by the clinician, radiologist, and pathologist, the tumor mass was inoperable and therefore an ultrasound-guided fine-needle aspiration biopsy (FNAB) was planned for further investigation. The patient was treated with paracetamol if she had a fever during the diagnosis process. Microscopic cytology and cell block analyses showed that the tumor cells were polymorphic, medium to large in size, and some cells had scanty cytoplasm, displacing the nuclei eccentrically, whereas it was relatively abundant in other cells. The nuclei were round, oval, or spindle-shaped with irregular membranes and distinct nucleoli (FIGURE 2). Immunocytochemistry assay showed positive expression of vimentin and negative expressions of cytokeratin and synaptophysin (FIGURE 3). The results of cytological examination followed by immunocytochemistry led us to the conclusion that the lung tumor was a pleuropulmonary blastoma.

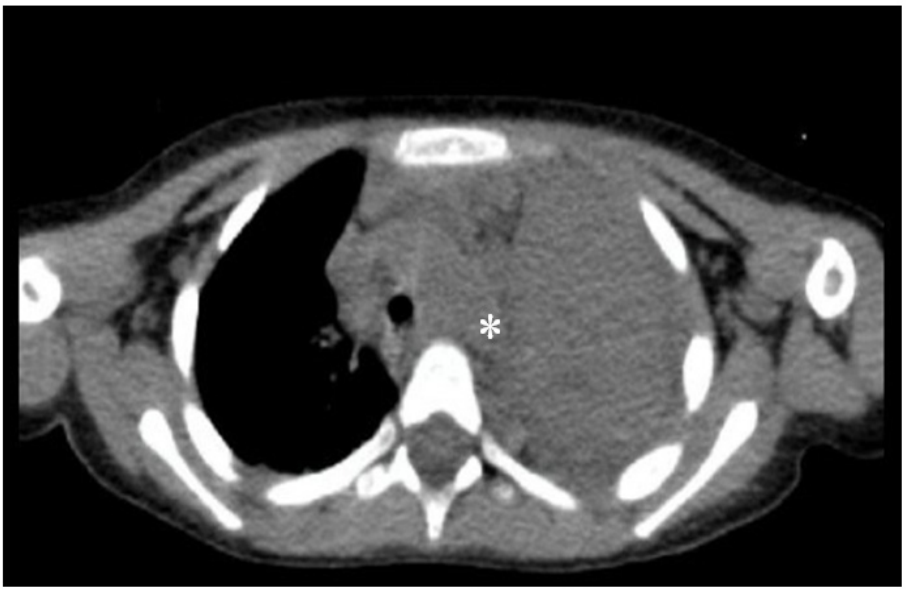

FIGURE 1. Axial section of CT scan of the chest. A solid cystic mass with an amorphous shape, irregular margin, and areas of necrosis was found in the left superior pulmonary lobe (*), sized 5.32 x 6.18 x 6.96 cm^3.

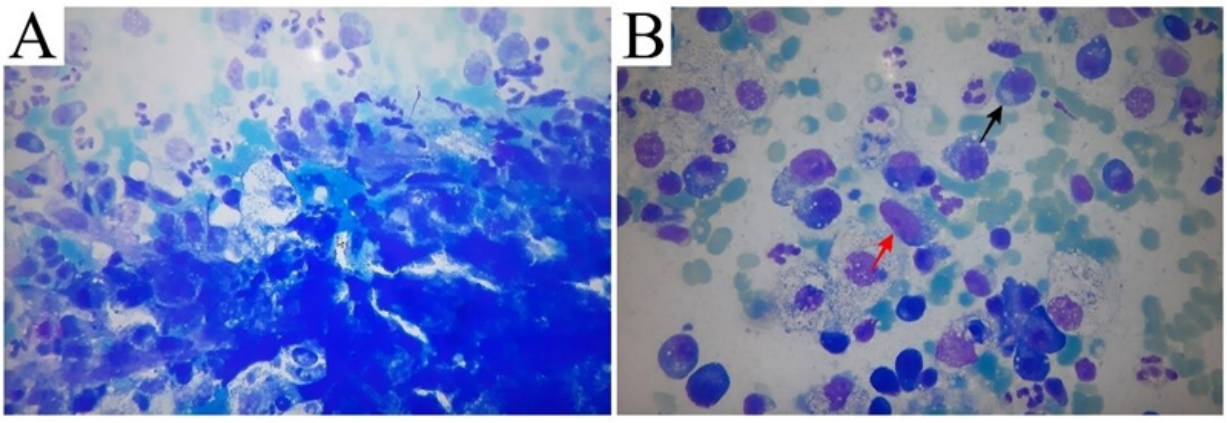

FIGURE 2. Cytologic examination of the pulmonary tissue. (A) Low-power photograph (100x) of cytological smear shows a dimorphic population of tumor cells. (B) High-power photograph (400x) of cytological smear shows a spindle-shaped cell with a high nuclear to cytoplasmic ratio, irregular membrane, distinct nucleolus (red arrow), and another type of round to oval cell with a high nuclear to cytoplasmic ratio, hyperchromatic nucleus, and distinct nucleolus (black arrow).

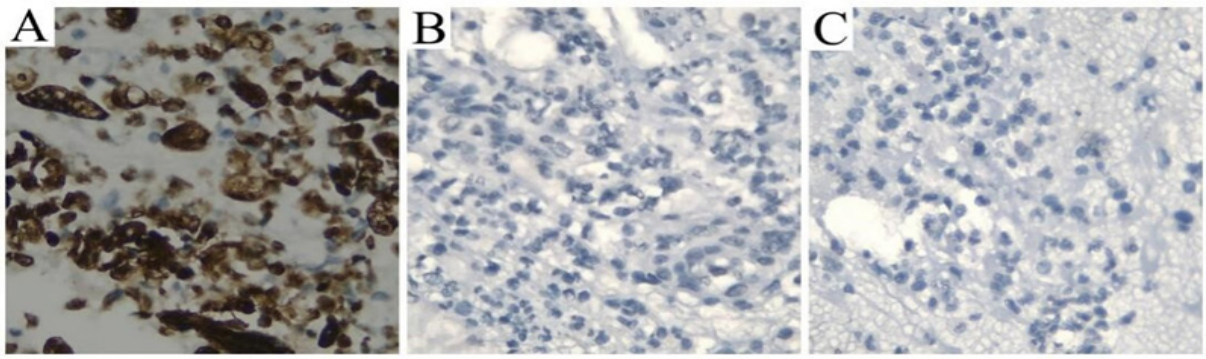

FIGURE 3. Immunocytochemistry staining of the pulmonary mass from cell blocks. High-power photographs (400x) of immunocytochemistry show diffusely positive cytoplasmic staining for vimentin (A), negative cytoplasmic stainings for cytokeratin (B), and synaptophysin (C).

After all examinations were performed and had been consulted to the Division of Medical Genetics, the patient was diagnosed with pleuropulmonary blastoma with Pfeiffer syndrome. The patient received chemotherapy with vincristine 0.8 mg, ifosfamide 500 mg, cyclophosphamide 400 mg combined with mesna 240 mg, and etoposide 80 mg in 6 cycles for 6 months. During chemotherapy, the patient also received leukokine drug after each chemotherapy cycle and paracetamol if the patient had a fever. Follow-up after 6 months of therapy showed improved patient condition and tumor size reduction by 37.5% based on CT scan evaluation (partial response according to RECIST 1.1 criteria).

