Clinical Microbiology

Performance of “RESIST-3 O.K.N. K-SeT” immunochromatographic assay for the detection of OXA-48 like, KPC, and NDM carbapenemases in Klebsiella pneumoniae in Turkey

Pınar Sağiroğlu a,b,*, Ufuk Hasdemir a, Gülşen Altıkanat Gelmez a, Burak Aksu a, Onur Karatuna c, Güner Söyletir a

a Marmara University School of Medicine, Department of Medical Microbiology, Istanbul, Turkey
b Private Melikgazi Hospital, Medical Microbiology, Kayseri, Turkey
c Acibadem University School of Medicine, Department of Medical Microbiology, Istanbul, Turkey

A R T I C L E   I N F O

Article history:
Received 26 April 2017
Accepted 5 February 2018
Available online 1 March 2018
Associate Editor: Elizabeth Marques

Keywords:
Klebsiella pneumoniae
Immunochromatographic test
NDM
KPC
OXA-48

A B S T R A C T

In this study, the performance of the "RESIST-3 O.K.N. K-SeT" (Coris BioConcept, Gembloux, Belgium) immunochromatographic assay was evaluated in 132 Klebsiella pneumoniae comprising 102 carbapenem resistant and 30 carbapenem susceptible isolates. Genotypically known isolates of Gram negative bacteria (n = 22) including various species were also tested by the assay as controls. The isolates tested by the immunochromatographic assay and also were run PCR for bla KPC, bla IMP, bla VIM, bla NDM, and bla OXA-48. The rates of bla NDM, bla OXA-48, and bla KPC in carbapenem resistant isolates were found at 52.9%, 39.2%, and 2.0%, respectively. Both bla NDM and bla OXA-48 were found in six (5.9%) isolates. The results of the assay showed 100% concordance with those obtained by PCR in 132 K. pneumoniae. The agreement between the two methods was found to be identical at the isolate level. The assay also correctly detected all genotypically known isolates of Escherichia coli, Serratia marcescens, Citrobacter freundii, Enterobacter cloacae, K. pneumoniae carrying bla KPC, bla NDM, and/or bla OXA-48. On the other hand, the assay did not exhibit any cross-reaction in control isolates harboring bla IMP and bla VIM. We conclude that the RESIST-3 O.K.N. K-SeT is a reliable, rapid, and user friendly test and we recommend it for routine diagnostic laboratories.

© 2018 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Introduction

Multi-drug resistant Klebsiella pneumoniae isolates are of great concern worldwide.1 Although carbapenems are widely used antibiotics in the treatment of infections caused by multi-drug resistant K. pneumoniae, nosocomial infections due to carbapenem resistant K. pneumoniae (CRKp) have also increased dramatically in the last decade.2 The main mechanism of carbapenem resistance in K. pneumoniae has been associated with the production of carbapenem hydrolyzing enzymes. Various types of class A, B, and D type carbapenemases have been identified in K. pneumoniae. KPCs are the most

* Corresponding author.
E-mail: drpinarsa@gmail.com (P. Sağiroğlu).
https://doi.org/10.1016/j.bjm.2018.02.002
1517-8382/© 2018 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
commonly seen class A carbapenemases and endemic in the USA, Colombia, Brasil, Argentina, Italy, Poland, China, Taiwan, Israel and Greece. Among class B carbapenemases, VIM and IMP have spread in many countries since the early 2000s and are endemic in Southern Europe and Asia-Pacific region. Another class B carbapenemase, NDM, has also spread rapidly and become a global threat since 2008. In class D, OXA-48 type carbapenemases have become prevalent among K. pneumoniae and other members of Enterobacteriaceae in Turkey, Morocco, Tunisia, Libya, Algeria, Egypt, and India. 

