Isolation and complete genome sequencing of the virulent phage vB_EcoS_XY3 infecting multidrug-resistant Escherichia coli

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Isolation and complete genome sequencing of the virulent phage vB_EcoS_XY3 infecting multidrug-resistant *Escherichia coli*

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Abstract

A virulent phage, named vB_EcoS_XY3, was isolated from hospital wastewater in Xiangyang, China. Its morphological characteristics, growth parameters, adsorption rate, and pH and temperature stability were determined. Phage vB_EcoS_XY3 was found to be able to infect *Escherichia coli* laboratory strains and also some multidrug-resistant *E. coli* strains. Its complete genome consists of 51,345 base pairs of double-stranded DNA with an average GC content of 55.24% and 85 putative protein-coding genes. Forty-four genes were annotated with known functions. These results will not only provide further insights into *E. coli* phages but also have implications for the development of potential biocontrol agents.

Escherichia coli is an opportunistic bacterial pathogen that causes a wide range of nosocomial infections. Under physiological conditions, *Escherichia coli* colonizes the gastrointestinal tract of humans [1], but it becomes pathogenic in patients when it ends up outside the gut – in wounds or in normally sterile fluids such as urine and blood, causing diseases such as urinary tract infections (UTIs) and sepsis [2–4]. The emergence of *E. coli* strains that are resistant to the most commonly used antimicrobials has often made it difficult to treat and eliminate infections. This poses a significant threat to public health and results in high morbidity, mortality, and financial costs [5, 6]. Bacteriophages (phages) have attracted attention as a means of controlling bacterial infections, especially those involving multidrug-resistant bacteria [7]. In this study, phage vB_EcoS_XY3 was shown to be able to kill some multidrug-resistant *E. coli* strains, strongly suggesting that it could be used as a component of phage cocktails to treat multidrug-resistant *E. coli.*

Phage isolation

A phage named vB_EcoS_XY3 was isolated from urban sewage samples near a hospital (32°05′N, 112°10′E) in Xiangyang, China, using *E. coli* K-12 MG1655 as the indicator strain, and the double-layer agar method was used to propagate this phage [8]. A crude phage suspension was concentrated with 10% polyethylene glycol 8000 and 1 M NaCl [9]. Sucrose density centrifugation was used for further purification. Finally, the purified phage was dialyzed in SM buffer (10 mM NaCl, 10 mM $MgCl_2$, 50 mM Tris–HCl, pH 8.0) at 4 °C.

The host range of the phage was determined using a double-layer agar plate test. The efficiency of plating (EOP) was used to determine the lytic activity of vB_EcoS_XY3 and its ability to infect different bacteria. Briefly, dilutions of phage suspensions and 100 µL of the log-phase test strain were mixed with 4 ml of LB medium with 0.7% agar and then poured onto bottom agar and left to incubate at 37 °C for 24 h. The EOP value was computed as the ratio of the PFU/ml on the test host to the PFU/ml on the natural host. Each strain was tested three times.

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Stability of the phage

For the thermal stability assay, the phage was incubated in a water bath at 4 °C, 25 °C, 37 °C, 50 °C, 60 °C, and 70 °C for 60 min, and the titer was determined. To determine the stability of vB_EcoS_XY3 at different pH values, the LB medium was adjusted to pH 2-13. After incubation for 60 min, the phage titer was determined. The double-layer agar method was used to determine the phage activity. The final results were expressed as the percentage of plaque-forming units remaining after treatment.

Efficiency of bacteriophage adsorption and one-step growth curve

A bacteriophage lysate was added to exponentially growing bacteria at an MOI (multiplicity of infection) of 0.1 (the optimal MOI, data not shown). During the incubation, samples were collected at an interval of 1 min and centrifuged at 12,000 *g* for 1 min to remove the cells. The supernatants were titrated immediately. The time when the phage lysate was added to the bacterial host strain was considered time zero, and the amount of unadsorbed phage was considered to be 100%. Other values were calculated compared to this value. A one-step growth curve assay was performed as described previously [10]. Briefly, an adsorption tube containing 0.1 ml of the phage lysate (4×10^6 PFU/ml) and 0.9 ml of host culture (2×10^8 CFU/ml) were mixed and then diluted to inhibit a successive infection of bacteria. Phage titers were measured by the double-layer agar method at 25, 30, 40, 50, 60, 70, 80 and 90 min. Each experiment was repeated three times.