DISCUSSION

There are three morphological stages in the formation of PPB, i.e., type I or purely multicystic, type II or mixed solid and cystic, and type III or purely solid stage.[7] Previous report of 50 cases of PPB shows a correlation between the morphological type of PPB and the median age at diagnosis, i.e., type I with a median age of 10 months, type II with a median age of 34 months, and type III with a median age of 44 months. In addition, the age at diagnosis correlates with the prognosis of PPB.[8] Type I PPB has a better prognosis, while type II and III PPB have a poor prognosis due to the frequent relapses and distant metastases, especially to the brain and bones. Due to its poor prognosis, PPB is aggressively treated with multimodal therapy, including surgery, chemotherapy, and/or radiotherapy. The combination of those therapies depends on the type and aggressiveness of the disease.[9] Clinically, PPB patients may present with chest or upper abdominal pain, dry cough, fever, dyspnea, tachypnea, fatigue, respiratory distress with or without an associated pneumothorax, hemoptysis, anorexia, malaise, or neurological symptoms resulting from brain metastases.[10]

PPB is often difficult to diagnose due to non-specific imaging findings. It may appear as a cystic lung mass and therefore it should be considered in the differential diagnosis of other benign cystic lung lesions on imaging findings. Early recognition and differentiation of PPB from congenital pulmonary airway malformations and other benign cysts are notably important due to increased survival rates at early diagnosis. On CT scan imaging, type I PPB emerges as a single or multicystic pulmonary lesion (ranging from 2 to 9 cm in diameter)

that causes a mass effect on surrounding structures, sometimes with mediastinal deviation toward the contralateral side. Type II PPB appears with both solid areas and air- or fluid-filled cavities.[11] Bleeding or infection in the intracavitary lesions may permeate the cysts and present as more solid areas. An enormous lesion can concur with pleural effusion and mediastinal shift to the contralateral side. Type III PPB appears as solid lesions with low attenuation on CT scan and heterogeneous enhancement pattern with contrast medium administration, with or without pleural effusion, atelectasis, and mediastinal deviation to the contralateral side. Necrotic areas often present without enhancement in contrast-enhanced CT scans. Whereas, the tumor masses are typically large and heterogenous on MRI imaging.[12] In our case, the patient was categorized as type II PPB based on CT scan results that showed a solid cystic mass and the time when first diagnosed in which type II PPB generally occurs in children over 2 years old (4 years old in our case). Based on previous studies, the long-term survival in this patient is less than 50% and the prognosis is poor. The recommended treatment for this type of PPB is aggressive surgery and chemotherapy.[13] Our patient had received chemotherapy in 6 cycles for 6 months and showed an improvement. The clinicians are discussing the next steps of therapy for this patient.

The histopathological pattern of PPB involves biphasic cell proliferation. One element comprises primitive cells with single round hyperchromatic nuclei, sometimes with clear nucleoli and sparse cytoplasm, causing high nuclear to cytoplasmic (N/C) ratios. Another major malignant component of PBB is mesenchymal spindle cells. Epithelial cells are not often found in this type of tumor, but the cells could present as entrapped benign epithelium or mesothelium. Focal mesenchymal differentiation is frequently identified as chondrosarcomatous, liposarcomatous, and particularly rhabdomyosarcomatous components. Cytomorphological findings of PPB from FNAB may conclude its histopathological elements.[14] In our case report, there are two major cell types, including the primitive blastemal cells and mesenchymal spindle cells, reflected in the fine needle aspiration smears. The blastemal cells appear as both solitary cells and cohesive aggregates with round to oval cells, high N/C ratio, hyperchromatic nuclei, and distinct nucleoli. The mesenchymal spindle cells appear mostly as individual scattered malignant cells. However, our specimens do not demonstrate any specific mesenchymal differentiation. In another previous case report, some additional components could be found in the aspiration smears, including the chondroid matrix, pleomorphic giant cells, and myxoid matrix.[15,16] Other tumors in the small round cell category should be considered as differential diagnoses in cytology samples.[17] The primitive neuroectodermal tumor originating within thoracic soft tissue is one of them. The small primitive malignant cells from both cases may be identical in the aspiration smears. Rosette-like structures may be related to primitive neuroectodermal tumors, which may also display a fibrillar background.[14] In our case, we did not find any rosette-like structure. Moreover, we exclude primitive neuroectodermal tumors in the differential diagnosis by synaptophysin immunocytochemistry that shows negative cytoplasmic expression. Other primitive embryonal malignant neoplasms in the lung also need to be considered in the differential diagnosis, including pulmonary blastoma. According to the WHO 2015 classification of lung tumors, pulmonary blastoma is separated from fetal adenocarcinoma (epithelium

only) and pleuropulmonary blastoma (mesenchymal only).[18] Pulmonary blastoma displays a biphasic histological pattern with both mesenchymal and epithelial components.[19] Immunocytochemistry on cell blocks from FNAB is notably helpful to exclude the differential diagnosis of pulmonary blastoma, in which cytokeratin and vimentin immunocytochemistry show positive cytoplasmic expression in tumor cells of pulmonary blastoma. In our case, only diffusely positive expression of vimentin is detected in tumor cells indicating a mesenchymal phenotype.

PPB is reported to be associated with the DICER1 mutations in 66% of the cases.[5] The patient has the possibility to develop malignancies in other organs when DICER1 mutations are detected during screening. In our case, the patient might have Pfeiffer syndrome which is a rare autosomal dominantly inherited disorder caused by mutations in the fibroblast growth factor receptor genes FGFR1 or FGFR2.[20] Unfortunately there are no previous studies showing a relationship between these two diseases since they have different mutations. Further investigation needs to be done on the genetic background of our patient.

CONCLUSION

We reported a case of pleuropulmonary blastoma in a 4-year-old girl based on cytopathological findings due to an inoperable tumor mass. Diagnosis of PPB using the cytology approach is very challenging, thus additional information from further immunocytochemical assay, radiological findings, and clinical examination is greatly helpful for diagnostic procedures.

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Pathological fracture in fibrous dysplasia: a case report

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ABSTRACT

Fibrous dysplasia (FD) is described as a growth disorder characterized by the progressive replacement of normal bone elements by fibrous-osseus tissue. Bones affected by FD is presented with bone weakening and prone to pathological fracture. We reported a case of FD in an 8-years-old boy, who came to the hospital with pain in the upper right thigh after falling with bumps in his head and foot. An X-ray revealed a deformity as a diasteal scalloping with radiolucency lesions on one-third proximal of the right upper femur, greater trochanter, and lesser trochanter, with complete transverse discontinuity in the distal deformity. No similar lesion was found in the other bones. Bone structure and trabeculation in the deformity area has decreased and the cortex was thinning. On magnetic resonance imaging (MRI), bone size was enlarged, hypointense on T1WI and inhomogeneous hyperintense on T2WI. Fibrous dysplasia with a pathological fracture is a rare case. The appearance on the X-ray was diasteal scalloping with a ground-glass radiolucency represented with Shepherd's crook deformity. This lesion was accompanied by a pathological fracture (fragility fracture) on distal lesion. Fibrous dysplasia which characterized by bone developmental anomaly gave an inhomogeneous hypointense on T1W1 and hyperintense on T2WI.