The rapid and accurate laboratory diagnosis of carbapenem-producer isolates is critical to enable the timely application of infection control measures. Standard susceptibility testing cannot specify the mechanism of carbapenem resistance. An ideal diagnostic method should be rapid and able to detect all types of threatening carbapenemases sensitively and specifically. Currently, there are various approaches to phenotypic tests based on different principles. These include inhibitor based approach (combination disk test, double disk synergy test, gradient diffusion strips), detection based on carbapenem hydrolysis (cloverleaf method, colorimetric assays, Carba NP, BlueCARBA, carbapenem inhibition method, starch-iodine assay, spectrometry, electrochemical assay). Although some of these methods exhibit high performance in diagnosis of KPC, IMP, VIM and NDM producers; none of them is highly sensitive and specific for detecting OXA-48 producers. This may lead to challenges in the diagnosis of OXA-48 producers especially in regions where this type of carbapenemases are prevalent. Moreover most of the methods described above are time-consuming. Recent studies have revealed promising results with immunochromatographic assays for easy and rapid detection of specific carbapenemases.

In this study we aimed to evaluate the performance of a newly marketed immunochromatographic assay, “RESIST-3 O.K.N. K-SeT™” (Coris BioConcept, Gembloux, Belgium) targeting OXA-48 like, KPC, and NDM type carbapenemases in a collection of carbapenem resistant K. pneumoniae.

**Materials and methods**

**Study isolates**

A total of 132 non-duplicated clinical K. pneumoniae isolates collected in two university hospitals between July 2014 and December 2016 were included. 102 out of 132 K. pneumoniae were resistant to ertapenem in routine testing by an automated antimicrobial susceptibility test system (VITEK® 2, bioMérieux, Marcy l’Etoile, France). The resting isolates were susceptible to carbapenems in routine testing.

**Reference and PCR control strains**

To assess the specificity of the new immunochromatographic assay, a collection (n = 22) of Gram negative bacteria which have been previously characterized by PCR and/or DNA sequencing for the presence of blaKPC, blaIMP, blaVIM, blaNDM, and blaOXA-48 genes were also studied with RESIST-3 O.K.N. K-SeT (Table 1). Escherichia coli ATCC 25922 were used as a reference strain in antimicrobial susceptibility testing.

**Bacterial identification**

Identification of the isolates was confirmed by MALDI-TOF mass spectrometry (VITEK® MS, bioMérieux, Marcy l’Etoile, France).

**Antimicrobial susceptibility testing**

Standard disk diffusion test was performed on all isolates to confirm the ertapenem susceptibility results obtained with the VITEK® 2 system. In addition, ertapenem MICs were determined by the gradient diffusion strip test (Etest®, bioMérieux, Marcy l’Etoile, France) in the isolates. The European Committee on Antimicrobial Susceptibility Testing (EUCAST v 6.0) standards used as interpretive criteria for antimicrobial susceptibility testing.

**Polymerase chain reaction**

Carbapenem encoding genes, blaKPC, blaIMP, blaVIM, blaNDM, and blaOXA-48, were investigated by in house single PCR method to confirm phenotypic test results in 132 K. pneumoniae isolates.

**Immunochromatographic assay**

All isolates (132 K. pneumoniae and 22 genotypically known Gram negative bacteria) were tested by the RESIST-3 O.K.N. K-SeT according to the manufacturer’s instructions. Briefly, a single colony on Columbia agar + 5% sheep blood (bioMérieux, Marcy l’Etoile, France) was suspended in 10 drops of lysis buffer. Three drops of the suspension were then added onto the test strip. The results were read with the naked eye within 15 minutes at room temperature (Fig. 1).

**Results**

A total of 132 K. pneumoniae isolates (102 CRKp and 30 CSKp) were evaluated for the presence of OXA-48 like, KPC, and NDM carbapenemases by the RESIST-3 O.K.N K-SeT immunochromatographic assay. The results of the RESIST-3 O.K.N. K-SeT assay were compared those obtained by PCR targeting these genes (Table 1). Ertapenem MICs in CRKp isolates ranged from 1 to >32 μg/mL (MIC<sub>90</sub> 32 μg/mL, MIC<sub>99</sub> >32 μg/mL) by the gradient diffusion strip test. Rates of blaNDM, blaOXA-48, and blaKPC, genes in CRKp isolates were found to be 52.9%, 39.2%, and 2.0%, respectively, by PCR. Combined positivity for both blaNDM and blaOXA-48 genes was found in six (5.9%) isolates. The carbapenemase genes blaIMP and blaVIM were not detected in any of 132 study isolates. The results of the RESIST-3 O.K.N. K-SeT assay showed 100% concordance with those obtained by PCR. The agreement between the two methods was found to be identical at the isolate level. The RESIST-3 O.K.N. K-SeT assay did not exhibit any cross-reaction in genetically known bacteria (n = 8) harboring blaIMP and blaVIM genes (Table 1). The results obtained with the assay also
Moreover, negative infections were observed in five carbapenem-susceptible E. coli isolates which were known negative for blaKPC, blaIMP, blaVIM, blaNDM, and blaOXA-48 genes (Table 1). False positive result was not observed in five carbapenem-susceptible E. coli isolates which were known negative for blaKPC, blaIMP, blaVIM, blaNDM, and blaOXA-48 genes (Table 1).

