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Original Article

Prevalence and antimicrobial susceptibility pattern of methicillin-resistant, vancomycin-resistant, and Panton-Valentine leukocidin positive *Staphylococcus aureus* in a tertiary care hospital Dhaka, Bangladesh

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ABSTRACT

Objectives: To observe the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA), and Panton-Valentine leukocidin (PVL)-positive *S. aureus,* this study was carried out in a tertiary care hospital in Dhaka, Bangladesh.

Materials and methods: S. aureus strains were recovered from 200 postoperative wound swab samples from patients hospitalized in Dhaka Medical College Hospital between July 2011 and June 2012. Methicillin resistance was determined by the oxacillin and cefoxitin disc diffusion method, the minimum inhibitory concentration (MIC) of oxacillin, and *mecA* gene detection. VRSA resistance was determined by the disc diffusion method, the MIC of vancomycin, and screening for the *vanA* and *vanB* genes. The PVL gene was also detected in MRSA strains.

Results: Fifteen of the 44 isolated strains of *S. aureus* were MRSA (2 of them were VRSA) and 29 were methicillin-sensitive *S. aureus.* All MRSA isolates were highly resistant to oxacillin (MIC $\geq$ 256 μg/mL). When compared with polymerase chain reaction (PCR), the sensitivity and specificity of the oxacillin disc diffusion method were 93.33% and 100% respectively; for the cefoxitin disc diffusion method and MIC of oxacillin both the sensitivity and specificity were 100%. Four (26.67%) MRSA isolates were positive for PVL genes which were also *mecA* positive. The MRSA strains were highly resistant to ciprofloxacin (93.33%), ceftriaxone (86.63%), azithromycin (73.33%), gentamycin (73.33%), and amoxiclav (66.67%). All (100%) MRSA strains were sensitive to linezolid and 86.67% were sensitive to vancomycin. The VRSA strains had an MIC $\geq$256 μg/mL for vancomycin and were positive for the *vanB* gene but negative for the *vanA* gene.

Conclusion: The results of this study provide insight into the high proportion of MRSA and presence of VRSA in Bangladesh.

1. Introduction

Infections caused by *Staphylococcus aureus*, especially methicillin-resistant *S. aureus* (MRSA), are emerging as a major public health problem in hospital and community settings, causing a wide range of diseases. The emergence and spread of both health care and community-associated MRSA has made infection control intervention and treatment challenging [1].

MRSA has evolved after acquiring the *mecA* gene that encodes the penicillin-binding protein 2a which confers resistance to methicillin and other β-lactam antibiotics [2]. The *mecA* is a highly conserved gene found only in methicillin-resistant strains with no allelic equivalent in methicillin-susceptible *Staphylococci*, making it a useful marker for β-lactam resistance [3]. Detection of the *mecA* gene is the gold standard for identifying MRSA [4] but this test is not available in many clinical laboratories in developing countries and is relatively expensive. Different methods have been developed for the detection

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of MRSA including minimum inhibitory concentrations (MIC), the oxacillin screening agar method, and disc diffusion testing. The major problem in routine screening is that MRSA strains are heterogeneous in the expression of resistance to β-lactam agents [5].

S. aureus produces numerous virulence factors, including Panton-Valentine Leukocidin (PVL), which is a pore forming cytotoxin more often identified in community-associated MRSA strains than hospital-associated strains [6]. PVL-positive Staphylococcal infection typically presents as a life-threatening infection of the skin and soft tissues, bone, or joints, although it can also lead to necrotizing pneumonia, a severe and often fatal condition involving primarily young, healthy patients [3,6].

In 1996, VISA (vancomycin-intermediate *S. aureus*) was first reported in Japan [7], and then in 2002 vancomycin-resistant *S. aureus* (VRSA) was first reported in the USA [8]. Subsequent isolation of VRSA from different countries has confirmed that the emergence of these strains is a global issue [9–12].

The aim of this study was to determine the prevalence of MRSA and VRSA in postoperative wound swabs using antimicrobial susceptibility profiles and to detect the PVL and *mecA* genes in MRSA as well as the *vanA* and *vanB* genes in VRSA.