ABSTRAK

Fibrous displasia (FD) dideskripsikan sebagai gangguan pertumbuhan yang ditandai dengan penggantian progresif dari elemen tulang normal oleh jaringan fibrosa. Fibrous displasia menyebabkan perbedaan ukuran antara tulang yang satu dengan yang lain. Tulang dengan FD lebih lemah karena terjadi penurunan komponen mineral tulang, sehingga rentan terhadap fraktur patologis. Dilaporkan kasus FD pada anak laki-laki usia 8 tahun, datang ke rumah sakit dengan keluhan nyeri di paha kanan atas setelah jatuh dengan benturan kepala dan kaki. Radiolografi X-ray ditemukan gambaran deformitas berupa diasteal scalloping dengan lesi radiolusensi pada tulang paha kanan atas sepertiga proksimal, trokanter mayor dan trokanter minor, disertai discontinuitas transversa komplit di area deformitas bagian distal. Tidak ditemukan lesi serupa di tulang yang lain. Struktur dan trabekulasi tulang di area deformitas menurun dan korteks menipis. Pada *magnetic resonance imaging* (MRI) didapatkan pembesaran ukuran tulang, hipointens pada T1WI dan hiperintens inhomogen T2WI. Fibrous displasia dengan fraktur patologis pada kasus ini merupakan kasus yang jarang terjadi. Pada X-ray lesi ini berupa diasteal scalloping dengan radiolusensi groundglass berbentuk *shepherd crook deformity*. Lesi ini disertai fraktur patologis jenis fraktur fragil pada bagian distal lesi. Pada T1WI, penyakit ini memberikan gambaran hipointens dan hiperintens inhomogen pada T2WI.

Keywords:
fibrous dysplasia;
ground glass lucent;
pathological fractures;
X-ray;
MRI

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INTRODUCTION

Fibrous dysplasia (FD) is a congenital skeletal disorder which can be associated with benign tumor. Lichtenstein first described FD in 1938 as a growth disorder characterized by the progressive replacement of normal bone elements by fibrous tissue.[1,2] Fibrous dysplasia is a developmental anomaly in which normal bone marrow is replaced by fibro-osseous tissue. This process can be localized to a single bone, a small segment, or affect the bone structure in a diffuse pattern.[2,3] Bones affected by FD present with bone weakening, making it abnormally fragile and prone to fracture due to minor trauma.[4] Muthusamy *et al*.[5] reported that the incidence of FD is 5 - 10% among benign tumors. Fibrous dysplasia divided into some types with prevalence of each type as follow, monostotic-type fibrous dysplasia (70 - 80%), polyostotic fibrous dysplasia (20 - 30%), McCune-Albright syndrome (2 - 3%), and Mazabraud's syndrome (very rare cases).[5] Fibrous dysplasia which is a common benign skeletal lesion, may involve one bone (monostotic) or multiple bones (polyostotic), and affect throughout the skeleton with a predilection for long bones, ribs, and craniofacial bones.[6]

Fibrous dysplasia is a congenital disorder characterized by bone marrow replacement with soft tissue. Monostatic fibrous dysplasia patients with rapid bone growth had a higher chance of malignant transformation. It is important to make malignant bone tumor as differential diagnosis of FD, such as osteosarcoma, osteoblastomas, or metastatic bone lesion. On the other hand, FD carries a very small risk to be sarcomatous transformation (1%).[7] In radio-imaging, FD can be described as pagetoid, sclerotic, and cyst like appearance.[8]

Fractures caused by FD are more frequent in childhood, between the age of 6-10 years and declining thereafter.[9] Pathological fractures can occur in more than fifty percent of the patients with polyostotic and monostotic FD.[10,11] Approximately, 5% of fibrous dysplasia were found in benign bone lesion, and monostatic form eight to ten more common than polyostotic.[12]

The etiology of FD is linked to the developmental failure of immature bone and irregular bone tissue leading to the formation of woven bone mass in abnormal fibrous tissue.[10] The defect is associated with a gene mutation encoding the subunit of the stimulatory G protein (*Gsα*) located at 20q13.2-13.3.[6] It was demonstrated that active mutation of *Gsα* in osteoblastic cells of patients with McCune-Albright syndrome and monostotic-type disease leads to constitutive activation of adenylate cyclase, increased cell proliferation, and inappropriate cell differentiation, resulting in overproduction of disorganized fibrotic bone matrix.[10]

Long bone fracture is the most common complication in FD. Proximal femur is the most common site of fracture which mainly caused by load transfer through weakened bone and repetitive microfracture that produce progressive varus and bowing known as Sherperd's crook deformity.[9] A coexisting aneurysmal bone cyst is a risk factor of pathologic fracture that causes bone weakened.[13]

Radiological examinations to diagnose FD are conventional X-ray, CT scan, and MRI. The common radiographic features in FD were radiolucent images,

as a picture of a decrease in the matrix of bone material, resulting in a reduction in the bone structure and strength. Fractures triggered by trauma can be the major risk for patients with expansion of cortical thinning and bowed shape of the bone. We presented a case report of FD in pediatric with specific features on X-ray and magnetic resonance imaging (MRI).

CASE

An 8-years-old boy came to the hospital with a chief complaint of pain in the upper right thigh after a fall with bumps on his head and foot. He was slipped and fell on his right foot. He was unable to walk properly. The upper thigh was swollen, and radiographs were obtained.

Conventional X-rays showed deformity with bone enlargement and ground glass lucency lesions on the one-third proximal of the right femur, greater trochanter, and lesser trochanter, accompanied by complete discontinuity with an irregular fracture line that crossed the distal deformity area with a callus formation. The ground-glass lucency lesion was well-demarcated, smooth edges, and sclerotic thickened layer (rind sign), and no soft tissue involvement was seen. The bone structure and trabeculation around the deformity area showed thinning of the bone cortex. The epiphyseal line was still evident without any abnormalities were seen in the femoral head or acetabulum. The lesion showed shepherd's crook deformity (FIGURE 1). The similar lesion was not found in the other bones.

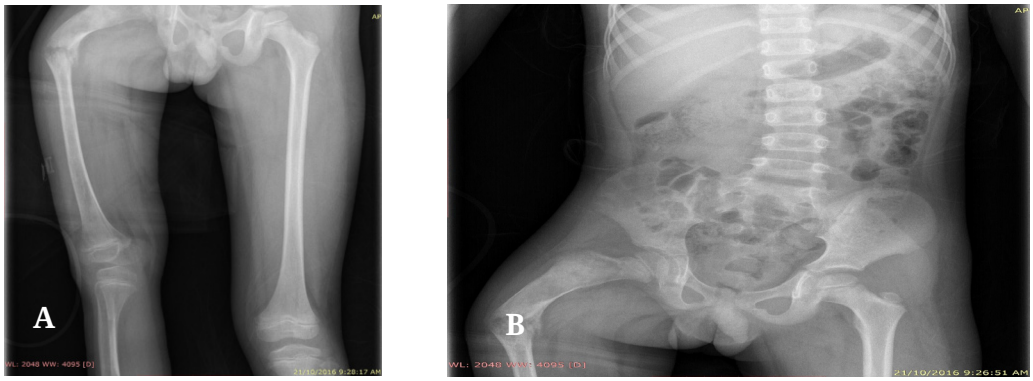

FIGURE.1. A. Lower limbs X-ray (anteroposterior view). B. Pelvic X-ray (anteroposterior view) A ground-glass expansive radiolucent lesion one-third proximal of the right femur gives a shepherd's crook deformity with a fracture in the distal le sion (a). Soft tissue was normal (b).

The MRI showed a widening bone lesion on T1WI with hypointense in the one-third proximal of the right femur, greater trochanter, and lesser trochanter, accompanied by signs of fracture in the distal lesion with hyperintensity at the edges of the fracture line and callus. On T2WI, the MRI shows an increase in the intensity of the inhomogeneous signal.