### Discussion

Infections due to carbapenemase producing K. pneumoniae pose great concerns since they are associated with high morbidity and mortality.1-3 Rapid and specific diagnosis of carbapenemase producers plays a crucial role in preventing the spread of CRKP among hospitalized patients. Although PCR is recommended as ‘gold standard’ in carbapenemase detection,5,18,27 in terms of laboratory use, it has several unfavorable properties that limit its usefulness in many routine diagnostic laboratories.5,27 These properties include difficulty in application, and the need for specific equipment and trained personnel.5,6,9 Moreover, PCR cannot reveal the expression state of a gene. The availability of a reliable, rapid, and a simple phenotypic assay to detect carbapenemases would be of great benefit. Furthermore, such a test should be able to identify most of the prevalent carbapenemases with epidemiological significance. Various phenotypic methods developed so far are considered as reliable assays especially in detecting
class A and B carbapenemases by many authors.\textsuperscript{5–11} However these methods remained unsatisfactory in the diagnosis of OXA-48 producers. A few immunochromatographic assays in the market have been shown as reliable and fast for detecting OXA-48 and/or KPC producers, separately.\textsuperscript{12–17} Unfortunately, these tests are not able to identify the most common carbapenemases, simultaneously. In this study, we evaluated the performance of a recently introduced RESIST-3 O.K.N. K-SeT immunochromatographic assay. It is the first assay in the market targeting the detection of KPC, NDM, and OXA-48 together.

In our CRKp collection, RESIST-3 O.K.N. K-SeT results exhibited excellent concordance with PCR, yielding 100% sensitivity for the tested isolates (Table 1). The assay also detected five NDM, three OXA-48 and one KPC producers correctly in genetically known collection of E. cloacae, E. coli, S. marcescens, C. freundii, K. pneumoniae (Table 1). Considering the specificity of the test, all carbapenem susceptible isolates of K. pneumoniae (n = 30) and E. coli (n = 5) and the isolates harboring bla\textit{IMP}, bla\textit{VIM} genes were found to be negative by the assay. These results indicate the high specificity (100%) of RESIST-3 O.K.N. K-SeT. The assay also detected coproduction of NDM and OXA-48 in clinical isolates harboring bla\textit{NDM} and bla\textit{OXA-48} genes. Recent studies have remarked the emergence of K. pneumoniae and other Enterobacteriaceae producing both carbapenemases from Turkey and some other countries.\textsuperscript{31–40} For that, our result was found to be significant in terms of the reliability of RESIST-3 O.K.N. K-SeT in detecting NDM and OXA-48 co-producers. We have a limited number of KPC producers in our collection. Therefore, it is not easy to reach a conclusion about the reliability of the RESIST-3 O.K.N. K-SeT\textsuperscript{4} test in KPC detection. As stated in many reports KPC is not an endemic carbapenemase in our country.\textsuperscript{3,28–30} Two recently published reports have shown that the RESIST-3 O.K.N. K-SeT exhibited the high performance in the detection of KPC producers as well as the producers of NDM and OXA-48.\textsuperscript{18,19} As concluded by the authors of these two studies we also suggest that the assay has a limitation in terms of detecting other carbapenemases such as IMP and VIM producers. We suggest that there is still a need for different assays in detecting the isolates producing carbapenemases other than KPC, NDM, and OXA-48.