2. Materials and methods

2.1. Samples

Two hundred swab samples were collected from infected wounds from postoperative patients admitted to Dhaka Medical College Hospital (DMCH) in Bangladesh over a 12-month period from July 2011 to June 2012. The age and sex of patients were recorded.

2.2. Inclusion criteria

Wound swabs collected from patients admitted to DMCH irrespective of age, sex, and antibiotic intake. Urine samples received in the Department of Microbiology of DMCH for culture and sensitivity irrespective of age, sex, and history of antibiotic intake were also included.

2.3. Ethical issues

Informed written consents were taken from each patient prior to collecting samples. Approval was obtained from the Research Review Committee and Ethical Review Committee of Dhaka Medical College according to the Declaration of Helsinki and national and institutional standards.

2.4. Isolation of S. aureus

S. aureus was identified by observing opaque colonies on blood agar media, Gram's staining, positive catalase and coagulase tests, and mannitol fermentation on mannitol salt agar [13].

2.5. Antimicrobial susceptibility testing

Standard disc diffusion techniques as recommended by the Clinical Laboratory Standards Institute (CLSI) were performed for susceptibility testing of oxacillin, cefoxitin, gentamycin, cotrimoxazole, ciprofloxacin, ceftriaxone, amoxiclav, vancomycin, and linezolid (oxoid, UK) for all *S. aureus*. [14].

2.6. Detection of MRSA and VRSA by the disc diffusion technique

Screening for methicillin-resistance was determined using the Kirby-Bauer disc diffusion method with 1 μg oxacillin and 30 μg cefoxitin discs. VRSA was detected by a 30 μg vancomycin disc. Three to five were emulsified into 3 mL of sterile normal saline. The turbidity of the suspension was compared with the 0.5 McFarland turbidity standard and the suspension was incubated on Mueller–Hinton agar plates at 37°C for 24 hours. An inhibition zone diameter of $\leq$ 10 mm around the oxacillin disc was considered resistance; 11–12 mm indicated intermediate, and $\geq$ 13 mm was considered sensitive. For the cefoxitin disc, an inhibition zone diameter of $\leq$ 21 mm was considered resistance and $\geq$ 22 mm was considered sensitive [14].

2.7. Detection of MRSA by the MIC of oxacillin and VRSA by MIC of vancomycin

The MIC of oxacillin by the agar dilution method was determined according to CLSI guidelines. An MIC of oxacillin $\geq$ 4 μg/mL was considered MRSA and $\leq$ 2 μg/mL was considered methicillin-sensitive *S. aureus* (MSSA) [14]. For preparation of the oxacillin stock solution, a 500-mg base of oxacillin was added to 50 mL distilled water to a concentration of 10 mg/mL. For each plate, 50 mL Mueller–Hinton medium was prepared and impregnated with 10 μL, 20 μL, 40 μL, 80 μL, 160 μL, 320 μL, 640 μL, or 1280 μL of the oxacillin stock solution to achieve concentrations of 2 μg/mL, 4 μg/mL, 8 μg/mL, 16 μg/mL, 32 μg/mL, 64 μg/mL, 128 μg/mL, or 256 μg/mL per plate, respectively.

For detection of VRSA, an MIC of vancomycin $\geq$ 16 μg/mL was considered resistant, $\leq$ 2 μg/mL was sensitive, and 4–8 μg/mL was VISA [14]. A vial of a 500 mg base of commercially available vancomycin injection was added to 50 mL distilled water to a concentration of 10 mg/mL. For each plate, 50 mL sterile Mueller–Hinton agar was prepared and impregnated with 10 μL, 20 μL, 40 μL, 80 μL, 160 μL, 320 μL, 640 μL, or 1280 μL of vancomycin stock solution to achieve a concentration of 2 μg/mL, 4 μg/mL, 8 μg/mL, 16 μg/mL, 32 μg/mL, 64 μg/mL, 128 μg/mL, or 256 μg/mL per plate, respectively.

2.8. DNA extraction

DNA was extracted using the boiling method. Bacterial colonies were suspended in 300 μL of distilled water and boiled for 10 minutes in a heat block, then placed on ice for 5 minutes. After centrifugation at 13,000 × *g* at 4°C for 5 minutes, the supernatant was placed in a microtube and kept at 4°C until used as a DNA template [15].

