

The Diagnostic Capacity of Three Phenotypic Techniques of Extended-Spectrum β -Lactamase Detection

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Article history:

Received: 27 Jan. 2022

Accepted: 16 Mar. 2022

Published: 29 Mar. 2022

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Abstract

Background: Early detection of extended-spectrum β -lactamases (ESBLs) producing bacteria is critical for infection prevention and control. Numerous phenotypic approaches and automated systems have been developed for detecting ESBL bacteria. However, there is a scarcity of data in Ethiopia regarding the most reliable, simple, and cost-effective methods for detecting ESBL-producing bacteria. This study, therefore, aimed to evaluate the diagnostic performance of three phenotypic approaches for detecting ESBL-producing bacteria.

Methods: In this study, 117 isolates of *Klebsiella pneumoniae*, *Escherichia coli*, *Klebsiella oxytoca*, and *Proteus mirabilis* were examined. Cefotaxime (30 μ g) and ceftazidime (30 μ g) were used for screening ESBL enzymes. A screening breakpoints of ≤ 27 mm and ≤ 22 mm were used for cefotaxime (30 μ g) and ceftazidime (30 μ g), respectively, as per the Clinical and Laboratory Standards Institute (CLSI) guidelines. All 117 strains were further confirmed by the Vitek 2 compact, double disk synergy, ESBL Epsilon test, and combined disk method. The combined disk method was adopted as the reference method.

Results: Out of 117 isolates, 90 (86%) had zone diameters of ≤ 27 mm and ≤ 22 mm for cefotaxime (30 μ g) and ceftazidime (30 μ g), respectively. The reference method detected 76 (65%) ESBL isolates out of 117 ones. From among the three techniques (i.e., double disk synergy, Vitek 2 compact, and ESBL Epsilon test), the double disk synergy method demonstrated overall sensitivity and specificity of 97.4% and 97.6%, respectively. Vitek-2, cefotaxime, and ceftazidime Epsilon test indicated indeterminate results of 6.8%, 6.8%, and 5.1% respectively.

Conclusion: Double disk synergy was found to have the highest sensitivity and specificity for detecting ESBL isolates with no indeterminate results.

Keywords: Double disk synergy, Combined disk, Gradient strip, Vitek 2 compact

Please cite this article as follows: Abdeta A, Bitew A, Fentaw S, Tsige E, Assefa D, Tigabu E, et al. The diagnostic capacity of three phenotypic techniques of extended-spectrum β -lactamase detection. Avicenna J Clin Microbiol Infect. 2022; 9(1):1-7. doi:10.34172/ajcmi.2022.01

Background

Extended-spectrum β -lactamases (ESBLs) are enzymes that are produced by bacteria causing resistance to a wide range of antibiotics including penicillins, cephalosporins, and aztreonam (1). Bacteria that produce ESBLs may also be resistant to unrelated and crucial antibiotics such as aminoglycosides, trimethoprim-sulfamethoxazole, and ciprofloxacin, making it difficult to treat critical patients (2). Oftentimes the infections caused by ESBL-producing Enterobacterales are associated with poor treatment outcomes (1,3). ESBLs hydrolyze the extended-spectrum cephalosporins and are becoming more common among Enterobacterales (4). The new breakpoints developed by European Committee on Antimicrobial Susceptibility

Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) for extended-spectrum cephalosporins have reduced the possibility of classifying an ESBL-producing Enterobacterales as susceptible to extended-spectrum cephalosporins (5,6). Therefore, identifying ESBL producing Enterobacterales is not compulsory for clinical outcome forecasting. However, for the purposes of epidemiological and infection prevention, it is very important to reduce its dissemination as well as monitor the distribution progress and the effectiveness of prevention strategies (5,6). Numerous phenotypic approaches have been developed for detecting ESBL-producing bacteria (7-13). The rising incidences of ESBLs-producing bacterial strains necessitate the development of low-cost, accurate,



and simple testing techniques for detecting these enzymes in Enterobacterales isolates for epidemiological purposes. In Ethiopia, there is a scarcity of data regarding the most reliable, uncomplicated, and cost-effective techniques for the laboratory detection of extended-spectrum and lactamase-producing bacteria. As a result, the current study aimed to evaluate the diagnostic accuracy of three phenotypic approaches for detecting ESBL-producing bacteria.