Besides the evaluation of RESIST-3 O.K.N. K-SeT performance, our work has also revealed some outputs regarding the prevalent carbapenemases in our CRKp collection. Accordingly, the most common carbapenemase has been NDM (52.9%), followed by OXA-48 (39.2%). These two carbapenemases have been found together in 5.9% of CRKp isolates. We consider these results to be very remarkable in terms of revealing the predominance of NDM in our CRKp collection. Almost all studies published from Turkey so far, have emphasized OXA-48 prevalence in the country.\textsuperscript{21,28,31,33} Although the presence of various reports from Turkey notifying the emergence of NDM in the country, this is the first report highlighting the very high rate of NDM in an extended collection of CRKp in our region.\textsuperscript{25–35} Moreover the detection of CRKp isolates harboring NDM together with OXA-48 was considered as a premonitory outcome of the study because it shows the spread of CRKp NDM and OXA-48 co-producers.\textsuperscript{31–40} On the other hand, this report has been the third one from Turkey presenting the isolation of KPC producing K. pneumoniae, so far.\textsuperscript{51,52} Kuakucu et al. also isolated KPC enzymes in two E. coli isolates in Turkey.\textsuperscript{43} In many other countries, including Turkey’s neighboring country Greece, the predominant enzyme type is KPC. The absence of any regional spread or institutional outbreaks due to KPC in Turkey may be associated with the already high burden of other resistance determinants among K. pneumoniae isolates, and the genetic failure of the sporadic KPC cases to transfer their plasmids carrying the \textit{bla}_{KPC} gene.

Finally, we conclude that the RESIST-3 O.K.N.K-SeT assay is a reliable, rapid, user-friendly test and we recommend it for routine diagnostic laboratories especially in regions where OXA-48 and NDM producers are prevalent.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Acknowledgments
We thank Cigdem Kaycan for granting the OXA-48, IMP-1 and VIM-4 positive strains. This study was supported by internal funding and partly supported by in-kind grant from Zemed Medikal Tekstil ve Dis Tic. Ltd. Sti., Istanbul, Turkey. This study was approved by the Marmara University Clinical Research Ethics Committee (Decision No: 09.2017.224).

REFERENCES

1. Tzouvelekis LS, Markogiannakis A, Psychogiou M, Tassios PT, Daikos GL. Carbapenemases in Klebsiella pneumoniae and other Enterobacteriaceae: an evolving crisis of global dimensions. Clin Microbiol Rev. 2012;25(4):682–707.
2. Lee C-R, Lee JH, Park KS, Kim YB, Jeong BC, Lee SH. Global dissemination of carbapenemase-producing Klebsiella pneumoniae: epidemiology, genetic context, treatment options, and detection methods. Front Microbiol. 2016;7:895.
3. Nordmann P, Dortet L, Poirel L. Carbapenem resistance in Enterobacteriaceae: here is the storm! Trends Mol Med. 2012;18(5):263–272.
4. Yong D, Toleman MA, Giske CG, et al. Characterization of a new metallo-beta-lactamase gene, \textit{bla}(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in \textit{Klebsiella pneumoniae} sequence type 14 from India. Antimicrob Agents Chemother. 2009;53(12):5046–5054.
5. Nordmann P, Gniadkowski M, Giske CG, Poirel L, Woodford N, Miriagou V. Identification and screening of carbapenemase-producing Enterobacteriaceae. Clin Microbiol Infect. 2012;18(5):432–438.
6. Aguirre-Quinoñero A, Martinez-Martinez L. Non-molecular detection of carbapenemases in Enterobacteriaceae clinical isolates. J Infect Chemother. 2017;23(1):1–11.
7. Bialvaei AZ, Kafli HS, Asgharzadeh M, Yousef Memar M, Yousefi M. Current methods for the identification of carbapenemases. J Chemother. 2016;28(1):1–19.
8. Hrabák J, Chudáčková E, Papagiannitsis CC. Detection of carbapenemases in Enterobacteriaceae: a challenge for diagnostic microbiological laboratories. Clin Microbiol Infect. 2014;20(9):839–853.
9. Hammoudi D, Ayoub Moubarek C, Karam Sarkis D. How to detect carbapenemase producers? A literature review of phenotypic and molecular methods. J Microbiol Methods. 2014;107:106–118.

