2017-A facile, rapid and sensitive detection of MRSA using a CRISPR-mediated DNA FISH method, antibody-like dCas9:sgRNA complex

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based detection approach has proved to be easy, fast, sensitive, and cost-efficient, it can be applied directly at the point of care to detect various pathogens as well as MRSA in this study.

1. Introduction

Methicillin-resistant Staphylococcus aureus (MRSA) is one of the most important multiresistant human pathogens; it is resistant to most commonly used antibiotics and causes a wide range of problems from mild skin infections to severe life-threatening diseases. Typically, MRSA causes infections such as pneumonia, bacteremia, endocarditis, necrotizing fascitis, and bone infections (Pantosti et al., 2012). The methicillin resistance rates of infectious S. aureus isolates are high, reaching 50% or greater, and MRSA infections are 4 times more likely to cause severe morbidity and mortality worldwide than methicillin-susceptible S. aureus (MSSA) infections (Diekema et al., 2001; Boucher et al., 2010). Due to the overuse and misuse of antibiotics as well as a lack of new drug development, MRSA has become endemic in devel-

oped countries, and more deaths are associated with MRSA in USA each year than HIV/AIDS, Parkinson's disease, emphysema, and homicide combined (Golkar et al., 2014). Therefore, rapid and reliable diagnosis of MRSA strains is crucial to guide effective patient treatment at early stages of infection and control the spread of MRSA infections. MRSA requires different types of antibiotics than MSSA, and timely administration of an appropriate treatment for infectious diseases has been associated with lower treatment costs and improved patient outcomes. Traditional culture-based methods for identifying MRSA are slow, requiring 24–48 h for results. Conventional techniques such as matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) (Wolters et al., 2011) and real-time polymerase chain reaction (RT-PCR) (Hagen et al., 2005) have been used to identify MRSA, but to obtain reliable results these techniques

require purification of the microbes to extract the target DNA. Therefore, further simplification of the detection procedures, a shorter detection time and a less labor-intensive procedure than conventional methods are required while still retaining high specificity of detection. The conventional fluorescence in situ hybridization (FISH) protocol based on oligo probes offers high-resolution detection of specific DNA in individual cells. DNA-FISH has found wide applications in cancer diagnosis, karyotyping, species specification, and genotyping but has major disadvantages, such as the laborious and time-consuming nature of protocols and the requirement for heat and chemical denaturation of the target DNA, leading to unsatisfactory results and hampering general utilization (Wang et al., 2012). Clustered regularly interspaced palindromic repeats (CRISPR) are a mechanism used by bacteria to protect themselves against phages by taking part of an invader's DNA sequence and inserting it into its own for future recognition and degradation (Barrangou et al., 2012; Luo et al., 2016). The CRISPR/CRISPR associated protein 9 (Cas9) system has become a revolutionary tool for targeted genome editing, Cas9, a double-stranded (ds) DNA nuclease, acquires sequence specificity when combined with a single-guide RNA (sgRNA) that can be easily programmed to recognize any target sequence (Harrison et al., 2014; LaFlamme, B, 2014; Qin et al., 2017; Ran et al., 2013; Unniyampurath et al., 2016). In vitro studies have revealed that the Cas9/sgRNA complex has a strong and stable affinity for its target DNA (Dickinson et al., 2016; Kozlov et al., 1981; Wilkinson et al., 2014). One Cas9 derivative (dCas9) lacks endonuclease activity, and the dCas9/sgRNA complex has been used for transcriptome modulation and visualization of genomic loci in live cells (Jinek et al., 2012; Chen et al., 2013). We hypothesized that the dCas9/sgRNA complex functions as a highly specific and efficient target DNA probe by acting similarly to an antibody for the targeting and labeling of DNA. SYBR green I (SG) is a well-known sensitive fluorescent dye used for dsDNA staining, and it has been successfully applied to DNA quantitation in solution, gels, real-time PCR, and cell chromosome staining due to the dramatic increase in its fluorescence emission upon interaction with dsDNA (Zipper et al., 2004). Herein, we report a simple, precise and rapid assay that can selectively detect MRSA strains using dCas9/sgRNA-SG I based DNA-FISH system.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise specified. AccuNanoBead Ni-NTA Magnetic Nanobeads and oligonucleotides were purchased from Bioneer. SYBR Green I was purchased from Lonza. N-(5-Amino-1-carboxypentyl) iminodiacetic acid (AB-NTA free acid) was purchased from Dojindo Molecular Technologies.

