HETERORESISTANT VANCOMYCIN INTERMEDIATE *STAPHYLOCOCCUS AUREUS* IN A TERTIARY CARE HOSPITAL

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ABSTRACT

Background and Objectives: The burden of heteroresistant Vancomycin Intermediate Staphylococcus aureus (HVISA) and the issue of reduced vancomycin sensitivity are still unknown in Pakistan. This study was carried out to determine antimicrobial sensitivity and detect hVISA in clinical isolates of MRSA by Glycopeptide Resistance Detection (GRD) E test. This was a cross-sectional descriptive study, performed at the Pathology Department, Postgraduate Medical Institute, Lahore, from May 2014 to May 2015.

Methods: The study was carried out on 41 MRSA isolates from different specimens collected from patients admitted in Lahore General hospital, Lahore. Antimicrobial susceptibility testing was performed on all isolates according to modified Kirby-Bauer method and interpreted according to CLSI 2015 criteria. After screening for methicillin resistance by cefoxitin disc method, MIC of vancomycin was determined by standard E test. Isolates with a vancomycin MIC of 1-2 µg/ml were screened for heteroresistance by Glycopeptide Resistance Detection (GRD) E-test.

Results: Majority of the MRSA isolates were recovered from pus 26 (63.4%) followed by tissue sample 04 (9.8%) and blood 03 (7.3%), two each form CVP tip, bronchoalveolar lavage and catheter tip (4.9%) and one each from CSF and pleural fluid (2.4%). Among the antibiotics, resistance to gentamicin was highest among MRSA isolates (73%), followed by TMP-SMZ (68%), ciprofloxacin (66%), erythromycin (51%), clindamycin (44%) and doxycycline (41%). All MRSA isolates in our study were sensitive to linezolid. Four (9.75%) MRSA isolates were confirmed as hVISA by E test GRD.The frequency of hVISA among MRSA isolates was higher in blood specimens (66.6%) followed by pus (7.69%).

Conclusion: The issue of heteroresistance is still unknown in Pakistan. This study is the first attempt at detecting heteroresistance VISA (hVISA) in MRSA isolates. Patients suffering from MRSA infection should be screened for hVISA so that prompt antimicrobial therapy can be initiated in order to avoid treatment failures. Further studies are required to address this issue and its clinical relevance in our country.

Key words: MRSA, heteroresistance, vancomycin, and GRD E test.

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) is the prototype of multi-resistant bacterial pathogens and is a major cause of hospital-acquired infections globally.[1] Vancomcyin has served as a keystone antibiotic for the treatment of serious MRSA infections for the last twenty years.[2]

Reduced Vancomycin susceptibility was first reported in Japan in 1996 and was designated as Vancomycin intermediate susceptible *Staphylococcus aureus* (VISA). Six years later, the first Vancomycin resistant *Staphylococcus aureus* (VRSA) was reported in the United States and since then, there have been 13 confirmed cases of VRSA worldwide.[3-5]

Another population of *Staphylococcus aureus*, known as heteroresistant VISA emerged in 1996, which is defined as the presence of subpopulation of VISA within the population of MRSA at the rate of one organism per 10^5 to 10^6 organisms.[3,7]

hVISA infection is associated with prolonged fever and bacteremia thus lengthening hospital stay and an inability of vancomycin to cure the infection.[8]

Major factors leading to emergence of VISA and hVISA phenotypes are cell wall changes, which include reduced turnover of the cell wall, decreased autolysis, and in certain instances, triggered cell wall synthesis. All of these events lead to decreased access of vancomycin to its functioning site, which is confined at the division septum.[9]

Infection caused by hVISA pose a clinical chall-

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enge, as these bacteria are usually not detected in the laboratory because these are considered vancomycin susceptible by routine MIC tests.[7]

Standardized susceptibility methods, such as CLSI broth micro dilution, agar dilution and standard E test methods fail to detect heteroresistant VISA probably due to small inoculums used or the relatively poor growth support on Mueller-Hinton agar plates, or a combination of both.[10]

Detection of hVISA, therefore, relies on the testing of a higher inoculum and techniques to promote the growth of this sub-population, such as prolonged incubation (usually 48 hours) or use of more nutritive media (e.g. brain heart infusion agar and Mueller-Hinton agar with 5% blood).[9]

PAP-AUC has been considered a gold standard but it is time-consuming, laborious and not suitable for routine microbiology laboratory.[1]

Alternatively, various screening methods, including macro E test method (MET), Glycopeptide Resistance Detection (GRD) E test and agar screening methods have been developed.[9]

The burden of heteroresistant Vancomycin Intermediate *Staphylococcus aureus* and the issue of reduced vancomycin sensitivity are still unknown in Pakistan.