2.9. Amplification of mecA and PVL genes

The *mecA* gene was amplified as described previously [16], using primers *mecA*-F: 5′-AAAATCGATGGTAAAGGTTGGC-3′ and *mecA*-R: 5′-AGTTCTGCAGTACCGGATTTTGC-3′. The DNA of the *S. aureus* ATCC 43300 and ATCC 25923 strains was used as positive and negative controls, respectively, for this polymerase chain reaction (PCR) assay of *mecA*. The PCR assay was performed in a total volume of 25 μL containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and 1 U of *Taq* DNA polymerase (Promega Corporation, USA). DNA amplification was carried out using the following thermal cycling profile: initial denaturation at 95°C for 10 minutes, 35 cycles of amplification (denaturation at 95°C for 45 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 1 minute), and a final extension at 72°C for 10 minutes in a thermal cycler (Mastercycler gradient, Eppendorf AG, Germany). PCR products were analyzed on 1.5% agarose gel with 0.53 Tris-borate-EDTA buffer. A 100-bp DNA ladder (Promega Corporation) was used

as the molecular size marker. The gels were stained with 1% ethidium bromide and visualized under UV light [16].

The PVL gene was amplified as described previously [6,15]. Primers *luk*SF- forward (5′- ATCATTAGGTAAAATGTCTGGA-CATGATCCA-3′) and *luk*SF- reverse (5′- GCATCAASTGTATTGGA-TAGCAAAAGC-3′) directed against the PVL S and F precursor genes (*luk*S/F-PV) were used. DNA amplification was carried out using the following thermal cycling profile: initial denaturation at 95°C for 10 minutes, 33 cycles of amplification (denaturation at 95°C for 30 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 1 minute), and a final extension at 72°C for 10 minutes in a thermal cycler (Mastercycler gradient, Eppendorf AG). The PCR products were analyzed on 1.5% agarose gel with 0.53 Tris-borate-EDTA buffer. A 100-bp DNA ladder (Promega Corporation) was used as the molecular size marker. The gels were stained with 1% ethidium bromide and visualized under UV light [15]. The DNA of the *S. aureus* ATCC 25923 strain was used as the negative control for the PVL gene assay.

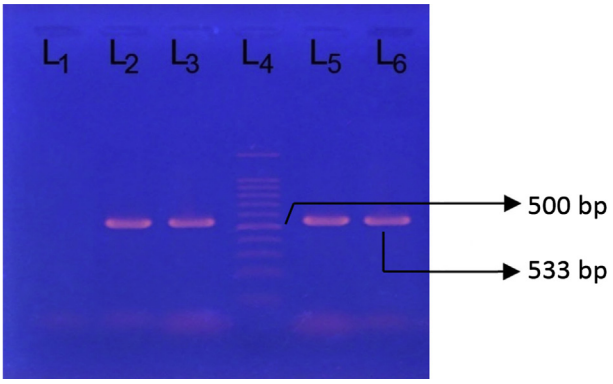

Figure 1. Amplified DNA of the *mecA* gene in *Staphylococcus aureus*. *Negative* control *S. aureus* ATCC 25923 (Lane 1) and positive control ATCC 43300 (Lane 2). Amplified DNA of 533 bp for the *mecA* gene (Lanes 3, 5, and 6). Hundred base pair DNA ladder (Lane 4).

2.10. Amplification of the vanA and vanB genes

The VRSA isolates were amplified using the primers for *vanA* (*vanA* F 5′-ATGAATAGAATAAAAGTTGCAATA-3′ and R 5′-CCCCTTTAACGCTAATACGATCAA-3′) and *vanB* (*vanB* F 5′- GTGA-CAAACCGGAGGCGAGGA-3′ and R 5′- CCGCCATCCTCCTGCAAAAAA-3′) [11,17]. The PCR assay was performed in a total volume of 25 μL containing 10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, 0.25mM each of deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and 1 U of *Taq* DNA polymerase (Promega Corporation). DNA amplification was carried out using the following thermal cycling profile: initial denaturation at 95°C for 10 minutes, 33 cycles of amplification (denaturation at 95°C for 45 seconds, annealing at 54°C for 45 seconds, and extension at 72°C for 1 minute), and a final extension at 72°C for 10 minutes in a thermal cycler (Mastercycler gradient, Eppendorf AG). The PCR products were analyzed on 1.5% agarose gel with 0.53 Tris-borate-EDTA buffer. A 100-bp DNA ladder (Promega Corporation) was used as the molecular size marker. The gels were stained with 1% ethidium bromide and visualized under UV light [11].

