Production of Bacteriocin Like Substances as Antipathogenic Metabolites by *Staphylococcus warneri* Isolated from Healthy Human Skin

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Abstract Antibiotic resistance is a serious problem of present world and development of viable alternative is urgent. The research work was designed to mitigate this problem. Different types of bacterial colony were isolated from skin of 30 healthy human and their antipathogenic activity was tested against 9 pathogens. The isolate showed activity against four pathogens- *Klebsiella pneumoniae* subsp. *pneumoniae*, *Klebsiella pneumoniae* subsp. *ozaenae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* was identified as *Staphylococcus warneri*. Variation was found in optimization of cultural conditions (incubation period, incubation temperature and pH) for the most potent antipathogenic metabolites production. *Staphylococcus warneri* showed most potent antipathogenic activity at pH 6 to pH 9, at an incubation period of 24h to 48h and at an incubation temperature of 37°C. Antipathogenic metabolites were then detected as bacteriocin like substances. Variation was observed with bacteriocin activity against different pathogens and was found most effective against *Klebsiella pneumoniae* subsp. *pneumoniae* and *Klebsiella pneumoniae* subsp. *ozaenae*. Samples containing bacteriocin like substances showed heat stability up to 80°C for 60 minutes and up to pH 8 for *Klebsiella pneumoniae* subsp. *pneumoniae* and *Klebsiella pneumoniae* sub sp. *ozaenae*. Papain treated cell-free supernatant did not show any bacteriocin activity, suggesting that the substances could be antimicrobial peptides. Solvent extraction of bacteriocin was performed by using chloroform where maximum bacteriocin activity was found in interface layer rather than aqueous and organic layer.

Keywords Antipathogenic Metabolites, Bacteriocin, Antimicrobial Peptide, Normal Microflora

Microbes that colonize the human body during birth or shortly thereafter, remaining throughout life, are referred to as normal flora [1]. A diverse microbial flora is associated with the skin and mucous membranes of every human being from shortly after birth until death [2]. Human skin is not a particularly rich place for microbes to live. This is an environment that prevents the growth of many microorganisms, but a few have adapted to life on our skin [3]. The effects of the normal flora are inferred by microbiologists from experimental comparisons between "germ-free" animals (which are not colonized by any microbes) and conventional animals (which are colonized with a typical normal flora). The overall beneficial effects of microbes are synthesis and excretion of vitamins, prevention of colonization by pathogens, antagonize other bacteria, stimulation of the development of certain tissues, development of gut associated lymphoid tissue, production of natural antibodies etc. [4]. Many mechanisms have been postulated by which Normal flora could produce antipathogenic activity. In addition to their competitive inhibition of the epithelial and mucosal adherence of pathogens and inhibition of epithelial invasion by pathogens, normal flora also show antipathogenic activity by producing antimicrobial substances or stimulating mucosal immunity. Generally normal flora produces three types of antimicrobial substances- H₂O₂, organic acid and Bacteriocin [5]. Bacteriocins are proteins or short polypeptides, which are generally only toxic to bacteria that are closely related to the producing strain [6]. Some researcher found that, [7]. *Staphylococcus epidermidis*, a major constituent of the normal microflora on healthy human skin protect skin by producing antimicrobial peptide. Another research work [8] on bacteriocin-like inhibitory substance (BLIS) which is produced by the Streptococcal normal flora of the nasopharynx were found active against pathogens *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae* and *Moraxella catarrhalis*. In our recent study, we have tried to investigate the ability of

1. Introduction

normal skin flora to produce antipathogenic metabolites and also tried to reveal the fact that whether the metabolites were bacteriocin like substances or not.

2. Material and Methods

Samples collection, isolation and purification

Swab samples were collected from the skin of upper arm, under arm, forearm, leg and forehead of 30 healthy human persons free from skin or any other infection during sampling time and with a history of not taking antibiotics or any other antimicrobial drugs in the previous six months. For the collection of normal skin flora, the person who met selection criteria was first taken to laboratory. With the help of sterile moisten cotton bud sample was taken from forearm, upper arm, under arm, forehead and leg of the selected healthy human persons. The swab stick was then soaked in 10ml sterile peptone water from which 1 ml sample was used for pour plate method [9] by using Mannitol salt agar medium and then incubated at 37°C for 24 to 48 hours. Following incubation, different types of colonies based on differences in size, shape, color and other colony characteristics, were selected and transferred to nutrient agar slant and were further purified by repeated streaking on nutrient agar plates.