Phage DNA purification and sequencing

The DNA of vB_EcoS_XY3 was extracted by using the phenol-chloroform method as described previously [11], and the pellet was resuspended into 50 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and stored at −20 °C. Genomic DNA was sequenced using an MGISEQ500 sequencer provided by the Beijing Genomics Institute (BGI) with an insert size of 350, in paired-end mode. The quality control of raw sequence data was performed using NGS QC Toolkit (version v2.3.3) [12]. Genome assembly was performed using SPAdes (version 3.13.1) [13].

Genome analysis

The genes encoding the putative tRNA and rRNA were detected using tRNAscan-SE-1.23 (http://lowelab.ucsc.edu/tRNAscan-SE/) [14] and RNAmmer 1.2 (http://www.cbs.dtu.dk/services/RNAmmer) [15]. RAST (http://rast.nmpdr.org/) was used to predict putative coding sequences. The average nucleotide identity (ANI) was calculated using OrthoANI based on the genome sequence [16]. In order to compare the sequence similarity of the two phage genomes with each other and with other homologous phage genomes, BLASTn and Clustal Omega analysis were performed [17]. BLASTp was used to annotate the functions of the ORFs and identify putative homologs that share similarities with the predicted phage proteins. Phage genome annotation was visualized using CGview [18]. Easyfig was used to visualize the comparison of the genomes of the phages vB_EcoS_XY3 and tunus [19]. Using BLASTp analysis, homologous major capsid protein sequences and terminase large subunit sequences of phages of the subfamily *Tempevirinae* were identified and collected from the GenBank database. Multiple sequence alignments of the major capsid protein (ORF34) and terminase large subunits (ORF28) were made using a ClustalW [20]. A phylogenetic tree was constructed by the neighbor-joining method in MEGA7 based on 1,000 bootstrap replicates [21].

On *E. coli* strain MG1655, phage vB_EcoS_XY3 formed clear round plaques that were about 3 mm in diameter (Fig. 1A). Morphological characteristics revealed by TEM observation indicated that vB_EcoS_XY3 consists of an isometric polyhedral head (61 nm in diameter, n = 3) and a noncontractile tail (179 nm in diameter, n = 3) (Fig. 1B). Five laboratory *E. coli* strains (K-12, Top10, DH5α, JM109 and BL21) and 24 clinical pathogenic *E. coli* strains were used for host determination. The results revealed that vB_EcoS_XY3 could infect 15 of the *E. coli* strains, including four laboratory strains and 11 multidrug-resistant strains, but the EOP values were quite different (Table S1).

The thermal and pH stability of the phage was then analyzed. The phage showed high tolerance to increased temperatures. There was no significant decrease in phage activity after a 60-min incubation at 50 °C, and even after a 60-min incubation at 60 °C, more than 50% of the phage remained viable (Fig. S1A). The phage lost its infectivity after incubation at 70 °C for 60 min. The phage also showed high resistance against a wide range of pH treatments (Fig. S1B), and it was stable when incubated at pH 4, as well as at pH 10. After incubating the phage at pH 12, it lost all infectivity. Phage vB_EcoS_XY3 adsorbs rapidly onto *E. coli* cells, with 40% of the phage particles already adsorbed within 5 min after mixing the phage lysate with the bacterial culture (Fig. S2A), and after 6 min, more than 90% of the phage particles had already adsorbed onto the cells. Particle formation in the cell was also found to be extremely rapid, as seen in the one-step growth curve shown in Fig. S2B. The latent period was approximately 25 min, and the burst size, which was determined based on the final concentration of the phage and the concentration of phage in the adsorption