2.2. Cloning, expression and purification of the Cas9 and dCas9 proteins

The Cas9 and dCas9 coding genes derived from Streptococcus pyogenes were amplified by polymerase chain reaction (PCR) using pCas9 and pdCas9 plasmids (addgene), respectively. The PCR products were cloned into the pET22b vector (Novagen) and transformed into the Escherichia coli BL21 Rosetta 2 (DE3). Cells were grown at 37 ° C in LB medium containing 50 g/ml kanamycin and 34 g/ml chloramphenicol until the optical density at 600 nm (OD600) reached 0.6. Protein expression was induced by supplementing with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 16 h at 16 ° C. After harvesting the cell by centrifugation, the pellets were washed in equilibrium buffer (50 mM Tris-HCl, 0.1 M NaCl, 10 mM EDTA, pH 8.0) followed by incubating in lysis buffer (50 mM Tris-HCl, 0.5 M NaCl, pH 8.0). The cell lysate was sonicated to reduce viscosity, and the soluble protein was obtained by centrifugation for 1 h at 16,000 g, applied to Ni-NTA agarose resin (Qiagen), washed, and eluted with
buffer (50 mM Tris - HCl, 0.5 M NaCl, 0.5 M Imidazole, pH 8.0). The purified proteins were dialyzed against phosphate buffer saline (PBS) and further purified by Q-Sepharose anion exchange chromatography (GE Healthcare).

2.3. Preparation of single-guide RNA (sgRNA)

The DNA template for the target sgRNA was synthesized as the 20-bp target specific sequence between the T7 promoter sequence and the scaffold template specific sequence. Amplification of the sgRNA-encoding template by PCR was performed and transcribed using the Guide-it sgRNA In Vitro Transcription system (Clontech) according to the manufacturer's instructions. In vitro transcribed RNA was phenol/chloroform extracted and followed by ethanol precipitation. The sgRNA concentration was quantified by UV absorbance, and the samples were kept at -80 ° C.

2.4. *In vitro* evaluation of the sgRNA efficacy

Purified Cas9 (100 ng) or dCas9 (100 ng) was incubated with mecA DNA (100 ng) and sgRNA (100 ng) in reaction buffer containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 mM NaCl, 5 mM MgCl, and 0.1 mM ethylene diaminete traacetic acid (EDTA) (pH 6.5) for 1 h at 37 ° C. Digested DNA or shifted DNA was analyzed by electrophoresis using 2% and 0.8% agarose gel, respectively.

2.5. Detection of MRSA using genomic DNA

Genomic DNA from cultured bacteria was purified using the Wizard Genomic DNA purification kit (Promega) according to the manufacturer's instructions, dCas9 protein (10 g) and sgRNAs (200 ng) were incubated with 20 g of genomic DNA in duplex buffer containing 100 mM potassium acetate and 30 mM HEPES (pH 7.5) for 1 h at 37 ° C. The ternary dCas9-sgRNA-target genomic DNA complexes were incubated with Ni-NTA magnetic nanobeads for 30 min. Unbound complex was removed by washing with buffer containing 0.05% Tween-20 and 100 mM imidazole in PBS (pH 8.0). The eluate was collected with elution buffer containing 500 mM imidazole in PBS (pH 8.0) and reacted with SG I to generate fluorescence signals. The fluorescence was measured at 494 nm (excitation) and 521 nm (emission) using a microplate reader. We performed in triplicate and calculated the average of fluorescence intensity and their standard deviation.

2.6. Detection of MRSA using cell lysates

Bacterial cells were grown in LB medium at 37 ° C until the optical density of the culture at 600 nm reached ~1.0. The enzymatic cell lysis was performed by adding lysozyme and lysostaphin for 1 h at 37 ° C. The cell lysates, dCas9 protein (10 g) and sgRNAs (200 ng) were incubated in duplex buffer for 1 h at 37 ° C. The Ni-NTA magnetic beads were added, mixed thoroughly by vortexing and incubated at 25 ° C with constant shaking for 30 min. The samples captured on the Ni-NTA magnetic beads were directly incubated with SG I at room temperature, and then, the fluorescence was measured using a micro-plate reader. All

experiments were performed at least in triplicate to confirm reproducibility, and all data are presented as mean ± standard deviation.

2.7. Microassay detection of MRSA and MSSA with dCas9/sgRNA-SG I

An epoxy-functionalized glass slide (Nanocs) was submerged in a small bath containing 0.01 M NTA in 0.1 M Tris-HCl (pH 8.0) and reacted overnight at room temperature. Then, it was washed in ethanol

and dried. The dried NTA-modified surface was immersed in a solution containing 0.1 M nickel chloride in 0.01 M Tris-HCl (pH 8.0) for 16 h at room temperature. The slide was washed with ethanol and dried under a mild nitrogen stream. Histidine6 tagged-dcas9/sgRNA (10 g) and sgRNA (200 ng) were immobilized on the Ni-NTA functionalized glass chip. After incubating for 2 h at room temperature, the surface was rinsed with ethanol and dried. Various genomic DNA of MRSA and MSSA were spotted on the glass slide for 1 h at room temperature. The slide was washed and bound genomic DNA was labeled with SG I.