Therefore, our study was aimed at detection of heteroresistance among clinical isolates of MRSA using GRD E test.

METHODOLOGY

The present study was carried out on 41 consecutive isolates of *Staphylococcus aureus* recovered from various clinical specimens including pus, blood, wound swab, urine, sputum and aspirates of patients admitted in Lahore General Hospital, Lahore.

All specimens were brought to microbiology laboratory, Department of Pathology, PGMI, Lahore for culture and sensitivity within two hours of collection. These specimens were inoculated on blood agar and MacConkey agar plates and incubated at 37°C for 24 hours.

Preliminary identification of *Staphylococcus aureus* was done by observing the colony morphology on blood agar plates and Gram staining. The isolates were subjected to catalase and coagulase tests for confirmation.

Antimicrobial susceptibility testing was performed on all isolates according to modified Kirby-Bauer method and interpreted according to CLSI 2015 criteria.

Methicillin resistance was detected by using cefoxitin disc diffusion method. MRSA ATCC 43300 and MSSA ATCC 25923 control strains were used. Interpretation was done according to CLSI criteria 2015.

All MRSA isolates were subjected to E test for determination of Vancomycin MIC. The MICs were interpreted according to CLSI guidelines 2015.

All MRSA isolates having MIC ≤ 2 µg/ml as determined by Vancomycin E strips were subjected to further testing for heteroresistance by using Glycopeptide Resistance Detection (GRD) E test.

1. Glycopeptide Resistance Detection (GRD) E test:

E-test GRD is a double-sided predefined gradient (0.5-32 µg/ml) of vancomycin and teicoplanin for the detection of VISA or hVISA phenotypes. MIC test strip GRD consists of a screening method and can be tested with 0.5 McFarland and Mueller-Hinton blood agar plates.

GRD E test strips, manufactured by Liofilchem Diagnostics were used. The strips were stored at -20°C.

A 0.5 McFarland turbidity standard suspension prepared from well isolated colonies of the bacterial isolates was swabbed onto the surface of Mueller-Hinton blood agar plates.

GRD E strips were applied on the Mueller-Hinton blood agar plates and incubated at 35°C for up to 48 hours.

The zone of the GRD E test strip was read at 24 hour and 48 hour of incubation. MIC showing complete inhibition of growth was recorded.

The isolate was considered a potential hVISA if the MIC was ≥ 8 µg/ml for either vancomycin or teicoplanin (Figure 1).

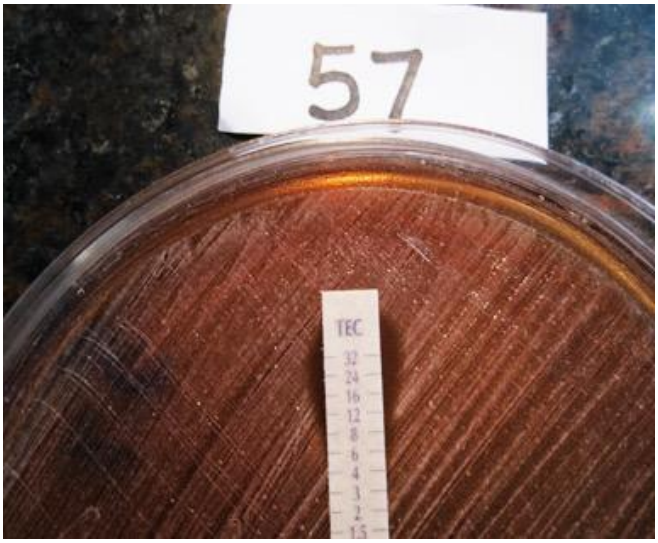

Fig. 1: *E test GRD showing a positive test (hVISA) MIC= 8 µg/ml*

Reference control strains used with each batch testing include ATCC 29213 (VSSA), ATCC 700698 (hVISA; Mu3) and ATCC 700699 (VISA; Mu50).