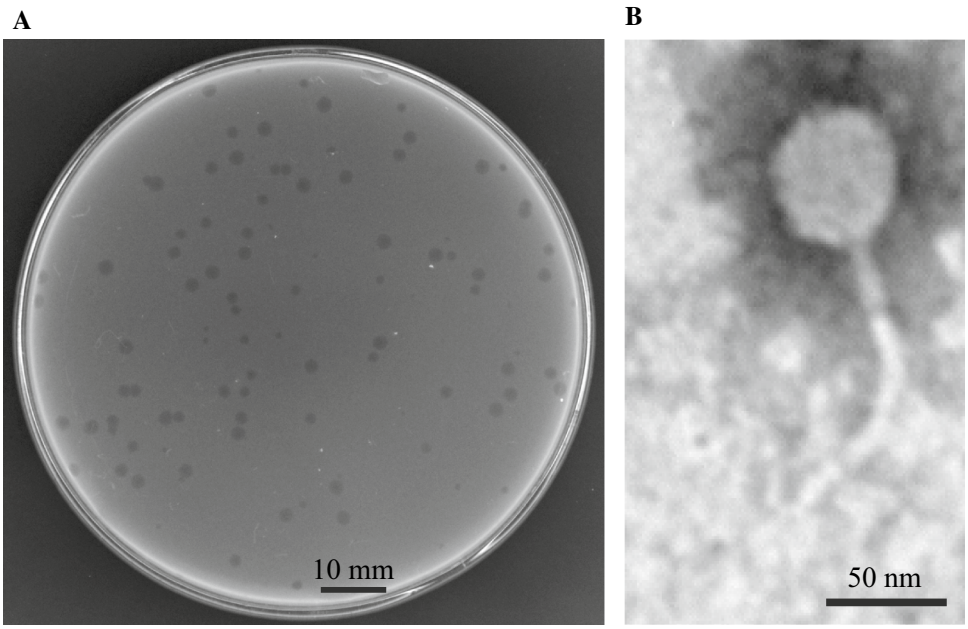

Fig. 1 Plaque morphology (**A**) and virion morphology (**B**) of phage vB_EcoS_XY3

tube, was about 750 PFU/ml, indicating rapid and efficient lytic development of the phage after absorbing to the host.

Genome sequencing revealed that the vB_EcoS_XY3 genome consists of 51,345 bp with a GC content of 55.24%. The vB_EcoS_XY3 genome was predicted to contain 85 open reading frames (ORFs), including 44 (51.8%) with known functions (Fig. S3). No tRNA or rRNA-encoding genes were detected. As shown in Fig. S3 and Table S2, the 44 predicted functional proteins were categorized into five functional groups: DNA replication/modification, host lysis, structural proteins, additional functions, and hypothetical proteins. Four genes were found to encode proteins involved in methylation, including methyltransferase type 11 (ORF19), DNA adenine methyltransferase (ORF63), DNA-cytosine methylase (ORF76), and putative cytosine DNA methylase (ORF77). ORF63 contains a Dam superfamily domain with a recognition site of GATC. ORF76 contains a Cyt_C5_DNA_methylase superfamily domain with a recognition site of CCWGG. Resistance systems in the host such as the R-M system can be overcome and the effectiveness of infection can be prolonged with the help of methylases encoded by the phage [22]. Several R-M sites encoded by *E. coli* MG1655 correspond to the predicted recognition site GATC of ORF63 and CCWGG of ORF76 in the phage genome, revealing the potential to overcome the host R-M system.