We acquired fluorescent images using the ChemiDoc MP imaging system (Bio-Rad). Fluorescent images of MRSA and MSSA detection were obtained under same analytical condition. We applied the same condition three times in one line. The optical density of each spot was analyzed using Image Lab Software, and their average and standard deviation were calculated.

3. Results and discussion

3.1. Characterization of dCas9/sgRNA-SG I based detection system

The mecA gene encoding an alternative penicillin binding protein is known to be responsible for resistance to methicillin (Chambers et al., 1985). SgRNA was designed to include a mecA gene recognition sequence immediately preceding a 5'-NGG-3' proto-spacer adjacent motif (PAM) sequence; thus, dCas9/sgRNA is capable of acting like an antibody that specifically binds to the genomic DNA of MRSA containing the mecA gene (Ryffel et al., 1992; Murakami et al., 1991). The dCas9/sgRNA complex is unable to recognize the genomic DNA of MSSA (mecA-negative); thus, the dCas9/sgRNA complex of CRISPR-dCas9 based system has efficient sequence-specific binding for the identification of MRSA vs. MSSA. The S. pyogenes Cas9 and dCas9 including an N-terminal 6X His tag were expressed in E. coli strain Rosetta (DE3) cells and were purified using a Ni-NTA column, followed by a Mono Q Ion-Exchange column (Fig. 51). We first designed sgRNAs containing approximately 20 bases specific to the mecA sequence and predicted the targeting efficiency of each sgRNA upon mixing with Cas9. The cleavage reaction of Cas9/sgRNA against plasmid DNA containing the mecA gene was performed and analyzed by agarose gel electrophoresis. In addition, the shift in mobility of dCas9/sgRNA upon binding to mecA DNA was monitored. Both designed sgRNAs (#1539 & #1545) in complex with Cas9 or dCas9 appeared to bind mecA DNA efficiently (Fig. S2). SgRNAs that lacked a mecA-specific sequence did not show a mobility shift in binding reactions with dCas9/sgRNA and mecA DNA (data not shown).

3.2. Rapid detection of MRSA using genomic DNA

To determine whether dCas9/sgRNA complex in combination with SG I is sufficient to detect MRSA in vitro, genomic DNA (10 g each) from several strains, including MRSA clinical isolates #78, #81, #82, and #84, MSSA clinical isolates #85, #88, and #94, and ATCC25923, were extracted and then incubated with dCas9/sgRNA (#1539) for 15 min at room temperature. After hybridization, a pull-down assay using Ni-NTA magnet beads to isolate the dCas9/sgRNA complex was performed, the unbound genomic DNA was removed by magnet-assisted washing, and then SG I was added. All clinical isolates were obtained from the BioNano Health Guard Research Center (H-GUARD). Using fluorescence spectroscopy, we examined the fluorescence change in the presence of genomic DNA of MRSA compared with that of control strains. The data revealed the great specificity of the dCas9/sgRNA-SG I based DNA-FISH system for MRSA detection with a marked fluorescence increase of approximately 10- to 16-fold relative to that for MSSA (MRSA#78: 1841; MRSA#81: 1772; MRSA#82: 1272; MRSA#84: 1409; MSSA#85: 86; MSSA#88:139; MSSA#94: 193; and ATCC25923: 77), suggesting the excellent potential of this

Fig. 1. Fluorescence detection of MRSA using dCas9/sgRNA- SG I. Fluorescence intensity of the genomic DNA of MRSA and MSSA (10 g each) after incubation with dCas9/sgRNA (#1539) for 1 h and Ni-NTA magnetic bead

separation, followed by the addition of SYBR Green I. Error bars represent the standard deviation of the mean.

Fig. 2. Changes in the fluorescence intensity of the dCas9/sgRNA- SG I based system. Various amounts of genomic DNA extracted from MRSA #78 were incubated with the dCas9/sgRNA (#1539) complex, followed by SG I treatment, and the fluorescence intensity at 494 nm was plotted versus the concentration of the target DNA (F_0: fluorescence intensity for 0 ng target DNA; F: fluorescence intensity for different concentrations of the target DNA).

Fig. 3. Fluorescence detection of MRSA using dCas9/sgRNA-SG I mediated DNA-FISH. Fluorescence intensity of dCas9/sgRNA after incubation with MRSA and MSSA cell lysates, Ni-NTA magnetic bead separation and the addition of SG I. Error bars represent the standard deviation of the mean.

novel method in diagnostics (Fig. 1). The limit of detection (LOD), determined from a standard curve using various amounts of genomic DNA extraction, was 31 ng/ml (Fig. 2).