10. van der Zwaluw K, de Haan A, Pluister GN, Bootama HJ, de Neeling AJ, Schouls LM. The carbapenem inactivation method (CIM), a simple and low-cost alternative for the carba NP test to assess phenotypic carbapenemase activity in gram-negative rods. Rohde H, ed. PLOS ONE. 2015;10(3):e0123690.

11. Tsakris A, Poulou A, Bogaerts P, Dimitroulla E, Pournaras S, Glupczynski Y. Evaluation of a new phenotypic OXA-48 disk test for differentiation of OXA-48 carbapenemase-producing Enterobacteriaceae clinical isolates. J Clin Microbiol. 2015;53(4):1245–1251.

12. Glupczynski Y, Evraud S, Ote I, et al. Evaluation of two new commercial immunochromatographic assays for the rapid detection of OXA-48 and KPC carbapenemases from cultured bacteria. J Antimicrob Chemother. 2016;71(5):1217–1222.

13. Dortet L, Jousset A, Sainte-Rose V, Cuzon G, Naas T. Prospective evaluation of the OXA-48 K-Set assay, an immunochromatographic test for the rapid detection of OXA-48-type carbapenemases. J Antimicrob Chemother. 2016;71(7):1834–1840.

14. Wareham DW, Shah R, Betts JW, Phee LM, Momim MHP. Evaluation of an immunochromatographic lateral flow assay (OXA-48 K-Set) for rapid detection of OXA-48-like carbapenemases in Enterobacteriaceae. J Clin Microbiol. 2016;54(2):471–473.

15. Rubio E, Zboromyrska Y, Pitart C, et al. Evaluation of a rapid immunochromatographic test for the detection of OXA-48 carbapenemase. Diagn Microbiol Infect Dis. 2017;87(3):266–267.

16. Fernández J, Fliete R, Rodriguez MR, Vazquez F. Evaluation of OXA-48 K-Set T: an immunochromatographic assay for rapid detection of OXA-48-producing Enterobacteriaceae. Diagn Microbiol Infect Dis. 2016;85(1):12–15.

17. Pasteran F, Denorme L, Ote I, et al. Rapid identification of OXA-48 and OXA-163 subfamilies in carbapenem-resistant gram-negative bacilli with a novel immunochromatographic lateral flow assay. J Clin Microbiol. 2016;54(11):2832–2836.

18. Wareham DW, Abdul Momim MHP. Rapid detection of carbapenemases in Enterobacteriaceae: evaluation of the resist-3 O.K.N. (OXA-48, KPC NDM) lateral flow multiplexed assay. J Clin Microbiol. 2017;55(4):1223–1225.

19. Glupczynski Y, Jousset A, Evraud S, et al. Prospective evaluation of the OKN K-SeT assay, a new multiplex immunochromatographic test for the rapid detection of OXA-48-like, KPC and NDM carbapenemases. J Antimicrob Chemother. 2017;72(7):1955–1960.

20. Matuschek E, Brown DF, Kahlmeter G. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. Clin Microbiol Infect. 2014;20(4):O255–O266.

21. Aktas Z, Kayacan CB, Schneider I, Can B, Midilli K, Bauernfeind A. Carbapenem-hydrolyzing oxacillinase, OXA-48, persists in Klebsiella pneumoniae in Istanbul, Turkey. Chemotherapy. 2008;54(2):101–106.

22. Livermore DM, Andrews JM, Hawkey PM, et al. Are susceptibility tests enough, or should laboratories still seek ESBLs and carbapenemases directly? J Antimicrob Chemother. 2012;67(7):1569–1577.

23. Aktas Z, Kayacan CB. Investigation of metallo-beta-lactamase producing strains of Pseudomonas aeruginosa and Acinetobacter baumannii by E-test, disk synergy and PCr. Scand J Infect Dis. 2008;40(4):320–325.

24. Perry JD, Naqvi SH, Mirza IA, et al. Prevalence of faecal carriage of Enterobacteriaceae with NDM-1 carbapenemase at military hospitals in Pakistan, and evaluation of two chromogenic media. J Antimicrob Chemother. 2011;66(10):2288–2294.

25. Pitout JD, Gregson DB, Poirel L, McClure J-A, Le P, Church DL. Detection of Pseudomonas aeruginosa producing metallo-beta-lactamases in a large centralized laboratory. J Clin Microbiol. 2005;43(7):3129–3135.