Material and Methods

Study Design, Area, and Period

This cross-sectional study was conducted from January 5 to June 30, 2020, at the National Clinical Bacteriology and Mycology Reference Laboratory of Ethiopian Public Health Institute, in which *Klebsiella pneumoniae*, *Escherichia coli*, *Klebsiella oxytoca*, and *Proteus mirabilis* isolates recovered from clinical specimens were examined.

Sample Size Determination

The CLSI EP09-A3 recommended using at least 40 and preferably 100 samples to compare two laboratory methods. Therefore, a total of 117 above-mentioned isolates were collected in order to compare laboratory methods of ESBL detection (14).

Bacterial Culture and Identification

During the study period, 265 clinical samples were collected. In the event of sample transit delays, appropriate transport media were used. The isolates used in this investigation were recovered from blood, bodily fluids, wounds, sputum, and urine specimens. All specimens were inoculated onto appropriate culture media and cultured at proper temperatures and incubation time according to the per laboratory standard operating protocols (15). The isolates were identified by performing traditional biochemical tests: triple sugar iron agar (Liofilchem, Italy), oxidase strips (Liofilchem, Italy), Simon's citrate agar

(Liofilchem, Italy), and lysine iron agar (Liofilchem, Italy). Indole production and motility using sulfide-indole-motility medium (Liofilchem, Italy). Urease production using a urea agar base supplemented with 40% urea solution (Oxoid Ltd., England) (15). Also, 117 isolates of *E. coli*, *K. pneumoniae*, *P. mirabilis*, *K. oxytoca* isolates were isolated during the study period (Figure 1).

Detection of Extended-Spectrum β -lactamases Producing Isolates

Screening of ESBL of Isolates

The disk diffusion method was adopted by using both cefotaxime (30 μ g) (Oxoid Ltd., England) and ceftazidime (30 μ g) (Oxoid Ltd., England) as indicator cephalosporins. A screening breakpoints of ≤ 27 mm and ≤ 22 mm were used for doing cefotaxime (30 μ g) and ceftazidime (30 μ g), respectively, on Mueller–Hinton agar (Oxoid Ltd, England) according to CLSI M100 guidelines (5). All 117 isolates were tested by confirmatory tests.

ESBL Phenotypic Confirmation Methods

Combined Disk Method (Reference Method)

The combined disk method was employed as the reference method. Ceftazidime 30 μ g (Oxoid Ltd., England), Ceftazidime clavulanate (30/10 μ g) (Oxoid Ltd., England), Cefotaxime 30 μ g (Oxoid Ltd., England), and cefotaxime-clavulanate (30/10 μ g) (Oxoid Ltd., England) were used on Mueller–Hinton agar in accordance with the CLSI guidelines 2020 (5) (Table 1). The plates were incubated at 37°C for 16–18 hours, and ESBL producers were defined as a 5-mm increase in zone diameter for indicator cephalosporins containing clavulanate vs indicator cephalosporins alone (5) (Figures 2 and 3). *K. pneumoniae* ATCC[®] 700603 and *E. coli* ATCC[®] 25922 control strains were used for checking the quality of all antibiotic disks