Fig. 4. Linear regression curve for the detection of MRSA. The change in the fluorescence intensity at 412 nm vs. the concentration of MRSA (log MRSA cfu/ml) was measured. Error bars represent the standard deviation of the mean.

3.3. Rapid detection of MRSA using cell lysates

The most conventional method to identify MRSA uses PCR, which is time-consuming and laborious. This method requires lysis of the bacterium, DNA extraction, and PCR, followed by gel electrophoresis, thus taking several hours to complete. We further evaluated our system

using cell lysates without an intervening DNA extraction protocol, which led to a definitive result within 30 min. Cell lysate samples (10^{10 cfu/ml) without further gene purification were incubated with dCas9/sgRNA (#1539), followed by a pull-down assay using Ni-NTA magnet beads, washing and fluorescence detection after the addition of SG I (Fig. 3). In the present detection method, the fluorescence signal increased more than 10-fold when evaluating the MRSA cell lysate as compared to MSSA. This difference in fluorescence intensity was sufficient to effectively detect the target, MRSA, in this study. The dCas9/sgRNA-SG I based DNA-FISH system allowed us to directly distinguish MRSA using cell lysates, confirming that the method can be used for the facile, rapid and specific detection of MRSA. The sensitivity of this system was then investigated by measuring the fluorescence intensity obtained with different concentrations of MRSA at room temperature (Fig. 4). The fluorescence intensity was significantly increased after the addition of cell lysate. A linear response to the MRSA concentration over an extended dynamic range of 10^{-10 CFU/ml was measured with a detection limit of 10 CFU/ml, which is more sensitive than the non-PCR-based methods reported (Lee et al., 2013; Malhotra-Kumar et al., 2010a, 2010b; Sherlock et al., 2010). Based on these results, we confirmed that the developed dCas9/sgRNA-SG I-based approach could be used for rapid and highly sensitive detection of MRSA. Unlike conventional gene diagnosis methods in which PCR must be accompanied or genes are isolated and analyzed, despite its excellent sensitivity of 10^0-10^5 CFU/ml, the simple sample

(a)

(b)

(c)

Fig. 5. (a) Microarray detection of MRSA by dCas9/sgRNA-SG I based system. Genomic DNA from MRSA clinical isolates (#78, #81, #82 and #84) and MSSA clinical isolates (#85, #88, #94, and ATCC25923) were separately applied to dCas9/sgRNA spotted on a Ni-NTA functionalized glass surface, followed by addition of SG I. (b) Fluorescence images of this system after detection of MRSA (left) and MSSA (right), respectively and (c) their fluorescence intensities corresponding to the fluorescence images. Error bars represent the standard deviation of the mean.

Scheme 1. Schematic representation of MRSA detection using the dCas9/sgRNA-SYBR Green I based system.

preparation is very convenient and highly beneficial for its practical application (Huang et al., 2015; Lee et al., 2013; Malhotra-Kumar et al., 2010a, 2010b; Malhotra-Kumar et al., 2010a, 2010b; Oh et al., 2013; Rossney et al., 2008).

3.4. dCas9/sgRNA-SG I based microarray for MRSA detection.

We further investigated the specificity of MRSA detection by using dCas9/sgRNA complex immobilized on the glass surface to confirm applicability to microarray technology. The dCas9/sgRNA (#1539) was spotted onto a Ni-NTA functionalized glass surface, and genomic DNA from MRSA clinical isolates and MSSA clinical isolates were applied to the dCas9/sgRNA (#1539) plotted glass slide, followed by SG I staining. As shown in Fig. 5, the MRSA specifically targeted the detection spot, whereas the fluorescence signals of MSSA were nearly at background levels. We confirmed that the dCas9/sgRNA-SG I based approach can be applied for an effective microarray technique to expand the potential applications of this method (Scheme 1).

4. Conclusions

In conclusion, we have demonstrated a dCas9/sgRNA-SG I based DNA-FISH system for the simple, rapid and highly sensitive detection of MRSA with a detection limit of 10 cfu/ml. This system employs dCas9/sgRNA complex, which acts similar to the antibody in conventional immunoassay systems, as the targeting material and SG I as a fluorescent probe, thus providing a relatively simple and expensive hybridization process for labeling. We confirmed that our system has the advantage of superior specificity for the target compared to conventional non-PCR-based methods (Lee et al., 2013; Malhotra-Kumar et al., 2010a, 2010b; Sherlock et al., 2010). In fact, our novel detection system introduces target sequence flexibility, detection accuracy, and efficiency to fluorescence generation. Furthermore, this dCas9/sgRNA-SG I-based system could be applied directly at the point of care for the rapid detection of MRSA because it is easy, sensitive, and affordable. Most importantly, our novel detection system is not limited to MRSA because the target of dCas9/sgRNA can be changed simply by altering the sgRNA sequence; thus, this system is widely applicable for many other targets.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2017.04.016.

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