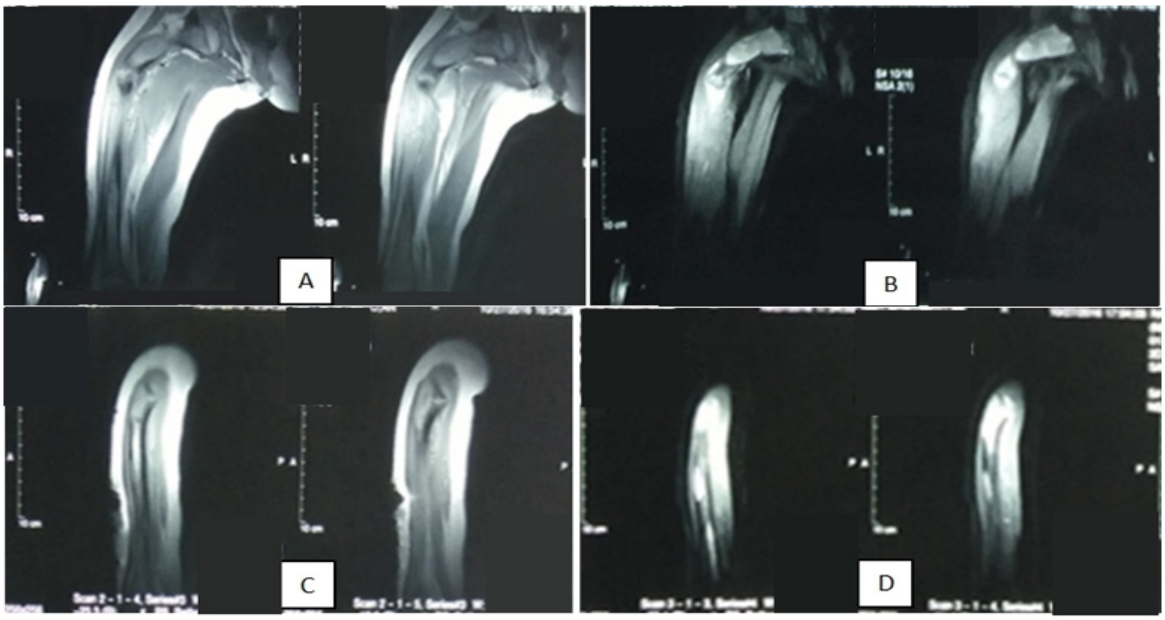

FIGURE 2. The MRI of one-third proximal right femur in FD with pathological fracture. T1W1 (A) and T2W1 (B) of MRI coronal section, T1W1 (C) and T2W1 (D) of MRI sagittal section.

In the image above, MRI showed the coronal section of the hypointense lesion on T1WI and inhomogeneous hyperintense on T2WI (FIGURE 2A and B), sagittal section (FIGURE 2C and D), accompanied by a pathological fracture in the distal lesion followed by a callus. Younger age patients with femoral lesions tend to have high potential mechanical deficit leading to fracture. The femoral bone weakened by FD was prone to fracture or deformity caused by high mechanical forces.

DISCUSSION

Fibrous dysplasia is a benign disorder that affects bone growth as marked with the replacement of bone with fibrous tissue.[5,6,14] Radiographically, FD was depicted as a radiolucent area that develops into the ground glass. The typical features of the disease are endosteal scalloping, bone expansion, and a sclerotic thickening reaction called the rind sign.[6,14] The MRI was more accurate for the detection and assessment of affected area by FD and was useful to determine a doubtful radiographic result of suspected FD.[15] Our case exhibited similar features with case reported by Hakim *et al.*[15] which presented radiolucency, endosteal scalloping, expansion, and the presence of a rind sign as the radiographic findings. In this case, we found a shepherd's crook deformity and a rind sign in the upper femur area, which is typical for FD.[4] In MRI finding, FD presented as hypointense on T1WI and can be either hyperintense or hypointense on T2WI. The signal intensity on T1 and T2WI images and the degree of contrast enhancement on TI images depend on bone trabeculae, cellularity, collagen, and cystic and hemorrhagic changes.[14]

The cystic changes in MRI were not found in our case, this condition was similar with previously case reported by Jee *et al.*[14] Among 13 MRI of FD cases, cystic changes were observed in two samples (15%). The imaging similarity was found between giant cell tumor and cystic fibrous dysplasia in the long bone; hence the understanding of the difference was required to avoid misdiagnosis. Cystic FD can be proven by biopsy.[16]

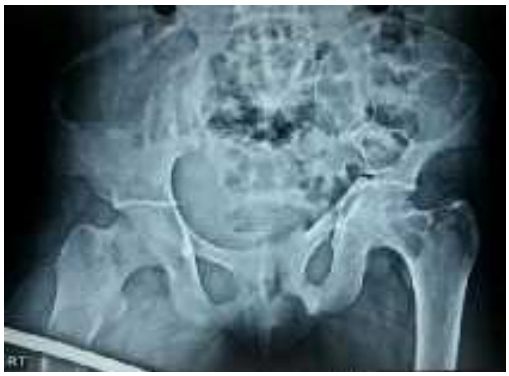

FIGURE 3. The pre surgery X-ray showed a pathological fracture and an expansive lucent lesion surrounded by a sclerotic lesion.[6]

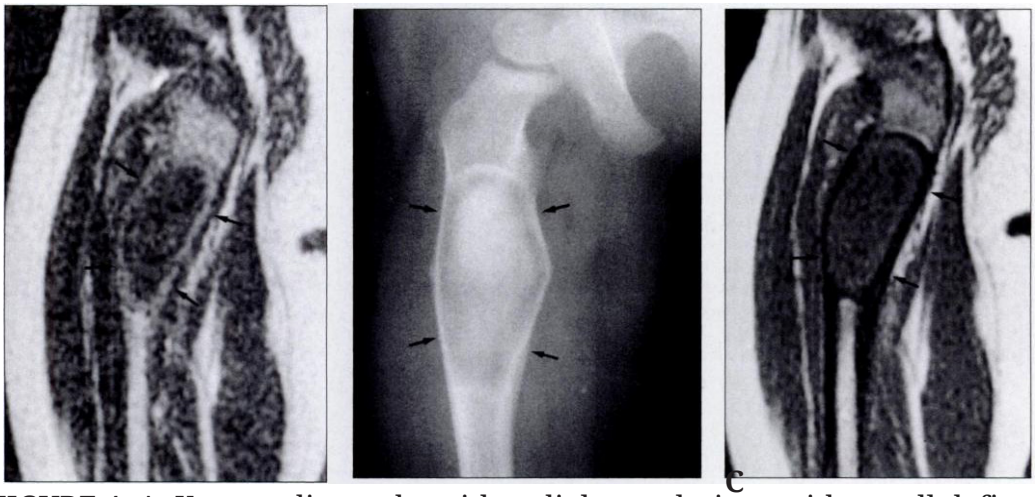

FIGURE 4. A. X-ray radiographs with radiolucent lesions with a well-defined sclerotic rim, B. T1WI, hypointense lesion, C. T2WI, hyperintense lesion.[14]

In this case, a complete pathological fracture with callus remodeling was discovered in the distal lesion (FIGURE 1). In certain conditions, fractures in FD patients are not associated with the bone condition caused by FD itself. Decreased bone mineral in FD should be considered as risk factor for fracture.[6,10,15,17] Pathological fractures should be suspected in pediatric patients with fracture-associated minor trauma, unusual fracture site, or abnormal bone process found on radiographs. Changes in normal bone biomechanics could be caused by intrinsic processes including changes in bone mineral density caused by bone tumors (both benign and malignant), illnesses such as osteogenesis imperfecta or infections, and extrinsic mechanisms including internal fixation, biopsy canal, and radiation. Load and changes in bone intensity both influence the risk of pathologic fracture.[18]

Pathological fracture is often associated with pain and deformity, which was divided into micro or macro fractures. Microfractures commonly occur in the trabecular bone of the metaphysis or corpus vertebrae, generally this condition is undetected and mostly immobile.[18] Characteristics changes in X-ray including pathognomonic ground-glass aspect with peripheral sclerotic reaction (rind sign), bone expansion, an indentation of areas inside the cortex (endosteal scalloping), and involved the femur represented the classical Shepherd's staff deformity as the result of repetitive microfractures.[18-20]