Data was entered and analyzed in SPSS 20.0 version. Continuous numerical variables like age are described as mean and standard deviation while categorical descriptive variables like gender, heteroresis-

tance, isolates with methicillin resistant *S. aureus* were presented as frequencies and percentages.

RESULTS

All isolates were recorded as Gram-positive, catalase and coagulase test positive and were resistant to cefoxitin.

The distribution of MRSA isolates among various clinical specimens was that majority of the isolates were recovered from pus 26 (63.4%) followed by tissue sample 4 (9.8%) and blood 3 (7.3%), two each form CVP tip, bronchoalveolar lavage and catheter tip (4.9%) and one each from CSF and pleural fluid (2.4%).

The resistance pattern of MRSA isolates to other antibiotics is shown in Table 1.

Table 1: *Antibiotic Resistance pattern of MRSA isolates (n = 41)*

Drugs	*Resistant*	
	Number	*Percentage*
Penicillin	41	100
Erythromycin	21	51.2
Clindamycin	18	43.9
Doxycycline	17	41.5
Gentamicin	30	73.2
TMP-SMZ	28	68.3
Ciprofloxacin	27	65.9
Linezolid	0	0

All the strains were 100% resistant to penicillin. Among other antibiotics, resistance to gentamicin was highest among MRSA isolates (73%), followed by TMP-SMZ (68%), ciprofloxacin (66%), erythromycin (51%), clindamycin (44%) and doxycycline (41%). All MRSA isolates in our study were sensitive to linezolid.

The frequency distribution of MIC values of vancomycin for 41% isolates of MRSA is shown in Figure 2. Twenty-nine (71%) isolates had a vancomycin MIC of 2 µg/ml, whereas for 12 (29%) isolates, the vancomycin MIC was 1 µg/ml.

The results of E test GRD are shown in Table 2. An isolate showing MIC value of ≥ 8 µg/ml was considered a possible hVISA. Four (9.75%) MRSA isolates were hVISA positive, whereas 37 (90.2%) had a MIC less than 8 µg/ml and therefore were taken as hVISA negative.

The frequency of hVISA among MRSA isolates was higher in blood specimens (66.6%) followed by pus (7.69%). However, the difference was statistically non-significant.

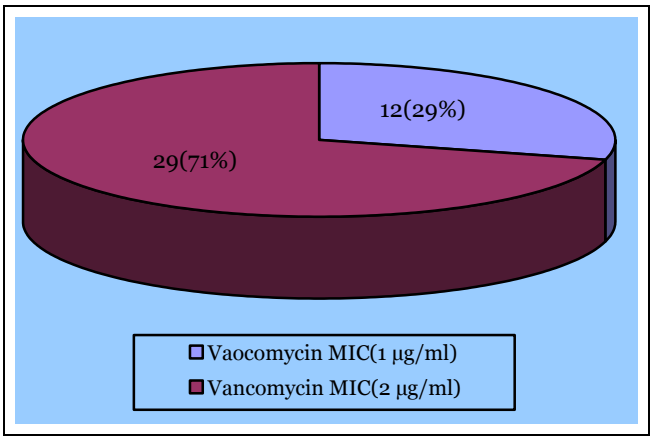

Fig. 2: *Distribution of MRSA isolates according to MIC for Vancomycin (n = 41).*

Table 2: *Frequency of hVISA among MRSA isolates as determined by E test GRD (n = 41).*

hVISA by Etest GRD	*No. of Isolates*	*Percentage (%)*
Positive*	04	9.75
Negative**	37	90.2

*MIC ≥ 8 µg/ml **MIC ≤ 8 µg/ml

DISCUSSION

Methicillin resistant Staphylococcus aureus (MRSA) is the prototype of multi-resistant bacterial pathogens and is a major cause of hospital-acquired infections globally. The emergence and spread of MRSA has changed drastically over the past decade. MRSA is not only resistant to methicillin, but it is also multi-drug resistant.[1]