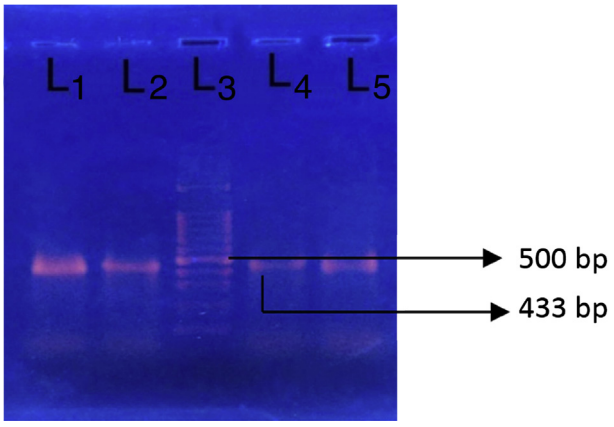

Figure 2. Amplified DNA of the PVL gene in *Staphylococcus aureus*. Amplified DNA of 433 bp for the PVL gene (Lanes 1, 2, 4, and 5). Hundred base pair DNA ladder (Lane 3). PVL = Panton-Valentine leukocidin.

3. Results

Fifteen of the 44 isolated strains of *S. aureus* (34.09%) were MRSA as identified by the cefoxitin disc diffusion method and the MIC of oxacillin, and all of them were *mecA* positive on PCR. Fourteen (31.82%) strains were resistant to methicillin based on the oxacillin disc diffusion method. Four (26.67%) of the 15 *mecA* positive strains were also positive for the PVL gene. MRSA colonization was highest in patients aged between 40 years and 55 years with male and female distributions of 73.33% and 26.67%, respectively.

Representative PCR amplified *mecA*, PVL, and *vanB* genes are shown in Figs. 1–3, respectively. The sensitivity, specificity, positive predictive value, and negative predictive value of the three phenotypic methods in comparison with the molecular method are shown in Table 1. The cefoxitin disc diffusion method was superior to the oxacillin disc diffusion method for detection of MRSA and was comparable to the MIC of oxacillin.

All the isolates were susceptible to linezolid. MRSA strains showed higher resistance than the other strains to all antimicrobials except for vancomycin (Table 2). All (100%) of the PVL positive strains were sensitive to amoxiclav, vancomycin, and linezolid, three (75%) were sensitive to azithromycin, two (50%) were resistant to ceftriaxone and gentamycin, and three (75%) were resistant to ciprofloxacin.

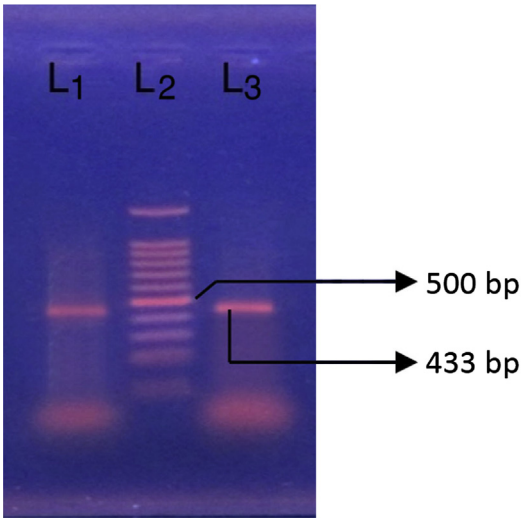

Figure 3. Amplified DNA of the *vanB* gene in *Staphylococcus aureus*. Amplified DNA of 433 bp for the *vanB* gene (Lanes 1 and 3). Hundred base pair DNA ladder (Lane 2).