The genome sequence of vB_EcoS_XY3 is highly similar to that of *E. coli* phage tunus (GenBank no. MN850638), and as determined by Clustal Omega analysis, their genomes share 97.72% identity. A genome sequence comparison of vB_EcoS_XY3 and tunus is shown in Fig. 2. Phage tunus contains 84 ORFs, which is one less than vB_EcoS_XY3. Eighty-one ORFs (96.4%) of vB_EcoS_XY3 showed more than 90% identity to those of tunus. We also determined ANI values for the 15 most closely related phages based on their genome sequences (Table S3). The ANI values for the comparison of vB_EcoS_XY3 with other phages ranged from 95.68% to 98.41%, indicating an extremely close relationship between them. Pairwise genome sequence alignments of vB_EcoS_XY3 and 14 other phages were conducted using BLASTn, and the results showed that they shared 97.14-98.52% identity. Phages with more than 95% genome sequence identity are considered members of the same species [23]. Therefore, vB_EcoS_XY3 should be included as a new member of the genus *Warwickvirus*, subfamily *Tempevirinae*. In a phylogenetic tree based on the large terminase subunit (ORF28), vB_EcoS_XY3 was in the same branch with the phages tunus and tonn (Fig. 3A), demonstrating their close relationship. A phylogenetic tree based on the major capsid protein (ORF34) revealed that vB_EcoS_XY3 formed a distinct clade with other members of the genus *Warwickvirus*, and it was also distant from phages of the genus *Hanrivervirus* (Fig. 3B). They all belong to subfamily *Tempevirinae*.

In the vB_EcoS_XY3 genome, markers of temperate bacteriophages, such as genes encoding integrase, recombinase, repressor, or excisionase were not identified [24]. No antibiotic resistance genes were found in vB_EcoS_XY3 [25]. Strictly virulent phages are preferred for phage therapy [26]. This study provides a promising candidate for phage cocktail therapy in the future.

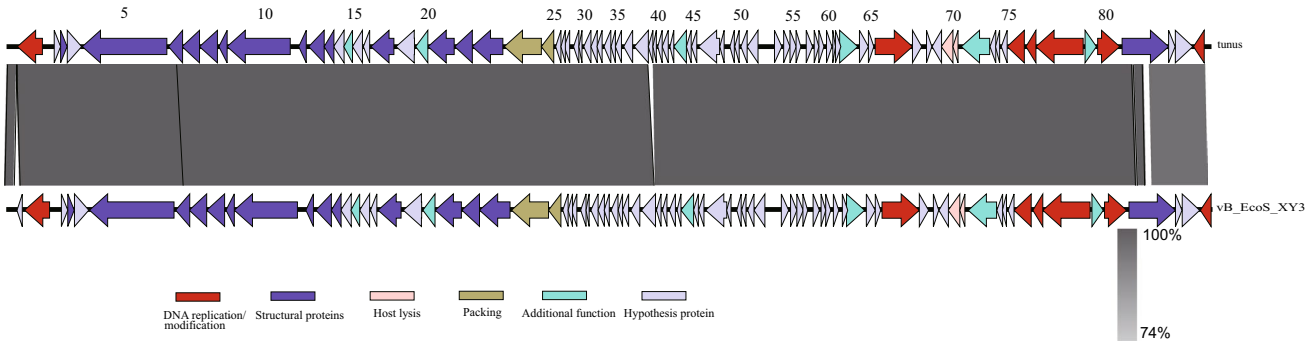

Fig. 2 Genome comparison of phage tunus and vB_EcoS_XY3. Arrows represent predicted ORFs, with the direction of the arrow indicating the direction of transcription. The colors indicate different functional groups of gene products: red, DNA replication/regulation; purple, structural protein; light red, host lysis; yellow, DNA packaging; blue, additional function; gray, hypothetical proteins. The percent nucleotide sequence identity values are shown in the shaded regions