Test pathogens

For our current study, some type strains of skin and intestinal pathogens were collected from Department of Microbiology, University of Chittagong, ICDDR, B and Chittagong Maa-Shishu O General Hospital (CMSOGH) to observe the inhibitory activity of the normal skin flora against these pathogens. The used bacterial test organisms were *Klebsiella pneumoniae* subsp. *ozaenae* (CMSOGH, urinary tract infection), *Klebsiella pneumoniae* subsp. *pneumoniae* (CMSOGH, urinary tract infection), *Salmonella typhi* (AEI14296), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (CRL, ICDDR,B), *Staphylococcus aureus* (ATCC 6538), *Streptococcus pyogenes* (CMSOGH, pus) *Bacillus subtilis* (BTTC17)

Primary screening for antimicrobial activity of the isolates

The isolated bacteria (normal flora) were subjected to primary screening procedures to find out the ability of the normal flora to produce antipathogenic metabolites by streak plate method [10]. The isolates (normal flora) were streaked over the surface of petridish containing pre-poured solidified Mueller Hinton media across one side of the plate & incubated at 37°C for growth and diffusion of antimicrobial metabolites in the growth medium. Then cultures of the test pathogens were streaked right angles to the previously inoculated isolate. The plates were incubated at 37°C for 24

hours. After the growth of the pathogens, the plates were observed for any zone of inhibition. A clear zone of inhibition appeared against only those organisms which were sensitive to the metabolites produced by the isolates. Those pathogens and respective isolates were selected for secondary screening.

Secondary screening for antimicrobial metabolite production by the isolates

Primarily selected isolates were subjected to the secondary screening procedures to ensure their antimicrobial activity against the respective pathogen. For this purpose, each normal flora was inoculated in 10ml sterile nutrient broth and incubated at 37°C for 24 hours. After incubation, culture broth was centrifuged at 12000 rpm for 15 minutes at 4°C and then filtered through membrane filter. After filtering off the biomass through .45µ cellulose nitrate filter paper, the antimicrobial activity of the crude filtrate obtained was tested against pathogens using disc diffusion [11] and well diffusion method [12]. The average clear zone greater than 10 mm was considered positive results [13].

3. Identification

Bacterial isolate (normal flora) which showed positive result on primary screening and secondary screening against maximum pathogens were subjected to biochemical tests and results were compared with standard description given in “Bergey’s Manual of Determinative Bacteriology”, 8th ed. [14] and 9th ed. [15]. The tests include Gram-staining, Spore staining, Acid-fast staining, starch hydrolysis, Voges Proskauer (V-P) Test, Production of hydrogen sulphide, Gelatin liquefaction test, Nitrate reduction test, Indole test, Deep glucose agar test, Catalase reaction, Methyl-red test, Fermentation test, Urease test, Motility test, Oxidase test. Cultural and physiological studies were also done. A rapid bacterial identification test kit BBL Crystal™ Identification Systems Gram-Positive Is Kit [16] was also used to identify species of bacteria.

Optimization of cultural conditions for antipathogenic metabolite production

Different environmental and cultural conditions may influence the antipathogenic metabolite production ability of the selected isolates. Considering this matter, in our present study, an attempt was made to optimize the cultural conditions that favor maximum antipathogenic metabolite production by isolated bacterial strain. To determine the effect of incubation period on antimicrobial metabolite production, 4 pieces 250 ml conical flask was labeled as 24h, 48h, 72h, 96h and 50 ml sterilized nutrient broth was taken on each flask. Selected normal flora strain was inoculated and incubated at 37°C for 24h, 48h, 72h, and 96h. After incubation, culture broth was centrifuged at 12000 rpm for