The last ten years has witnessed a remarkable rise in cases of MRSA in Pakistan.[11-13] Although the incidence of MRSA differs from one region to another, Safder et al in 2003 reported the incidence of MRSA in various hospitals of Pakistan in a range of 10 – 65%.[14]

Multiple phenotypes with decreased sensitivity to glycopeptides are on the rise due to increased vancomycin usage in critically ill patients with MRSA infections.[15] It is possible that vancomycin treatment failures against putatively vancomycin-susceptible *Staphylococcus aureus* are due to undiagnosed VISA or hVISA.[16]

The resistance pattern of MRSA isolates to various antibiotics is shown in Table 1. Antimicrobial susceptibility testing was performed by modified-Kirby Bauer method. All the strains were 100% resistant to penicillin. Among other antibiotics, resistance to gentamicin was highest among MRSA isolates (73%), followed by TMP-SMZ (68%), ciprofloxacin (66%), erythromycin (51%), clindamycin (44%) and doxycycline (41%). All MRSA isolates in our study were sensitive to linezolid.

Similar findings were reported in studies carried out by different researchers in Pakistan as well as abroad. MRSA was generally multidrug resistant with increased resistance to gentamicin, ciprofloxacin, erythromycin and clindamycin.[17,18]

In our study, 12 (29%) isolates showed a vancomycin MIC of 1 µg/ml while remaining 29 (71%) isolates showed a vancomycin MIC of 2 µg/ml. Vancomycin MICs distribution among MRSA isolates can vary among institutions. Edwards et al and Joana et al reported majority of MRSA isolates in their study showing a vancomycin MIC in the range of 1 µg/ml with a lesser percentage inhibited at 2 µg/ml.[19,20] The trend of increasing vancomycin MIC is alarming because high vancomycin MICs in the susceptible range has been associated with treatment failures and poor clinical outcomes.[21] Researchers in Pakistan have observed similar trends of increasing vancomycin MIC.[22,23]

A number of laboratory tests have been used to detect hVISA. These include Macro E test method (MET), which uses a higher inoculum (2 McFarland standard), screening agars containing varying concentration of vancomycin, specialized MIC test strips (E test GRD) and population analysis profile- area under the curve (PAP-AUC).[9]

The present study employed E test GRD for the detection of heteroresistance among Methicillin-resistant *Staphylococcus aureus* in our setup.

Four MRSA isolates were hVISA positive (9.75%) as determined by GRD E test.

The rates of hVISA vary from region to region. In Australia, hVISA and VISA have been increasingly reported since 2001 and range from 9.3% to 12%.[24]

Wang et al, at their institution in China in 2013 detected 20.2% hVISA among 122 MRSA isolates.[25] In France, Filleron et al (2011) detected 8.3% hVISA among cystic fibrosis patients who were either colonized or infected with MRSA.[26]

Prevalence of hVISA was 4.6% at a Malaysian hospital.[21] The prevalence of hVISA in a study conducted by Chaudhari et al in India in 2014 was 6.9% amongst 58 MRSA isolates.[27]

More studies at different institutions are required in order to define the burden of hVISA in our country and its clinical implications. As there is no single genetic mechanism for detection of hVISA, we have to rely on the phenotypic detection methods. It is the need of time to establish methods for detection of hVISA and determining its prevalence rates, especially isolates from patients in whom vancomycin therapy fails to treat the infection.

It is ***concluded*** that the phenomenon of heteroresistant VISA is still unknown in Pakistan. This study is the first attempt at detection of hVISA among Methicillin resistance Staphylococcus aureus. Our study detected four hVISA among 41 MRSA isolates with a vancomycin MIC in the susceptible range (≤ 2 µg/ml). Further research should be conducted at various institutions to find out the burden of this problem in our country. Patients suffering from MRSA infection should be screened for hVISA so that prompt antimicrobial therapy can be initiated in order to avoid treatment failures.

Owing to the excessive usage of vancomycin therapy for infections caused by MRSA, screening for hVISA should be made part of the infection control practice.

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Authors' Contribution

SQ: Data collection, literature search, materials & methods, result writing, contributed to discussion writing. IJ: Conceived the idea, contributed in literature search, materials & methods, result and discussion writing. SM: Contributed in literature search, writing results based on analysis. MSA: Analyzed the data, reviewed the manuscript and gave expert opinion.