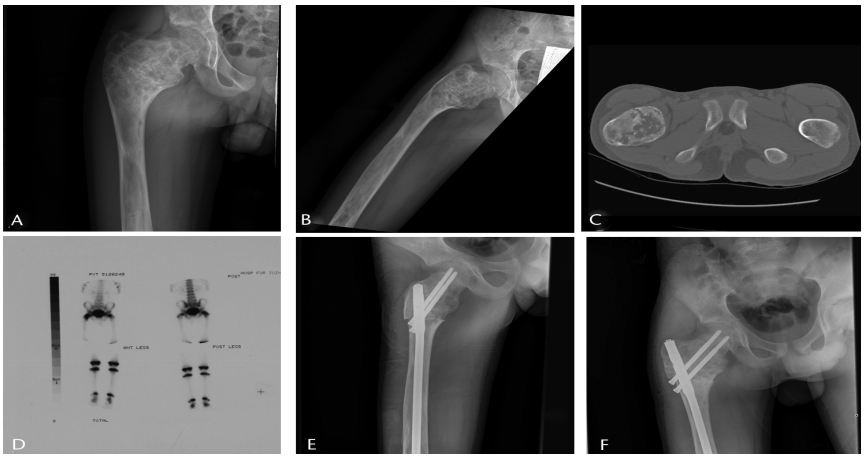

FIGURE 5. In a 14-year-old male patient with sudden onset of right hip pain, radiographs A and B at presentation show Shepherd deformity and pathological fracture of the proximal femur.[18]

The pathological macrofracture in this case was seen on a distal lesion, whereas the microfracture was seen in the lesser trochanter and column of the femur forming shepherd's crook deformity (FIGURE 1). de Mattos *et al.*[18] reported that pain, size of the lesion (> 2.5 cm in width or > 3.5 cm in length) and cortical destruction (≥ 50%) are not independently predicted the risk factors of fracture. Further study is required to determine the risk factor of pathologic fracture.

CONCLUSION

Fibrous dysplasia with a pathological fracture, in this case, is a rare case. On conventional X-ray radiographs, the lesion showed endosteal scalloping with a ground-glass radiolucency, and shepherd's crook deformity, occurring on the right upper femur in an 8-years-old boy. The lesion is accompanied by a rind sign, which defined as the presence of a sclerotic area around the lesion. In this case, the lesion is accompanied by a pathological fracture (fragility fracture). Fibrous dysplasia gives an inhomogeneous hypointense on T1W1and hyperintense on T2WI. Hyperintense signal inhomogeneity on T2WI image indicates an internal condition of thinning bone within the cortex of the disease. It is important to recognize FD radiological findings because of the higher chance of malignant transformation. Therefore, biopsy is needed to exclude any malignant bone tumor.

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Potential skin problems of diabetes mellitus patients: a review

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ABSTRACT

Diabetes mellitus (DM) is one of the common metabolic disorders, and a major part of chronic diseases, the prevalence of which tends to increase due to multifactor. Blood vessels, kidneys, lungs, and skin are among the organs that are affected. The first problem that arises, or commonly exists among one-third of diabetics, are problems with their skin, although skin lesions may develop along with the progress of the disease, or can occur during the later phase of DM. The prevalence and symptoms of skin problems in type 1 DM (T1DM) and type 2 DM (T2DM) are often unclear, and at the beginning of the course of the diseases they often go undiagnosed. Several theories regarding the pathophysiology of DM can be used as a logical reference for the early identification and diagnosis of skin problems, aimed at preventing the worsened condition. The use of skin autofluorescence (SAF) and AGEs reader in several cases of skin problems, can also be an important marker as an adjunct to predict the possibility and progressiveness of DM. Skin problems linked to patients with DM can be categorized as strongly related to diabetes, non-specific and related to DM, skin infection in DM, and skin problems due to diabetic medication. With the current COVID-19 pandemic, there are additional demands for more critical investigation of skin problems in patients with DM. The skin problems that occur in DM may need to be examined from the early stage and it is necessary to inhibit the progression of skin problems, as well as to consider the need for multidisciplinary DM therapy.

ABSTRAK

Diabetes mellitus (DM) merupakan salah satu gangguan metabolisme yang sering terjadi, dan merupakan penyakit kronis dengan prevalensi yang cenderung meningkat, dengan penyebab multifaktorial. Pembuluh darah, ginjal, paru-paru, dan kulit termasuk di antara organ-organ yang dapat terpengaruh. Masalah pertama yang muncul, atau yang biasa dialami oleh sepertiga penderita diabetes, adalah permasalahan pada kulit, lesi kulit yang terjadi dapat berkembang seiring dengan perkembangan penyakit, atau bahkan dapat terjadi pada fase lanjut dari DM. Prevalensi dan gejala gangguan kulit pada DM tipe 1 (DMT1) dan DM tipe 2 (DMT2) seringkali tidak jelas, bahkan sering tidak terdiagnosis. Beberapa teori mengenai patofisiologi DM dapat dijadikan sebagai acuan logis untuk identifikasi dini dan diagnosis masalah kulit, yang bertujuan untuk mencegah terjadinya kondisi yang semakin memburuk. Penggunaan *skin autofluorescence* (SAF) dan AGEs *reader* pada beberapa kasus masalah kulit, dapat menjadi penanda penting untuk memprediksi kemungkinan dan progresivitas DM. Masalah kulit yang berhubungan dengan penderita DM dapat dikategorikan berhubungan erat dengan diabetes, non-spesifik dan berhubungan dengan DM, infeksi kulit pada DM, dan masalah kulit akibat pengobatan diabetes. Dengan adanya pandemi COVID-19 saat ini, diperlukan investigasi yang lebih kritis terhadap pemeriksaan permasalahan kulit pada pasien DM. Permasalahan kulit yang terjadi pada DM perlu diperiksa sejak dini, untuk menghambat progresivitas, serta mempertimbangkan perlunya terapi DM secara multidisiplin.

Keywords:
diabetes mellitus;
skin problems;
pathophysiology;
multidisciplinary therapy;
dermophaty

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INTRODUCTION

Diabetes mellitus (DM) is related to several physiological symptoms, including skin symptoms. The main symptoms of DM include increased blood sugar level and insulin resistance, more importantly, changes in glucose level.[1-3] Type 1 DM (T1DM) is indicated by the existence of autoantibodies circulating within the cytoplasmic protein in β cell, which causes gradual damage of β-islet immune-mediated in the pancreas, while patients with type 2 DM (T2DM) have chronic hyperglycemia, with defects in glucose, protein, and fat metabolism that are normally accompanied by an increase in insulin resistance, which is age-related, genetically predisposed, and linked to obesity.[1,4]

The T2DM is the most common form of DM disease accounting for around 90% of all diabetes cases worldwide. It was predicted by the World Health Organization (WHO) within thirty years (2000-2030), and could be one of the highest-ranking causes of death worldwide. The WHO further predicts that DM will affect more than 21 million people of Indonesia's population in 2030. Result of an investigation on DM prevalence in Indonesia in 2018, conducted by Basic Health Research (*Riset Kesehatan Dasar*/Riskesda), there are 8.5% or around 20.4 million people suffered from DM.[5] The International Diabetes Federation (IDF) explained that T2DM prevalence will rise from 10.3 million in 2010-2017 to become 16.7 million in 2045.[5] According to data from the United States National Health Interview Survey, the age and sex-adjusted prevalence of T2DM in Caucasians ranges between 3.8% to 6.0%, which is lower than Asian Americans (4.35% to 8.2%) in the United States from 1997 to 2008. Asian Indians, in particular, have the highest diabetes risk. Adult DM was found in 17 percent of Asian Indians, 15% of Native Americans/Alaska natives, 8% of non-Hispanic whites, 13% of non-Hispanic blacks, and 10% of Hispanic Latinos.[6,7] The occurrence of the pattern and the types of DM are various, with around 32% of diabetic symptoms associated with cutaneous problems.[8] In different regions worldwide, the cutaneous involvement prevalence in T1DM and T2DM varies between 51.1% - 97%.[9]

The data are very limited regarding the correlation between the two types of diabetes in the initial conditions of skin problems without skin damage. The skin problems of people who suffer from T1DM and T2DM are often not properly or underdiagnosed, e.g. dry skin and pruritus.[9] Possibly, there is a limited number of well-conducted investigations that have been published on this topic linking DM with skin problems.