15 minutes at 4°C and then filtered through. 45µ Cellulose nitrate membrane filter. After filtering off the biomass, using 50 µl and 100 µl of crude filtrate per well the antipathogenic activity was tested against respective pathogens by well diffusion method. The clear zone diameter was measured to determine the antimicrobial activity. To observe the influence of pH on the production of antipathogenic metabolites 8 pieces 250 ml conical flask was labeled as, pH 5 to pH 10. 50 ml sterilized nutrient broth was taken on each flask and pH was adjusted according to the label in aseptic condition. Selected normal flora strain was inoculated and incubated at 37°C for optimum incubation period. After incubation, preparation of cell free supernatant and determination of antimicrobial activity was done by following the same method of incubation period optimization. To evaluate the impact of temperature on antipathogenic metabolite production, 3 pieces 250 ml conical flask was taken and labeled as 27°C, 37°C and 45°C. 50 ml sterilized nutrient broth was adjusted to optimum pH in aseptic condition and taken on each flask. Selected normal flora strain was inoculated and incubated at 27°C, 37°C and 45°C for optimum incubation period. After incubation, preparation of cell free supernatant and determination of antimicrobial activity was done by following the same method of incubation period optimization.

4. Detection of Bacteriocin like Substance Production

Preparation of Bacteriocin samples

Isolated bacterial strain was cultivated in its appropriate culture medium (Brain Heart infusion Broth, Hi Media Ltd) and conditions. Extraction of bacteriocin was carried out using the method of Schillinger and Luke [17]. According to this method, cells from the culture medium were removed by centrifugation at 5000 rpm for 10 minutes at 4°C followed by filtration through .45µ Cellulose nitrate membrane filter. The pH of the supernatant was adjusted to 6.5 with 5M NaOH and 5M HCl to exclude the antimicrobial effect of organic acid. Inhibitory activity from hydrogen peroxide was eliminated by the addition of 5 mg/ml catalase. The supernatant was serially diluted (2 fold dilution) with filter-sterilized 20mM phosphate buffer and stored at -20°C.

Bacteriocin bioassay

Antimicrobial activity against indicator organisms was determined by a well diffusion assay. Pre-poured Brain Heart Infusion (BHI) agar plates were overlaid with BHI soft agar containing indicator culture of *Staphylococcus aureus*. Wells of 6 mm in diameter were cut into the agar plate with a cork borer and 100 µl of the culture supernatant fluid was placed into each well. The plates were incubated overnight at 37°C. After incubation, arbitrary units (AU) of bacteriocin

activities were calculated according to spot-on-lawn method[18]. The antimicrobial activity of the bacteriocin is defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and is expressed in activity units per ml (AU/ml). That means,

$$\text{AU / ml} = \frac{2^n \times 1000 \mu\text{l}}{\text{Amount of sample per well } (\mu\text{l})}$$

Determination of Bacteriocin activity against test pathogens

Bacteriocin activity of the skin isolates against test pathogens was determined by the same process of bacteriocin bioassay just replacing the indicator organism with the test pathogens. The activity units (AU) of bacteriocins against the pathogens were also calculated.

Characterization of Bacteriocin

The bacteriocin samples were characterized with respect to their stability at or sensitivity to different temperatures, pH, and susceptibility to denaturation by enzyme.

Heat sensitivity of Bacteriocin

To observe the heat stability, bacteriocin samples were prepared from culture supernatants of the selected bacterial isolate by following the method of Schillinger and Luke [17]. Then samples were then dispensed at 10 ml per test tube and exposed to various temperatures such as 40, 60, 80 and 100°C in circulatory water bath and 121°C in autoclave. Aliquot volumes were then removed after 0, 30, 60 and 90 minutes and assayed for bacteriocin activity.

pH sensitivity of Bacteriocin

For the determination of pH stability, samples of bacteriocin were prepared from culture supernatants and pH was adjusted to 3, 4, 5, 6, 7, 8 and 9 with 5N HCl or 5N NaOH. After incubating for 4 hours at room temperature, the bacteriocin samples were adjusted to pH 6.5 and assayed for bacteriocin activity.