REFERENCES

1. Alsulami SO, Al-Doghairther HA, Iyer AP. Detection of Methicillin Resistant *Staphylococcus aureus* isolated from Nasal Carriage of Health Care Workers by Polymerase chain Reaction. J Environ Agric Sci. 2017; 5 (3): 288-293.
2. Phillips CJ, Wells NA, MaQrtinello M, Smith S, Woodman RJ, Gordon DL. Optimizing the detection of methicillin-resistant *Staphylococcus aureus* with elevated vancomycin minimum inhibitory concentrations within the susceptible range. Infect Drug Res. 2016; 9: 87-92.
3. Hiramatsu K, Aritaka N, Hanaki H, Kawasaki S, Hosoda Y, Hori S, Fukuchi Y, Kobayashi I. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. The Lancet. 1997 Dec. 6; 350 (9092): 1670-3.
4. Mirza HC, Sancak B, Gür D. The Prevalence of Vancomycin Intermediate *Staphylococcus aureus* and Heterogenous VISA among Methicillin-Resistant Strains Isolated from Pediatric Population in a Turkish University Hospital. Microb Drug Resist. 2015; 21 (5): 537-544.
5. Walters M, Lonsway D, Rasheed K, Albrecht V, McAllister S, Limbago B, Kallen A. Investigation and control of Vancomycin-resistant *Staphylococcus aureus*: A guide for Health Departments and Infection Control Personnel. 2015. Available at: *http://www.cdc.gov/hai/pdfs/VRSA-Ivestigation-Guide-05_12_2015.pdf*.
6. Monaco M, Pimentel de Arujo F, Cruciani M, Coccia EM, Pantosi A. Worldwide Epidemiology and Antibiotic Resistance of *Staphylococcus aureus*. In: Current topics in Microbiology and Immunology. Springer, Berlin, Heidelberg. 2016: https:// doi.org/10.1007/82_2016_3.