Table 1
Sensitivity, specificity, PPV, and NPV of disc diffusion methods and MIC compared with *mecA* gene for detection of MRSA.

Methods	No. of MRSA	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Oxacillin disc diffusion	14	93.33	100	100	96.67
Cefoxitin disc diffusion	15	100	100	100	100
MIC of oxacillin	15	100	100	100	100

MIC = minimum inhibitory concentrations; MRSA = methicillin-resistant *Staphylococcus aureus*; NPV = negative predictive value; PPV = positive predictive value.

Table 2
Antimicrobial susceptibility pattern of *Staphylococcus aureus* and MRSA strains.

Antibiotics	*Staphylococcus aureus* ($n = 44$)		MRSA ($n = 15$)	
	Resistant	Sensitive	Sensitive	Resistant
Ceftriaxone	15 (34.10)	29 (65.90)	2 (13.33)	13 (86.67)
Ciprofloxacin	25 (56.82)	19 (43.18)	1 (6.67)	14 (93.33)
Azithromycin	18 (40.91)	26 (49.09)	4 (26.67)	11 (73.33)
Amoxiclav	13 (29.55)	31 (70.45)	5 (33.33)	10 (66.67)
Gentamycin	14 (31.82)	30 (68.18)	4 (26.67)	11 (73.33)
Oxacillin	14 (31.82)	30 (68.18)	1 (6.67)	14 (93.33)
Cefoxitin	15 (34.10)	29 (65.90)	0 (0.00)	15 (100.00)
Vancomycin	2 (4.55)	42 (95.45)	13 (86.67)	2 (13.33)
Linezolid	0 (0.00)	44 (100.00)	15 (100.00)	0 (0.00)

Data are presented as *n* (%).
MRSA = methicillin-resistant *Staphylococcus aureus*.

The MIC for vancomycin of one of the two VRSA strains was > 256 μg/mL and the other was 256 μg/mL. Both were positive for the *vanB* gene and neither was positive for the *vanA* gene. Both VRSA strains were sensitive to linezolid, one was sensitive to gentamycin and the other was sensitive to azithromycin (Table 3). No VISA was observed.

4. Discussion

Infections caused by drug resistant *S. aureus* are increasing worldwide due to the emergence of MRSA and VRSA. The prompt and accurate detection of MRSA and VRSA isolates could be useful for determining the appropriate treatment strategy and subsequent effective management and control of corresponding infections.

We found that the prevalence of MRSA was 34.09%, which correlates with previous reports from other regions of Bangladesh, Pakistan, and India [18–21]. However, some studies have reported much higher rates of 64–88% [17,22]. The exact cause of the relatively low prevalence of MRSA in the present study in comparison to other countries is not known but it is possible that the prevalence of MRSA is still low in Bangladesh. Moreover, the samples in the present study were taken from postoperative patients with infected wounds. The prevalence could have been higher if the samples had been taken from patients in the burn unit or casualty ward. The prevalence of MRSA varies significantly in different regions, which suggests a need for periodic evaluation of MRSA [23]. The increasing prevalence of MRSA is a threat to management of cases all over the world, including Bangladesh.

Table 3
Antimicrobial resistance pattern of VRSA strains.

Patient	MIC of VA (μg/mL)	Antimicrobials								
		CIP	CRO	AZM	CN	AMC	OX	FOX	VA	LZD
1	>256	R	R	R	S	R	R	R	R	S
2	256	R	R	S	R	R	R	R	R	S

AMC = amoxiclav; AZM = azithromycin; CIP = ciprofloxacin; CN = gentamycin; CRO = ceftriaxone; FOX = cefoxitin; LZD = linezolid; MIC = minimum inhibitory concentration; OX = oxacillin; VA = vancomycin.

As described in other studies [24,25], our MRSA isolates were resistant to most commonly used antibiotics. All MRSA isolates were sensitive to linezolid and 86.67% were sensitive to vancomycin, similar to other studies. This suggests that these drugs could be suitable treatment options [19,20,26]. As these drugs are expensive and sometimes not available in the local pharmaceutical market, treatment options in Bangladesh are limited for infections caused by MRSA.