Gradient Test Method/ Epsilometer Test

Strips containing cefotaxime/cefotaxime + clavulanic acid

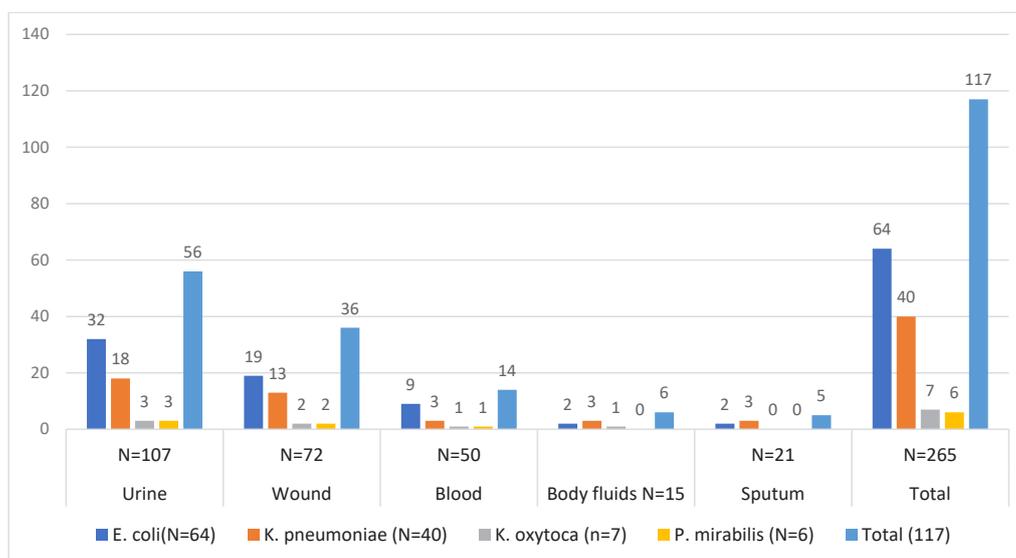


Figure 1. Distribution of Different Isolates Among Clinical Specimens.

Table 1. Distribution of ESBL Producing Enterobacteriales by Reference Method

Methods		<i>E. coli</i> (n=64)	<i>K. pneumoniae</i> (n=40)	<i>K. oxytoca</i> (n=7)	<i>P. mirabilis</i> (n=6)	Total (117)	Percent
CD CTX/CTL	ND	0	0	0	0	0	0.00
	Positive	39	30	3	3	75	64.10
	Negative	25	10	4	3	42	35.90
CD CAZ/CAL	ND	0	0	0	0	0	0.00
	Positive	40	30	3	3	76	65.00
	Negative	24	10	4	3	41	35.00

Abbreviations: CAZ, ceftazidime; CTX, cefotaxime; ND, Indeterminate; ESBL, extended-spectrum β -lactamase.

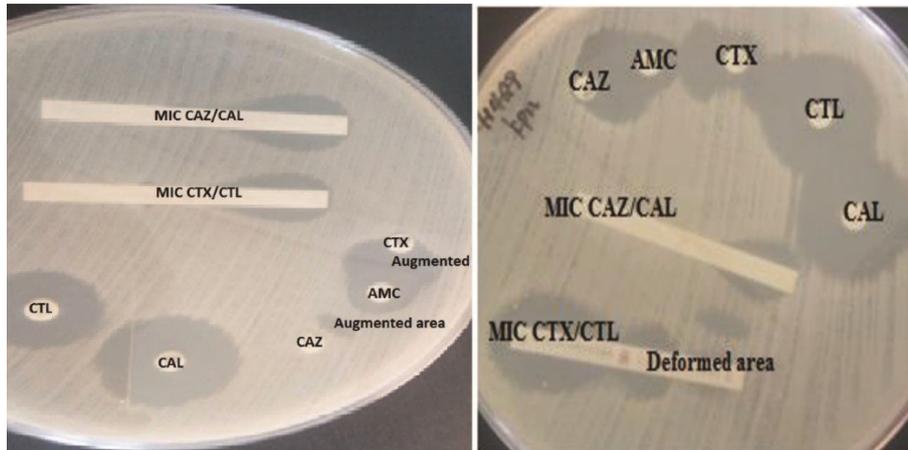


Figure 2. *Klebsiella Oxytoca* (left) and *Klebsiella Pneumoniae* (Right) Positive for ESBL by DDS, ESBL E-test/MIC, and Combination Disk Methods on Muller Hinton Agar. Abbreviations: MIC, minimum inhibitory concentration; CAZ, ceftazidime; CTX, cefotaxime; CAL, ceftazidime/ clavulanic acid; CTL, cefotaxime/ clavulanic acid; AMC, amoxicillin/clavulanic acid; DDS, double-disk synergy.