Enzyme sensitivity of Bacteriocin

The sensitivity of the bacteriocin to enzymes was also checked. Bacteriocin samples, from cell-free culture supernatant fluid of different isolates, at pH 6.5 was treated separately with papain at a final concentration of 1.0 mg/ml. Samples of bacteriocin were incubated with the enzyme for 2 hours at 37°C. After that, samples of bacteriocin were heated for 3 minutes at 100°C to inactivate enzyme activity and assayed for bacteriocin activity [19].

Solvent extraction of bacteriocin and its' bioassay

The extraction of bacteriocin was done by the sample made from cell-free culture supernatant. The extraction was performed following slightly modified method of Burianek and Yousef (2000) [20]. The culture supernatant (100mL) was stirred vigorously for 20 minutes with equal volume of chloroform and transferred to separation funnel. The aqueous layer, organic layer and the interface layer between the aqueous and organic phases were harvested separately and the residual chloroform was eliminated by rotary vacuum evaporator. Then the bacteriocin activity of the interface layer, aqueous and organic layers was measured separately against the respective pathogen and the indicator organism and the result was expressed in AU/ml.

5. Results and Discussion

Isolation from skin

In our present study, 10 normal bacterial floras from the skin of upper arm, under arm, forearm, leg and forehead of 30 healthy persons were isolated based on their colony characteristics and abundance on the selective culture medium, Mannitol Salt Agar and subjected to primary screening by cross streaking method.

Primary Screening

After primary screening, one bacterial strain isolated from Under Arm (UndA) showed inhibitory activity against maximum pathogens and it was selected for secondary screening. It showed inhibitory activity against *Klebsiella pneumoniae subsp. pneumonia*, *Klebsiella pneumoniae subsp. ozaenae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Secondary screening

Well diffusion and disc diffusion method was used in secondary screening and our selected Isolate showed remarkable result against those four pathogens (Table 1)

Table 1. Production of inhibitory metabolites by selected isolates against pathogens in secondary screening.

Pathogen	Diameter of zone of inhibition (mm)			
	Aliquots of sample in well diffusion method		Aliquots of sample in disc diffusion method	
	100µl	50µl	80µl	50µl
<i>Klebsiella pneumoniae subsp. ozaenae</i>	17	12	16	11
<i>Klebsiella pneumoniae subsp. pneumonia</i>	20	14	18	13
<i>Staphylococcus aureus</i>	22	16	21	15
<i>Pseudomonas aeruginosa</i>	21	13	22	14

Note: Incubation temperature: 37°C and incubation time: 24–48 hours.

Identification of selected isolates

The selected skin isolate was tested for their morphological, cultural and biochemical characters. The characters were then compared with the standard description given in “Bergey’s Manual of Determinative Bacteriology”, 8th and 9th Edition [14] [15]. The characteristics of each isolates are summarized in Table 2. Data present in Table 2 indicated that the isolate UndA belongs to the genus *Staphylococcus* and found closely related to the species *Staphylococcus warneri*, while compared with the standard description given in “Bergey’s Manual of Determinative Bacteriology”, 9th ed. [15]. For further confirmation, rapid Identification kit BBL Crystal™ Identification Systems Gram-Positive Kit was used. (Fig 1) and found same result with .956 confidence.

Figure 1. Rapid Identification through BBL Crystal Identification kit

Table 2. Cultural, morphological and biochemical characteristics of selected isolate

Parameter	Observation	Parameter	Observation	Parameter	Observation
Biochemical tests		Fermentation		Agar Colony	
Oxidase	—	Glucose	+	Form	Punctiform
Catalase	+	Lactose	—	Elevation	Convex
Deep glucose agar	Aerobic	Sucrose	—	Margin	Entire
Motility	—	Fructose	+	Surface	Smooth
Starch hydrolysis	—	Mannitol	—	Color	Off white
Gelatin hydrolysis	+	Arabinose	—	Staining and Gram staining	Morphology Gram positive
Nitrate reduction	—	Xylose	—		
Citrate utilization	+	Inulin	—		
Voges-Proskauer	+	Raffinose	—		
Motility test	—	Galactose	—		
Methyl red	—	Salicin	—	Size	1.85-2.4µm
Urease	+	Maltose	+	Shape	Coccoid
H ₂ S production	+	Cellubiose	+	Form	Single/pair and cluster
Indole	—				

Optimization of cultural conditions for the production of antipathogenic metabolites with maximum activity

Production of metabolites from microorganism varies with the variation in incubation period, incubation temperature, pH, etc. In present investigation, the optimum cultural conditions (incubation period, incubation temperature, pH etc) were studied with the isolate *Staphylococcus. warneri* (**Table 3**).