In this study, MRSA strains had an MIC of oxacillin ≥ 256 μg/mL. Some authors from Iran reported that most MRSA strains are highly resistant to oxacillin (MIC ≥ 256 μg/mL), similar to our study [17]. The higher MIC of oxacillin in the present study might be due to the fact that common antibiotics are sold over the counter in Bangladesh and anybody can buy them without a doctor's advice. This might cause development of resistance to antibiotics.

Some authors from Bangladesh reported a PVL positive *S. aureus* rate of 15.25%, but no PVL was detected in MRSA strains [27]. However, in this study, the PVL gene was detected in four (26.67%) of the MRSA isolates which were hospital acquired MRSA. In the present study, however, MSSA strains were not screened for the PVL gene. Therefore, a surveillance mechanism should be set up to detect PVL genes in both MRSA and MSSA in Bangladesh. Usually PVL positive strains are found in community acquired MRSA but the PVL gene is not restricted to community acquired strains [28]. Some authors from India reported that 68–85% of MRSA strains were positive for the PVL gene, which is much higher than in the present study [21,29]. The higher percentages in those studies might be due to misuse of antibiotics causing selective pressure for development of resistant strains along with the PVL virulence factor in those countries. The presence of PVL is associated with increased virulence of certain strains of *S. aureus* and is the cause of necrotic lesions involving the skin and mucosa which are very difficult to manage, such as necrotic hemorrhagic pneumonia. Therefore, early detection of the PVL gene in *S. aureus* may be important to manage cases and to assess the outcome after treatment.

In the present study, both the sensitivity and specificity of the cefoxitin disc diffusion method and the MIC of oxacillin were 100% when compared with PCR and these results are similar to those in other studies [30–32]. The sensitivity and specificity of the oxacillin disc diffusion method were 93.33% and 100% respectively in the present study, which correlates with another study [33]. We showed the cefoxitin disc diffusion method correlated better with the presence of the *mecA* gene. Cefoxitin is a potent inducer of the *mecA* regulatory system and is being widely used as a marker for *mecA* gene-mediated methicillin resistance [4]. Some reports showed almost no discrepancy between cefoxitin susceptibility testing and PCR results for the *mecA* gene [23,34]. The oxacillin disc diffusion method is used to identify MRSA in most laboratories in Bangladesh. However, false negative as well as false positive results may be obtained using this method. This problem can be overcome by using cefoxitin discs instead of oxacillin discs to detect MRSA.

VRSA had not been detected previously in Bangladesh but the present study showed its emergence, which is a problem in treatment. Both VRSA isolates in the present study were resistant to several antimicrobials, but not linezolid, which is in accordance with other reports [10]. In the present study, one VRSA strain had an MIC of vancomycin of 256 μg/mL and the other was > 256 μg/mL, whereas VRSA isolated in other countries had MICs 32–1024 μg/mL [9–11,35]. In the present study, however, VRSA strains were not tested for MICs > 256 μg/mL. Most VRSA isolated worldwide carries the *vanA* gene. However, in our study, neither strain was positive for

the *vanA* gene but both strains were positive for the *vanB* gene. Some authors from India, Cairo, and Iran have reported *vanB* gene-positive VRSA [12,36,37]. The prevalence of the *vanA* gene among VRSA isolates has been reported in some studies worldwide. The reason behind the absence of *vanA* gene in the present study might be differences in the geographical distribution of *van* genes in VRSA. The low number of isolated VRSA strains might also be the reason. In the present study, no VISA was identified.

5. Conclusion

Multidrug-resistant *S. aureus,* including MRSA, is increasing at DMCH in Bangladesh. Some of the MRSA are PVL positive. To the best of our knowledge, this is the first report of VRSA identified by phenotype, the MIC of vancomycin, and *vanB* gene detection in Bangladesh. A decreased sensitivity of MRSA and VRSA to commonly used antibiotics has been observed. This study suggests that efficient control protocols should be adopted in hospitals to prevent the transfer of these strains between patients. Moreover, the rational use of effective antibiotics and prevention of their sale over-the-counter should also be considered.

Acknowledgments

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