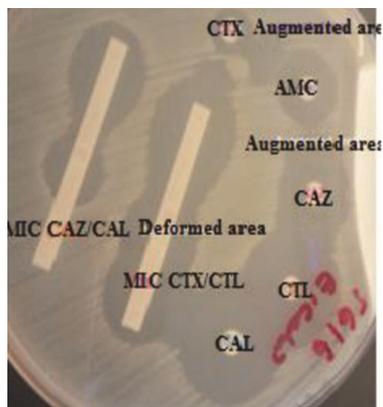


Figure 3. ESBL Positive *E. Coli* by Double Disk Synergy, ESBL E-test/MIC, and Combination Disk Methods. Abbreviations: MIC, minimum inhibitory concentration; CAZ, ceftazidime; CTX, cefotaxime; CAL, ceftazidime/ clavulanic acid; CTL, cefotaxime/ clavulanic acid; AMC, amoxicillin/ clavulanic acid; ESBL, Extended-spectrum β -lactamases.

and ceftazidime/ceftazidime + clavulanic acid (Liofilchem, Italy) were used. Cefotaxime ≥ 0.5 , cefotaxime/cefotaxime + clavulanic acid ratio ≥ 8 or CAZ ≥ 1 , CAZ/CAL ratio ≥ 8 or distortion of the cefotaxime, and ceftazidime were considered ESBL positive. Cefotaxime < 0.5 , or cefotaxime/cefotaxime+clavulanic acid ratio < 8 , or ceftazidime < 1 , or ceftazidime/ceftazidime + clavulanic acid ratio < 8 was considered ESBL negative. Cefotaxime > 16 , cefotaxime + clavulanic acid > 1 , ceftazidime > 32 , and ceftazidime + clavulanic acid > 4 were considered inconclusive results (16) (Figures 2 and 3).

Double-disk Synergy Test (DDST)

Third-generation cephalosporins (cefotaxime and ceftazidime) were applied along with a third-generation cephalosporins disk containing clavulanic acid (amoxicillin-clavulanic acid) (Oxoid Ltd., England) (Oxoid Ltd., England). Enhanced inhibition zones around either of the disks in the direction of the clavulanic acid disk were considered ESBL positive. As for cephalosporin 30 μ g disks, the gap between the disks was 20mm from center to center (6) (Figures 2 and 3).

VITEK 2 System (bioMérieux)

VITEK 2 system antimicrobial susceptibility testing cards were used for doing (AST-GN86) (bioMérieux, Durham, North Carolina, USA). The results were analyzed using Vitek-2 compact software version 7.0, and The result was interpreted by the Vitek-2 compact software knowledge data base called the advanced expert system, which is used to interpret the antimicrobial susceptibility testing results. (17).

Quality Control

Klebsiella pneumoniae ATCC 700 603 and *E. coli* ATCC 25 922 Standard American type culture collection strains were used as quality control strains.

Statistical Analysis

The detection power of each method was evaluated based on its sensitivity, specificity, and positive and negative

predictive values. Cohen's kappa value was used to measure the agreement between each phenotypic technique and reference method. Crosstabulation was used to calculate Cohen's kappa value and *P* value. Cohen's kappa values of 0.61-0.80 and 0.81-1.00 were used as good and very good agreement scores, respectively. *P* values ≤ 0.05 were considered statistically significant. The data were presented in tables and figures.

Results

In this study, 265 clinical specimens from five different clinical specimens were processed during the studied period. Out of the given number, 117 isolates of *E. coli*, *K. pneumoniae*, *P. mirabilis*, and *K. oxytoca* were recovered. *E. coli* was the most prevalent isolate, accounting for 64 (54.7%) of the isolates, followed by *K. pneumoniae* 40 (34.2%), *K. oxytoca* 7 (6%), and *P. mirabilis* 6 (5.1%). Out of 265 clinical specimens, most of the bacterial isolates were recovered from urine specimens (47.9%), then from wound (30.8%), blood (12%), body fluids (5%), and sputum (4.3%) (Figure 1). Out of 117 isolates, 90 (86%) had zone diameters of ≤ 27 mm and ≤ 22 mm for cefotaxime (30 μ g) and ceftazidime (30 μ g), respectively. Even though only 86 isolates showed reduced susceptibility to indicator cephalosporins (cefotaxime (30 μ g) and ceftazidime (30 μ g)), all 117 isolates were tested by confirmatory tests. The reference method

detected (n = 76/117, 65%) ESBL positive strains among 86 isolates with reduced susceptibility to cephalosporins (Table 1).

Vitek 2 Compact ESBL Results

Vitek 2 compact showed sensitivity (88.2%), specificity (82.9%), positive predictive value (90.5%), and negative predictive value (85%) with indeterminate results for 6.84% of the strains (Tables 2 and 3). Vitek 2 compact also showed a good measure of agreement with the reference method (kappa value = 0.709, *P* value = 0.001) (Table 3).