As our body's most identifiable organ, the skin often displays the first signs of a metabolic disorder. It can also be used as an effective marker to indicate the risk of DM, as well as to determine the effectiveness of the therapy. As a non-invasive method, the use of skin autofluorescence (SAF) and AGEs reader in several cases of skin problems, can be an important marker that can be used to predict the possibility of DM and the progressiveness of the DM disease.[10]

Skin problems linked to patients with DM can be categorized according to the following descriptions: (1) skin problems strongly related to DM; (2) skin problems non-specific and related to DM; (3) skin infection in DM; and (4) skin problems due to diabetic medication.[9,10] Although there are few criteria with little or limited specificity. One study evaluated 100 patients with T1DM and T2DM in Egypt, with one skin lesion at least, in a one-center cohort study, and the most common cases were cutaneous infection, followed by pruritus.[9] Another study showed a higher rate of skin disease in T2DM vs T1DM (75.6 vs 41%), for which the differences in

lesions between diabetes types are still unknown. A similar study found further results indicating that the prevalence of skin problems was higher in patients with T2DM,[9] and should be observed in the initial stage, without regard to the manifestations of the disease and the diabetes type.[9,10]

This review has several purposes: (a) to remind clinicians about the DM pathology process in a concise manner, (b) to comprehend the effects of diabetes on cutaneous indicators associated with T1DM and T2DM, and (c) to demonstrate the importance to perform early-stage surveillance using a multidisciplinary method in order to prevent unintended consequences with poor avoidable outcomes.

DISCUSSION

Pathophysiology of DM on skin

Changes in skin function and clinical skin changes in patients with DM are very complex.[11] Briefly, some of DM mechanisms that cause skin lesions are directly from the hyperglycemia state (pathologic glucose level)/direct increase of glucose level and indirect mechanism via glycation of lipid, protein, nucleic acid, which induce the development of advanced glucose end products (AGEs) that are linked to the skin manifestations of DM.[10-12] Formation of AGEs via several pathways further induce among others: formation of reactive oxygen species (ROS), harmful ROS clearance, which upsets the function of the intracellular matrix (ICM) and extracellular matrix (ECM) protein.[10] AGEs also transform the collagen, [10,11] usually type I and type IV which are susceptible to glycation,[10] and cause the impairment of skin elasticity that can contribute to skin problems, e.g. skin aging extrinsically.[10,11] Even though, non-enzymatic glycosylation is a normal process of aging,[3] the skin of patients with DM often has signs of premature skin aging.[10] When such condition persists, it can lead to the development of a micro and macroangiopathy diabetic condition, which can cause tissue hypoxia, and ultimately will be followed by nerve damage, also neuropathy, retinopathy, and nephropathy.[1,12]

Skin problems in DM

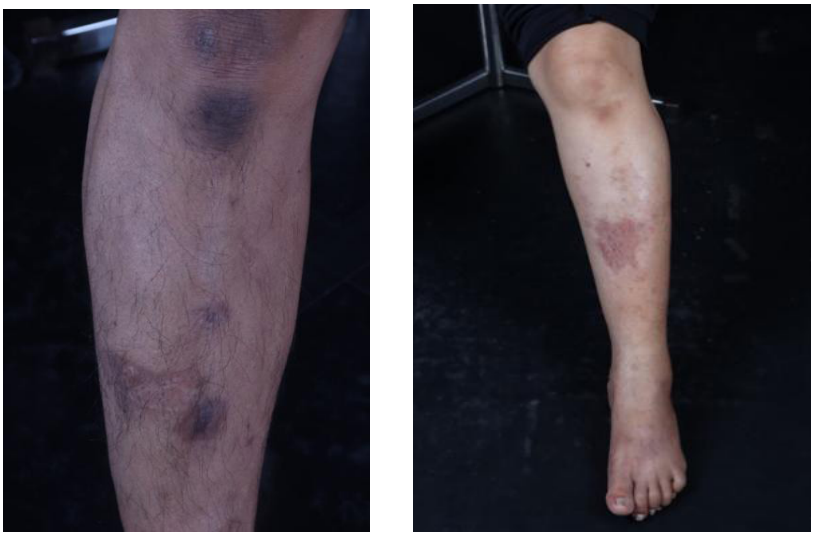
Generally, the cause of diabetes-related skin problems is unknown, it is most likely due to the direct effects of hyperglycemia and hyperlipidemia. However, as the disease progresses, it can cause damage to the vascular, neurologic, and immune systems, which can lead to skin problems.[4,8,13] Skin symptoms may appear before a DM diagnosis and be due to the disease, or they can appear during the course of diabetes as a result of diabetes complications or antidiabetic treatment side effects.[10,13] The most common skin problems in 100 patients with T2DM diabetes aged 34 to 76 years, according to one study in 2020, were fungal infections (55%).[13] The following is a list of the most common skin problems that patients with DM face, regardless of whether they have T1DM or T2DM.

Skin problem strongly associated with diabetes

Diabetic dermopathy

Diabetic dermopathy (DD) is an asymptomatic condition, which affects a significant proportion of males, with a mean age of 50 years, also known as “shin spots”. While DD affects between 7-70% of all DM patients, actually it is not a particular lesion of the DM disease, since non-diabetic patients as many as 20% have similar lesions.[14] Atrophic skin lesions as a clinical sign, involve irregular red papules or plaques that are pink to brown within 0.5-1 cm in diameter, numerous, bilateral, asymmetrical of the lower extremity, extensor surface of the

lower legs, and forearms.[4,15] In several studies, microvascular complications have been discovered to be serious in patients with DM,[14] including retinopathy, nephropathy, and neuropathy.[4,12,15] This disorder typically does not need any treatment and will recover on its own in around 1-2 years.[16] FIGURE 1A shows a diabetic dermopathy lesion in a male patient.



A B

FIGURE 1. A. Diabetic dermopathy on right lower leg (Source: Photography Unit, Dermatovenereology Outpatient Care Clinic, Dr. Sardjito General Hospital) and B. Necrobiosis lipoidica (Source: authors)

Necrobiosis lipoidica

The pathogenesis of necrobiosis lipoidica (NL) is unknown, but changes in microangiopathy and hypoxia may be to blame. Necrobiosis lipoidica affects 0.3% of patients with DM, and women tend to develop the disease. The history of a family with DM is seen in 43% of patients with NL.[7] The yellow appearance of the lesions in the central region is most likely due to dermal thinning, which makes subcutaneous fat more noticeable.[14,17] Necrobiosis lipoidica is characterized by a sharply developed anthropic in the center,[19] and telangiectatic plaque with a glazed appearance containing yellow-brown color, with NL that resembles granuloma annulare (GA).[17,19] A few cases reported the risks of the change into squamous cell carcinoma.[20] FIGURE 1B shows the lesion of NL (before biopsy).