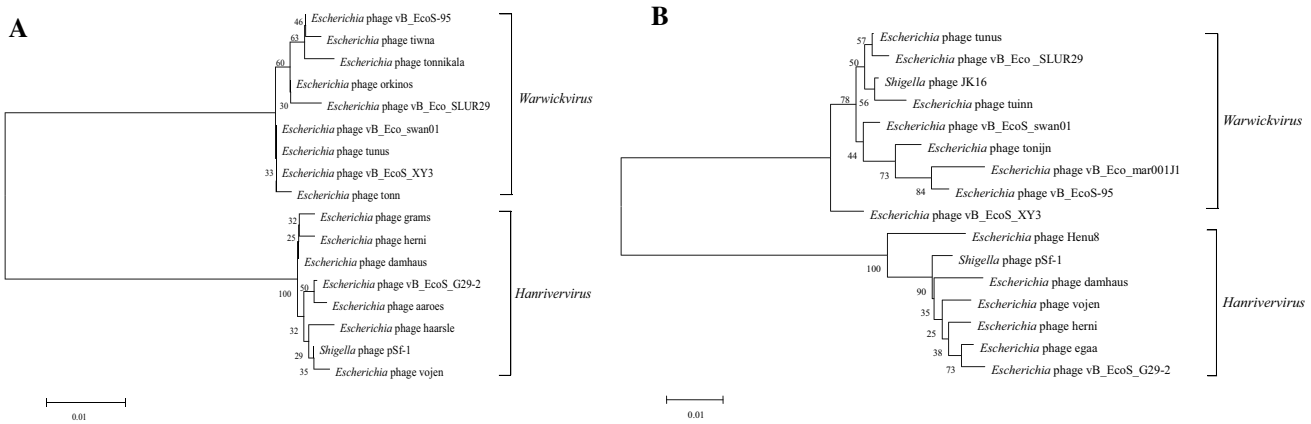

Fig. 3 Phylogenetic relationship of phage vB_EcoS_XY3 to other phages. MEGA 7.0 was used to build neighbor-joining trees based on amino acid sequences with 1000 bootstrap replicates. (**A**) Phylogenetic tree based on the amino acid sequences of the terminase large subunits. (**B**) Phylogenetic tree based on the amino acid sequences of the major capsid protein

Nucleotide sequence accession number

The GenBank accession number for phage vB_EcoS_XY3 is MN781674.

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Author contributions PF, QZ and MS conceived and designed the experiments. PF, QZ, LS, QX, HX and SC performed the experiments. PF, QZ, HX, SC and QX analyzed the data. PF wrote the paper. PF, QZ, HX, SC, QX, XS and MS revised the paper. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Jang J, Hur HG, Sadowsky MJ, Byappanahalli MN, Yan T, Ishii S (2017) Environmental *Escherichia coli*: ecology and public health implications—a review. J Appl Microbiol 123(3):570–581. https://doi.org/10.1111/jam.13468