Double-Disk Synergy Test

DDST using ceftazidime and amoxicillin with clavulanic acid showed sensitivity (97.4%), specificity (97.6%), positive predictive value (98.7%), and negative predictive value (95.2%). DDST using cefotaxime and amoxicillin with clavulanic acid showed sensitivity (96.1%), specificity (97.6%), positive predictive value (98.6%), and negative predictive value (93.8%) (Table 3). The double-disk synergy showed a very good measure of agreement with the reference method (kappa value = 0.926-0.944, *P* value = 0.001) (Tables 2 and 3).

ESBL Detection Using E-test

The ESBL E-test ceftazidime/ceftazidime + clavulanic acid strips showed sensitivity, specificity, positive predictive

Table 2. Distribution of ESBL Among Enterobacterales by Other Methods

Methods		<i>E. coli</i> (n=64)	<i>K. pneumoniae</i> (n=40)	<i>K. oxytoca</i> (n=7)	<i>P. mirabilis</i> (n=6)	Total (117)	Percent
DDS CAZ	ND	0	0	0	0	0	0
	Positive	39	30	3	3	75	64.1
	Negeative	25	10	4	3	42	35.9
DDS CTX	ND	0	0	0	0	0	0
	Positive	39	30	2	3	74	63.2
	Negeative	25	10	5	3	43	36.8
ESBL E CAZ	ND	1	3	1	1	6	5.1
	Positive	37	28	3	3	71	60.7
	Negeative	26	9	3	2	40	34.2
ESBL E CTX	ND	2	3	2	1	8	6.8
	Positive	37	27	3	3	70	59.8
	Negeative	25	10	2	2	39	33.4
ESBL VITEK 2 AST GN-86	ND	3	2	2	1	8	6.8
	Positive	37	27	3	3	70	59.8
	Negeative	25	10	2	2	39	33.4

Abbreviations: DDS, double-disk synergy; CAZ, Ceftazidime; CTX, Cefotaxime; ND, Indeterminate; E, Epsilonometer; ESBL, Extended-spectrum β -lactamase.

Table 3. Statistical Analysis of Different Phenotypic Methods of ESBL Detection

Phenotypic Methods	%Sensitivity	%Specificity	%PPV	%NPV	Kappa Value	<i>P</i> Value
DDS CAZ	97.4	97.6	98.7	95.2	0.944	0.001
DDS CTX	96.1	97.6	98.6	93.8	0.926	0.001
Vitek 2	88.2	82.9	90.5	85	0.709	0.001
ECAZ	93.4	95.1	100	97.5	0.877	0.001
ECTX	90.8	92.7	98.6	97.4	0.827	0.001

Abbreviations: DDS, double-disk synergy; CAZ, ceftazidime; CTX, cefotaxime; E, Epsilonometer; PPV, positive predictive value; NPV, negative predictive value.

value, and negative predictive values of 93.4%, 95.1%, 100%, and 97.5%, respectively, and with indeterminate results for 5.1% of the strains. The sensitivity, specificity, positive predictive value, and negative predictive value of the cefotaxime/cefotaxime+clavulanic acid strips were 90.8%, 92.7%, 98.6%, and 97.4%, respectively, and with indeterminate results for 6.84% of the strains (Tables 2 and 3). The ESBL E-tests showed a very good measure of agreement with the reference method (kappa value = 0.827-0.877, P value = 0.001) (Tables 2 and 3).

Discussion

The new extended-spectrum cephalosporin and aztreonam breakpoints reduced the possibility of determining ESBL producers susceptible to these antibiotics. However, timely detection of ESBL-producing Enterobacterales is also very important for early designing and implementing control strategies (1,2).