Acanthosis nigricans

Acanthosis nigricans (AN) is frequent in the general population, but darker-skinned people tend to have a higher prevalence, and it is higher among females than males. In some cases, increased androgen production may contribute as has been shown in other etiologies of malignancy, and is most often seen in prediabetic,[15] and obese T2DM patients.[4,15,17] Lesions appear as smooth, hyperkeratotic, velvety plaques, and hyperpigmented skin, chiefly involving folds in the body, i.e. neck, axillae, and flexures,[4,14,15] meanwhile there is a rare case also found on acral area,[18] most of the cases are linked to obesity and insulin resistance. FIGURE 2 shows AN in the neck and right axillae of a woman.

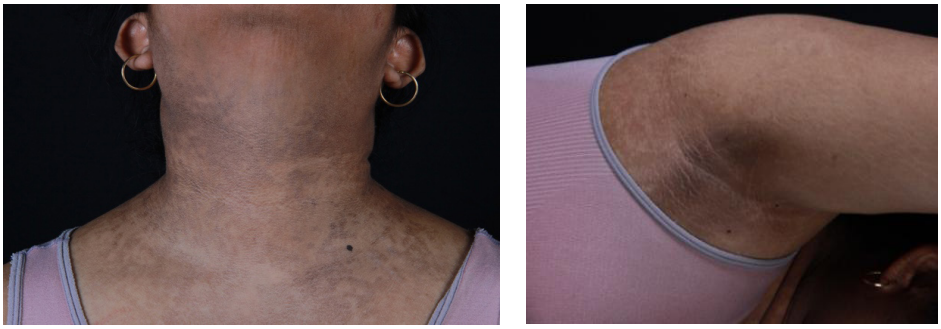


FIGURE 2. Acanthosis nigricans (Source: Photography Unit, Dermatovenereology Outpatient Care Clinic, Dr. Sardjito General Hospital).

Diabetic thick skin and stiff skin

Usually asymptomatic, this type of lesion affects the fingers and hands, as well as the back of the neck and upper back. There are three types: asymptomatic lesions, where we can calculate the thickness of the skin, clinically evident thickening on fingers and hands, and diabetic scleroderma, where we can see thickening on the fingers and hands. In a common syndrome, the skin of the upper back and posterior neck thickens noticeably, spreading to the lumbar and deltoid regions. The progression of this disorder began with stiffness in the metacarpophalangeal and proximal joints of the interphalangeal joints, as well as progressivity and reduced joint motion.[14] The diabetic hand syndrome affects anywhere from 8 to 50% of diabetics, which commonly affects people more than 60 years old.[21] Pathogenesis requires biochemical changes in dermal collagen and mucopolysaccharides. Receptors for AGE products (RAGEs) activate protein kinase C, which stimulates many inflammatory and fibrogenic growth factors and cytokines,[15] while increased deposition and improper degradation of these constituents cause clinical syndromes, which are possibly linked to the formation of AGEs.[4,21]

Diabetic bullae

These lesions usually affect older patients with DM, with more males than females, while the etiology of bullae is unclear.[21]The lower legs and feet, as well as the hands and fingers, can be affected by nonscarring subepidermal bullae up to several centimeters in diameter on a noninflamed base, particularly on the acral area. These lesions are rare, but they are considered to be a distinct diabetes marker, which may take several weeks to recover without scarring.[21]

Skin problem non-specific and related to DM

Skin tags

According to one report, skin tags or acrochordon were related to an atherogenic lipid profile, including in cases where low levels of high-density lipoprotein (HDL) cholesterol were found. In a study of a large quantity of patients with acrochordon, 8% of them had reduced glucose tolerance, > 25% suffered from DM,[14] while in another study, 57 (26%) of 216 patients with skin tags had noninsulin-dependent DM. These skin tags usually affect the face, neck, axilla, back, armpit, and trunk.[15,17] Skin tags, which are also known as

acrochordon, appear soft, small, skin-colored or hyperpigmented and pedunculated lesions, to be a marker for diabetes, independent of obesity, women, and acanthosis nigricans.[17,21]

Granuloma annulare

The connection between granuloma annulare (GA) and DM is weaker than the one between NL and diabetes,[4] around 50% of NL patients have DM,[19] although single lesion can resolve spontaneously.[21] The skin lesions of GA are usually symmetrically distributed along the distal region of the extremities and sun-exposed skin, with skin-colored or red borders,[16] usually with an annular lesion.[19,21] The lesion may be generalized or localized; in diabetics, the generalized distribution appears to be more common and thought to be associated with diabetes.[21] While a correlation between GA and DM has been proposed, no definitive evidence of such a link has been found.[14,19,21] However, recent studies identified patients with DM account for 9.7% of patients with localized GA and 21% of patients with generalized GA. The cause of the disease is unknown.[4,21]

Eruptive xanthoma

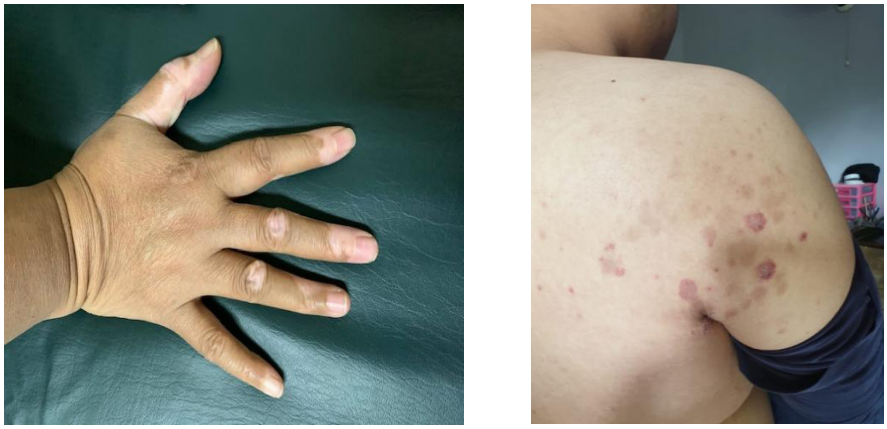
In patients with DM, this form of skin lesion, eruptive xanthoma (EX) is linked to high triglyceride levels,[4] eruptive xanthomas form a crop of yellow papules with erythematous halo, and typically appear on the buttocks and extensors.[14,21] Controlling carbohydrate and lipid metabolism have the potential to overcome these lesions.[4,14,21]

Pruritus

There are several detailed studies about generalized pruritus,[15] generalized pruritus has been reported in around 3% to 50% of patients with DM, one study observed around 25% of diabetics,[13,17] another study of pruritus found in around 18.4% to 27. 5% can be the initial symptom of DM, although without initial skin lesion.[22] There are few comprehensive studies on the association with DM,[17] and a recent study from Japan reported an increased prevalence of truncal,[15] pruritus may be used as a marker for polyneuropathy,[15,17] and the dysfunction of the symphatic nerves that causes hypohydrosis and dry skin condition.[22] However, there was no correlation with hemoglobin A1c (HbA1c) performance.[17] Instead, uremic pruritic (UP) patients who usually have a poor glycemic control have a correlation with HbA1c.[23]

Vitiligo

This pigmentary disorder, an acquired autoimmune disorder, which lacks intact melanocytes has been detected on the skin and has been observed in association with DM and thyroid diseases.[18,24] The prevalence in the general population is around 1%.[24] One study conducted in 2020, among 120 subjects diagnosed with DM, there are 5.8% cases with vitiligo.[25] FIGURE 3A shows a case of stable vitiligo on the right hand, especially on the right acral area (right hand) of a 53 years age woman.