2. Ron EZ (2010) Distribution and evolution of virulence factors in septicemic *Escherichia coli*. Int J Med Microbiol 300(6):367–370. https://doi.org/10.1016/j.ijmm.2010.04.009
3. Wiles TJ, Kulesus RR, Mulvey MA (2008) Origins and virulence mechanisms of uropathogenic *Escherichia coli*. Exp Mol Pathol 85(1):11–19. https://doi.org/10.1016/j.yexmp.2008.03.007
4. Russo TA, Johnson JR (2003) Medical and economic impact of extraintestinal infections due to *Escherichia coli*: focus on an increasingly important endemic problem. Microbes Infect 5(5):449–456. https://doi.org/10.1016/S1286-4579(03)00049-2
5. van Hout D, Verschuuren TD, Bruijning-Verhagen PCJ, Bosch T, Schürch AC, Willems RJL, Bonten MJM, Kluytmans JAJW (2020) Extended-spectrum beta-lactamase (ESBL)-producing and non-ESBL-producing Escherichia coli isolates causing bacteremia in the Netherlands (2014–2016) differ in clonal distribution, antimicrobial resistance gene and virulence gene content. PLoS One 15(1):e0227604–e0227604. https://doi.org/10.1371/journal.pone.0227604
6. Bloom DE, Black S, Salisbury D, Rappuoli R (2018) Antimicrobial resistance and the role of vaccines. Proc Natl Acad Sci USA 115(51):12868–12871. https://doi.org/10.1073/pnas.1717157115
7. Moelling K, Broecker F, Willy C (2018) A wake-up call: we need phage therapy now. Viruses. https://doi.org/10.3390/v10120688
8. Gu J, Liu X, Li Y, Han W, Lei L, Yang Y, Zhao H, Gao Y, Song J, Lu R, Sun C, Feng X (2012) A method for generation phage cocktail with great therapeutic potential. PLoS One 7(3):e31698. https://doi.org/10.1371/journal.pone.0031698
9. Govind R, Fralick JA, Rolfe RD (2006) Genomic organization and molecular characterization of *Clostridium difficile* bacteriophage PhiCD119. J Bacteriol 188(7):2568–2577. https://doi.org/10.1128/JB.188.7.2568-2577.2006
10. Kropinski AM (2018) Practical advice on the one-step growth curve. Methods Mol Biol 1681:41–47. https://doi.org/10.1007/978-1-4939-7343-9_3
11. Yuan Y, Gao M, Wu D, Liu P, Wu Y (2012) Genome characteristics of a novel phage from *Bacillus thuringiensis* showing high similarity with phage from *Bacillus cereus*. PLoS One 7(5):e37557. https://doi.org/10.1371/journal.pone.0037557
12. Patel RK, Jain M (2012) NGS QC toolkit: a toolkit for quality control of next generation sequencing data. PLoS One 7(2):e30619. https://doi.org/10.1371/journal.pone.0030619
13. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA (2012) SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19(5):455–477. https://doi.org/10.1089/cmb.2012.0021
14. Lowe TM, Chan PP (2016) tRNAscan-SE on-line: integrating search and context for analysis of transfer RNA genes. Nucleic Acids Res 44(W1):W54–W57. https://doi.org/10.1093/nar/gkw413
15. Lagesen K, Hallin P, Rodland EA, Staerfeldt HH, Rognes T, Ussery DW (2007) RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res 35(9):3100–3108. https://doi.org/10.1093/nar/gkm160
16. Lee I, Ouk Kim Y, Park SC, Chun J (2016) OrthoANI: an improved algorithm and software for calculating average nucleotide identity. Int J Syst Evol Microbiol 66(2):1100–1103. https://doi.org/10.1099/ijsem.0.000760
17. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD, Higgins DG (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol 7:539. https://doi.org/10.1038/msb.2011.75
18. Stothard P, Wishart DS (2005) Circular genome visualization and exploration using CGView. Bioinformatics (Oxf, Engl) 21(4):537–539. https://doi.org/10.1093/bioinformatics/bti054
19. Sullivan MJ, Petty NK, Beatson SA (2011) Easyfig: a genome comparison visualizer. Bioinformatics (Oxf, Engl) 27(7):1009–1010. https://doi.org/10.1093/bioinformatics/btr039
20. Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 31(13):3497–3500. https://doi.org/10.1093/nar/gkg500
21. Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33(7):1870–1874. https://doi.org/10.1093/molbev/msw054
22. Murphy J, Mahony J, Ainsworth S, Nauta A, van Sinderen D (2013) Bacteriophage orphan DNA methyltransferases: insights from their bacterial origin, function, and occurrence. Appl Environ Microbiol 79(24):7547–7555. https://doi.org/10.1128/aem.02229-13
23. Adriaenssens E, Brister JR (2017) How to name and classify your phage: an informal guide. Viruses. https://doi.org/10.3390/v9040070
24. Abel Carrias TJW, Waldbieser Geoffrey C, Mead David A, Terhune Jeffery S, Liles Mark R (2011) Comparative genomic analysis of bacteriophages specific to the channel catfish pathogen *Edwardsiella ictaluri*. Virol J 8(1):6. https://doi.org/10.1186/1743-422X-8-6
25. Joensen KG, Scheutz F, Lund O, Hasman H, Kaas RS, Nielsen EM, Aarestrup FM (2014) Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli*. J Clin Microbiol 52(5):1501–1510. https://doi.org/10.1128/jcm.03617-13
26. Nilsson AS (2014) Phage therapy—constraints and possibilities. Upsala J Med Sci 119(2):192–198. https://doi.org/10.3109/03009734.2014.902878