Vitek 2 Compact System

In our study, the Vitek 2 compact method demonstrated a sensitivity and specificity of 88.2% and 82.9%, respectively. This result was in line with the findings from Wiegand et al study in which the sensitivity and specificity were 86% and 78%, respectively (13), and from Thomson et al study in which the sensitivity and specificity were 89% and 85%, respectively (18). However, following researchers reported sensitivity results that were comparable to ours, but documented higher specificity results: Singh et al: sensitivity 91.8% and specificity 97.24 % (19); Robin et al: sensitivity 91.8% and specificity 100% (20); and Young et al: sensitivity 92% and specificity 100% (21). The following researchers, on the other hand, reported higher sensitivity and specificity for Vitek 2 compact: Spanu et al: sensitivity 98.1% and specificity 99.7% (10); Hackman et al: sensitivity 98.5% and specificity 98.9% (22); Sorlózano et al: sensitivity 100% and specificity 99.3% (23); and Valenza et al: sensitivity 100% and specificity 96% (24). Garrec et al reported sensitivity and specificity of 50% to 79% (25) for different Vitek 2 cards, which were much lower than those found in our study. This inconsistency may have been attributed to the differences in geography, selected bacterial isolate groups, and adopted Vitek 2 compact cards.

ESBL E-tests

According to our study results, the sensitivity and specificity for ESBL E-tests were 92% and 98%, respectively, when both MIC ceftazidime and cefotaxime were used. Our results in this regard were consistent with findings from the studies by following researchers: Linscott and Brown: sensitivity 97% and specificity 94% (26); Singh et al: sensitivity 88.52% and specificity 100% (18); Brown et al: sensitivity 88.9% and specificity 100% (27); Cormican et al: 100% for both sensitivity and specificity (7); Ho et al: sensitivity 96% and specificity 100% (28); and Sorlózano et al: sensitivity 100% and specificity 99.3%. Our results,

however, were only partially consistent with findings from the studies by following researchers since they reported relatively lower sensitivity: Vercauteren et al: sensitivity 84% and specificity 100% (29); and Yang et al ESBL E-test: sensitivity 84.0% and specificity 100% (30). The inconsistency between the results may have been attributable to the utilized bacterial isolates, since we only used *K. pneumoniae*, *K. oxytoca*, *P. mirabilis*, and *E. coli*, unlike other researchers, the geographical difference. In the current study, ESBL E-test yielded indeterminate results of 5.1% to 6.8%, which were lower than the results in a study by Garrec et al (28) reporting indeterminate results of 11% to 49% for the ESBL E-test. This inconsistency may have been explained by the fact that they utilized other organisms in addition to *K. pneumoniae*, *E. coli*, *K. oxytoca*, and *P. mirabilis* as well as employed MIC cefepime strips, in addition to MIC ceftazidime and cefotaxime strips.

Double-Disk Synergy Methods

In this study, the overall sensitivity and specificity of the DDST were detected to be 97.37% and 97.56%, respectively. Our findings are partially consistent with the findings of other researchers who investigated the diagnostic capacity of double disk synergy methods using ESBL and non-ESBL producing bacteria (ranging from 79% to 97% and 94% to 100%, respectively), which were initially confirmed using PCR methods(11,23,24,31,32). However, our study findings contradicted the results from the studies by Yang et al reporting sensitivity of 96.0% and specificity of 69.2% for the double-disk synergy method (25), and by De Gheldre et al determining sensitivity of 89% and specificity of 92% (33). The observed inconsistency was likely due to the different Enterobacterales strains used (in this study, only *K. pneumoniae*, *K. oxytoca*, *P. mirabilis*, and *E. coli* were used, whereas *K. aerogenes*, *P. vulgaris*, *Providencia stuartii*, and others were applied in the studies by other researchers), regional differences, and so on.

Conclusion

In sum, Vitek-2 compact and ESBL E cefotaxime/cefotaxime with clavulanic acid yielded inconclusive findings of 6.8 percent. In addition, ESBL E ceftazidime/ceftazidime with clavulanic acid produced 5.1 percent indeterminate findings. However, the double-disk synergy method was shown to detect more ESBL producers with no indeterminate results. Therefore, it was concluded that the double-disk synergy method was more reliable, simpler, and less expensive method which also required no specialized equipment or considerable expertise.

Acknowledgments

The authors would like to appreciate the Ethiopian Public Health Institute for donating supplies for this study, as well as Dr. Adane Bitew and Dr. Eyasu Tigabu for their assistance.

Conflict of Interests

The authors declare that they have no conflict of interests.

Ethical Approval

The Ethical clearance of this study was obtained from the Ethical Review Committee of the Department of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University (DRERC/474/19/MLS). In addition, the Ethiopian Public Health Institute granted an official permission.

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