A B

FIGURE 3. A. Acral vitiligo and B. Psoriasis in the right back of the upper arm (Source: authors)

Psoriasis

Psoriasis is a skin disorder, which can be categorized as a chronic inflammatory skin disease,[24,26] observed in around 125 million of the population, worldwide.[26] Various symptoms, such as an erythematous plaque with scaly areas of the skin,[24] which is linked to a number of risk factors.[24, 26] Among individuals with psoriasis, it has been discovered that there is a higher frequency condition of obesity, hypertension, and diabetes in a one cohort study,[26] besides the cardiometabolic syndrome it was also found that other diseases were associated with psoriasis, e.g.: bowel disease, cancer, etc.[27] Several studies reported that in the population, the obese people with psoriasis are around twice, while one study more specifically discovered that this prevalence was also the case for T2DM.[28] A retrospective cohort study in 2018 revealed that children who suffered from psoriasis tend to have a higher chance for comorbidities, have excess body weight and some have a cardiometabolic syndrome, including DM, hypertension, etc.[29] Therefore, children with psoriasis, who are usually obese, have the higher risk of the development of DM in the future. FIGURE 3B shows psoriasis in the right back of the upper arm in an obese man.

Skin infection in diabetes

TABLE 1 shows the more common and serious skin infections in diabetic patients, indicating that skin infection disorders are linked to impaired glycemic control in patients with DM,[4,14,15,21] affecting 20% to 50% of patients and are more frequent in T2DM patients,[14] but both T1DM and T2DM have the risk of skin infection.[30] Infection is thought to be caused by a connection between hyperglycemia, neutrophil chemotaxis, phagocytes, and killer T-cells, which act abnormally,[18] and can be identified as generalized immunologic defects in diabetics.[30] Bacteria, fungi, and yeast cause the majority of infections,[4] which typically affect patients with uncontrolled DM.[19,21,30]

TABLE 1. Bacterial and Fungal Infections in DM

Bacteria	Fungal and Yeast
• Eritrasma caused by: *Corynebacterium minutissimum*	• Caused by Candida, dermatophyte
• *Staphyloccocus aureus or β-hemolytic strepcocci* cause condition of erisipelas, impetigo, folliculitis & others	The rare infections include:
• Invasive *group B streptococcus*	• Infection by Phycomycetes cause Mucormycosis
• Invasive *group A streptococcus*	• Clostridium species cause anaerobic cellulitis
• Malignant external otitis most frequent caused by *Pseudomonas aeruginosa*, can be fatal	• Caused by Dermatophyte: *Trichopyton rubrum, Trichopyton mentagrophytes, Epidermophyton flocosum*

One study on helminth infection and DM was undertaken in Indonesia, which took place in a semi-urban coastal area of Nangapanda, a sub-district of Ende District of Flores Island, Indonesia. This region has been found to be endemic for soil-transmitted helminth (STH) infections in previous studies. The research looked at how helminthiasis affects insulin resistance. The study examined the relationship between worm infection status, immune condition of the people, and metabolic factors, with the connectivity of whole-body metabolic condition and inflammation to be established.[31]

Another study revealed that Helminth-derived antigens present, therefore possible novel therapies to treat obese people and obesity with the connection of the metabolic diseases i.e.: insulin resistance and T2DM through the induction of several protective mechanisms. In the conclusion, filarial infection and antigens would protect against the onset of T1DM and reduce diet-induced insulin resistance. Identification of those regulatory mechanisms, as well as helminth-derived products that cause them, may provide a powerful tool for combating autoimmune and metabolic diseases, which are becoming a growing public health concern.[32]

Skin problems in DM in connection with COVID-19 infection

Corona virus disease-2019 (COVID-19) pandemic that has occurred since the beginning of 2020, has drawn more attention to the need for more serious treatment of patients with DM, which is possibly linked to skin problems. COVID-19 is a viral infection that causes severe acute respiratory syndrome (SARS-CoV-2). It is important to consider that there are strong associations between acute and chronic inflammation, the receptors, and the pathogenic association between DM and COVID-19. One example of association is that chronic hyperglycemia causes chronic complications of diabetes.[33]

The COVID-19 infection exacerbates the stress of DM by releasing glucocorticoids and catecholamines into the bloodstream, causing glycemic regulation to deteriorate also there is an increase in the formation of AGEs in many tissues, as well as a worsening prognosis.[33] According to recent research, aging patients who have DM, are at higher risk to contract COVID-19, and have a higher mortality rate.[33,34]

In order to understand the pathomechanism of SARS-CoV-2 on diabetic skin comorbidity, it is crucial to

prevent and treat the difficult conditions of the skin and soft tissue problems in COVID-19 in patients with DM.[33,34] Among aging patients with DM, chronic ulcers and diabetic foot are two examples of complications of skin problems and soft tissue which are frequently found. The possible pathomechanism of SARS-CoV-2 is currently unknown, despite some research findings. Researchers have speculated that the pathomechanisms can be: a lack of blood glucose control, which SARS-CoV-2 causes blood glucose instability; increases an angiotensin-converting enzyme-2 (ACE2). Expression of ACE2 (found in skin, tissues, lungs, and other organs), which can predispose people with diabetes to become infected with SARS-CoV-2, possibly influencing tissue that expresses an ACE2, such as skin; then, impaired angiogenesis: the occurrence of leukopenia and thrombocytopenia, as well as an increase in D-dimer levels in DM patients with ischemia and hypoxia condition; and SARS-inflammatory CoV-2's response, and cytokine storm can distress the response of inflammation in patients with DM, intruding the normal skin and soft tissue microenvironment.[34]

The skin signs of SARS-CoV-2 infection according to one study with 375 sample cases, are in the form of vesicular eruption that appears in the initial stage of the disease, and tend to be a specific sign.[35] Covid-19 presents many aspects in the skin, of which the specific manifestations have connections with the severity of the disease.[36] The manifestations can be classified into: vesicular eruption as described above; urticarial lesions, maculopapular eruption, livedo or necrosis,[35] exanthematous eruptions, and acral purpuric nodule resemble to idiopathic perniosis (chilblains), which can also be a specific symptom in the later stages.[36] A study about skin manifestation of Covid-19 explains that there are differences in morphology and prevalence of skin manifestations in COVID-19. Among Europeans, pseudo-chilblains were the most common skin manifestations of COVID-19, but only once reported in Asia. However, understanding the scientific skin manifestations in patients with COVID-19 is still evolving.[37]

Skin problems due to diabetic medication

Insulin injection

Insulin injections under the skin are linked to a variety of localized changes. Lipohypertrophy is the most typical case of the local adverse effect of insulin injection, affecting approximately 30% of diabetic patients who use insulin injection, in the injection site,[24] liposuctions on this site may have a satisfactory cosmetic result.[38] However, lipoatrophy is less common in DM patients with regular insulin injection,[38] but more common in young women who suffered from diabetes.[21] Skin lesions are mostly found where insulin is injected. The reaction can be quick (a few hours) or delayed (within a day), and appear as a local allergic reaction e.g.: erythema, induration and pruritus, as well as a systemic reaction. [21,24,38] Such subcutaneous allergic reaction to insulin is quite rare with only 1% of DM patients with insulin medication,[24] and with the introduction of new insulins, cutaneous adverse reactions tend to decrease.[38]

Oral medications

Several oral diabetic medications can cause cutaneous adverse drug reactions in particular patients, even though this is a rare case.[24,39] There are first and second generations of sulfonilurea. The first-generation sulfonilurea has brought more cutaneous reactions and photosensitivity reactions. Possible cutaneous reactions are linked to sulfoniluria treatment,

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