26. Cole JM, Schuetz AN, Hill CE, Nolte FS. Development and evaluation of a real-time PCR assay for detection of Klebsiella pneumoniae carbapenemase genes. J Clin Microbiol. 2009;47(2):322–326.

27. Nordmann P, Cuzon G, Naas T. The real threat of Klebsiella pneumoniae carbapenemase-producing bacteria. Lancet Infect Dis. 2009;9(4):226–232.

28. Carrér A, Poirel L, Yilmaz M, et al. Spread of OXA-48-encoding plasmid in Turkey and beyond. Antimicrob Agents Chemother. 2010;54(3):1369–1373.

29. Poirel L, Potron A, Nordmann P. OXA-48-like carbapenemases: the phantom menace. J Antimicrob Chemother. 2012;67(7):1597–1606.

30. Grundmann H, Glaser C, Albigier B, et al. Occurrence of carbapenemase-producing Klebsiella pneumoniae and Escherichia coli in the European survey of carbapenem-resistant Enterobacteriaceae (EuSCAPE): a prospective, multinational study. Lancet Infect Dis. 2017;17(2):153–163.

31. Alp E, Perçin D, Dolakoğlu S, et al. Molecular characterization of carbapenem-resistant Klebsiella pneumoniae in a tertiary university hospital in Turkey. J Hosp Infect. 2015;84(2):178–180.

32. Kilic A, Baysallar M. The first Klebsiella pneumoniae isolate co-producing OXA-48 and NDM-1 in Turkey. Ann Lab Med. 2015;35(3):382–383.

33. Çakar A, Akyoun Y, Gür D, et al. Characterization of carbapenem-resistant Klebsiella pneumoniae strain from Istanbul, Turkey. J Clin Microbiol. 2010;48(10):33990.

34. Bargoutgu A, El otmani F, Lakbakkhi el yaagoubi F, Talimi M, Zerouali K, Timinouni M. First report of a Klebsiella pneumoniae strain co-producing NDM-1 VIM-1 and OXA-48 carbapenemases isolated in Morocco. APMIS. 2013;121(7):675–677.

35. Balm MND, La M-V, Krishnan P, Jureen R, Lin RTP, Teo JWP. Emergence of Klebsiella pneumoniae co-producing NDM-type and OXA-181 carbapenemases. Clin Microbiol Infect. 2013;19(9):E421–E423.

36. Doi Y, O’Hara JA, Lando JF, et al. Co-production of NDM-1 and OXA-232 by Klebsiella pneumoniae. Emerg Infect Dis. 2014;20(1):163–165.

37. Stella Uwaezuzue N, Kieffer N, Iregbu KC, Nordmann P. First report of NDM-1 and NDM-1 from a clinical Klebsiella pneumoniae isolate from Nigeria. Int J Infect Dis. 2017;61:1–2.

38. Lázaro-Perona F, Sarria-Visa A, Ruiz-Carrascoso G, Mingorance J, García-Rodríguez JM, Gómez-Gil R. Klebsiella pneumoniae co-producing NDM-1 and OXA-48 carbapenemases isolated from a patient with prolonged hospitalisation. Int J Antimicrob Agents. 2017;49(1):112–113.

39. Labarca J, Poirel L, Özdamar M, Turkoglu S, Hakko E, Nordmann P. KPC-producing Klebsiella pneumoniae, finally
targeting Turkey. New Microbes New Infect. 2014;2(2):50–51.

42. Poirel L, Yilmaz M, Istanbullu A, Arslan F, Mert A, Bernabeu S, et al. Spread of NDM-1-producing Enterobacteriaceae in a neonatal intensive care unit in Istanbul, Turkey. Antimicrob Agents Chemother. 2014;58(5):2929–2933.

43. Kuskucu MA, Karakullukcu A, Ailiken M, Olu B, Mete B, Aygun G. Investigation of carbapenem resistance and the first identification of Klebsiella pneumoniae carbapenemase (KPC) enzyme among Escherichia coli isolates in Turkey: a prospective study. Travel Med Infect Dis. 2016;14(6):572–576.