7. Satola SW, Farley MM, Anderson KF, Patel JB. Comparison of detection methods for heteroresistant vancomycin-intermediate *Staphylococcus aureus*, with the population analysis profile method as the reference method. J Clin Microbiol. 2011 Jan 1; 49 (1): 177-83.
8. Leonard SN, Rossi KL, Newton KL, Rybak MJ. Evaluation of the E-test GRD for the detection of *Staphylococcus aureus* with reduced susceptibility to Glycopeptides. J Antimicrob Chemother. 2009 Mar; 63 (3): 489-492.
9. Howden BP, Stinear TP, Allen DL, Johnson PD, Ward PB, Davies JK. Genomic analysis reveals a point mutation in the two-component sensor gene graS that leads to intermediate vancomycin resistance in clinical Staphylococcus aureus. Antimicrob Agents Chemother. 2008 Oct. 1; 52 (10): 3755-62.
10. Ford BA. Identification of Low-Level Vancomycin Resistance in *Staphylococcus aureus*in the Era of Informatics. J Clin Microbiol. 2016; 54 (4): 836-839.
11. Khan S, Rasheed F, Zahra R. Genetic Polymorphism of *agr* Locus and Antibiotic Resistance of *Staphylococcus aureus* at two hospitals in Pakistan. Pak J Med Sci. 2014 Jan-Feb; 30 (1): 172-176.
12. Kaleem F, Usman J, Sattar A, Amanat ST, Hassan A, Omair M, Khalid A, Riaz M. Current status of vancomycin susceptibility against methicillin resistant *Staphylococcus aureus* (MRSA) strains: A study at two tertiary care hospitals of Pakistan. Afr J Microbiol Res. 2012 Aug. 30; 6 (33): 6243-6.
13. Riffat M. Prevalence, Antibiotic Susceptibility Pattern and Demographic Factors Related to Methicillin Resistant *Staphylococcus aureus* in Lahore, Pakistan. Int J Microbiol Adv Immunol. 2014 Jun. 23; 2 (3): 45-48.
14. Arshad MU, Akhtar RW, Irum S. Prevalence of MRSA containing *SCCmec* types isolated from a tertiary care hospital, Rawalpindi. J Infect Mol Biol. 2015; 3 (4): 81-85.
15. Fitzgerald JR. Evolution of *Staphylococcus aureus* during human colonization and infection. Infect Genet and Evol. 2014; 21: 542-547.b
16. Moreillon P, Bizzini A, Giddey M, Vouillamoz J, Entenza JM. Vancomycin-intermediate *Staphylococcus aureus* selected during vancomycin therapy of experimental endocarditis are not detected by culture-based diagnostic procedures and persist after treatment arrest. J Antimicrob Chemother. 2012 Mar. 1; 67 (3): 652-60.
17. Indian Network for Surveillance of Antimicrobial Resistance (INSAR) group. Methicillin resistant *Staphylococcus aureus* (MRSA) in India: Prevalence & susceptibility pattern. Ind J Med Res. 2013 Feb. 1; 1 37 (2): 363.
18. Bari F, Wazir R, Haroon M, Ali S, Ali I, Rahman H, Ullah A, Khattak AM, Qasim M. Frequency and Antibiotic Susceptibility Profile of MRSA at Lady Reading Hospital, Peshawar. Gomal J Med Sci. 2015 Mar. 31; 13 (1).
19. Edwards B, Milne K, Lawes T, Cook I, Robb A, Gould IM. Is vancomycin MIC "creep" method dependent? Analysis of methicillin-resistant *Staphylococcus aureus* susceptibility trends in blood isolates from North East Scotland from 2006 to 2010. J Clin Microbiol. 2012 Feb. 1; 50 (2): 318-25.
20. Joana S, Pedro P, Elsa G, Filomena M. Is vancomycin MIC creep a worldwide phenomenon? Assessment of *Staphylococcus aureus* vancomycin MIC in a tertiary university hospital. BMC Research Notes, 2013 Feb. 19; 6 (1): 65.
21. Sit PK, Teh CSJ, Idris N, Sam IC, Omar SFS, Sulaiman H, Thong KL, Kamarulzaman A, Ponnampalavanar S. Prevalence of Methicillin-resistant *Staphylococcus aureus* (MRSA) infection and the molecular characteristics of MRSA bacteremia over a two-year period in a tertiary teaching hospital in Malaysia. BMC Infect Dis. 2017; 17: 274. Doi.org/10.1186/s12879-017-2384-y.
22. Perveen I, Majid A, Kanwal S, Naz I, Sehar S, Ahmed S, Raza MA. Prevalence and antimicrobial susceptibility pattern of methicillin-resistant Staphylococcus aureus and coagulase-negative Staphylococci in Rawalpindi, Pakistan. Br J Med Med Res. 2013 Jan. 1; 3 (1): 198.
23. Arshad F, Javed I, Mushtaq S, Anwer S. Emergence of Vancomycin Intermediate *Staphylococcus aureus* (VISA) in a Tertiary Care Hospital in Lahore: An Alarming Situation. Intern J Pathol. 2015; 13 (3): 89-94.
24. da Costa TM, Morgado PGM, Calvalcante FS, Damasco AP, NouérSA, dos Santos KRN. Clinical and Microbiological characteristics of Heteroresistant and vancomycin-Intermediate *Staphylococcus aureus* from Bloodstream Infections in a Brazilian Teaching Hospital. PLoS ONE. 2016; 11 (8). Doi: 10.1371/journal.pone. 0160506.
25. Wang Y, Hu YJ, Ai XM, Xu HT, Sun TY. Prevalence and clinical prognosis of heteroresistant vancomycin-intermediate *Staphylococcus aureus* in a tertiary care center in China. Chin Med J. 2013 Feb; 126 (3): 505-9.
26. Filleron A, Chiron R, Reverdy ME, Jean-Pierre H, Dumitrescu O, Aleyrangues L, Counil F, Jumas-Bilak E, Marchandin H. Utility of the Etest GRD for detecting *Staphylococcus aureus* with reduced susceptibility to glycopeptides in cystic fibrosis patients. Eur J Clinical Microbiol Infect Dis. 2012 Apr. 1; 31 (4): 599-604.
27. Chaudhari CN, Tandel K, Grover N, Sen S, Bhatt P, Sahni AK, Praharaj AK. Heterogeneous vancomycin-intermediate among methicillin resistant *Staphylococcus aureus*. Med J Armed Forces India, 2015 Jan. 31; 71 (1): 15-8.