Table 3. Optimization of different cultural condition (incubation period, pH, temperature) for maximum antipathogenic metabolite production

Optimization parameter		Pathogen							
		<i>Klebsiella. pneumoniae</i> subsp. <i>pneumonia</i>		<i>Klebsiella. pneumoniae</i> subsp. <i>ozaenae</i>		<i>Stap. aureus</i>		<i>Pseudomonas. aeruginosa</i>	
		Diameter of zone of inhibition(mm)		Diameter of zone of inhibition (mm)		Diameter of zone of inhibition (mm)		Diameter of zone of inhibition (mm)	
		100µl	50µl	100µl	50µl	100µl	50µl	100µl	50µl
Incubation period	24h	20	14	17	12	22	16	21	13
	48h	23	17	21	17	19	14	17	11
	72h	19	13	15	11	15	11	13	10
	96h	14	11	11	8	10	7	10	7
	pH 5	--	--	12	9	14	10	16	10
pH	pH 6	--	--	16	11	18	12	20	13
	pH 7	12	9	20	16	20	16	17	10
	pH 8	17	13	17	12	17	11	15	7
	pH 9	21	15	11	7	12	9	11	--
	pH 10	16	11	--	--	--	--	--	--
Temperature	25°C	14	11	14	10	17	12	17	11
	37°C	21	15	21	17	20	16	20	13
	45°C	17	12	20	16	14	10	16	10

Detection of Bacteriocin like substance

In our present investigation, we tried to determine whether the metabolites found from normal microflora contain any bacteriocin like substance or not. For this purpose, we used Brain heart infusion broth for bacteriocin production²¹ and bacteriocin was extracted by following the method of Schillinger and Luke[17]. From this study, we found that the isolates-*Staphylococcus. warneri* produced bacteriocin like substances and showed different activities against different pathogens or indicator organism(**Fig 2 & Fig 3**) where lowest zone of inhibition was found 8mm for *Staphylococcus. aureus* and *Pseudomonas. aeruginosa*. For *Klebsiella. pneumoniae* subsp. *pneumoniae* and *Klebsiella. pneumoniae* subsp. *ozaenae* it was 9 mm. Chaimanee [21] described isolates- *Lactobacillus. fermentum* and *Streptococcus bovis* showing bacteriocin activity 40 AU/ml against pathogens *Staphylococcus. agalactiae* and *Staphylococcus. dysgalactia* by following the same method which is closely related to the result we obtained.

Figure 2. Activity of bacteriocin produced by the *Staphylococcus. warneri*

Figure 3. Activity of bacteriocin produced by the isolate *Staphylococcus. warneri* against *Klebsiella. pneumoniae subsp. ozaenae*

Characterization of Bacteriocin

In our present investigation, we tried to characterize the bacteriocin sample with respect to their sensitivity to different pH, temperature and enzyme papain.

Heat sensitivity of Bacteriocin

Samples of bacteriocin were exposed to various heat treatments such as 40, 60, 80, 100 and 121°C for the periods of 0, 30, 60 and 90 minutes and then assayed for bacteriocin activity. Data presented in **Fig 4 to Fig 7** showed that bacteriocin produced by *Staphylococcus. warneri* was prominent for its' heat stability up to 80°C. It retained its' full activity even after 30 minutes of treatments at this

temperature and one fourth of activity after 60 minutes of treatment at the same temperature against *Klebsiella. pneumoniae subsp. pneumoniae* and *Klebsiella. pneumoniae subsp. ozaenae*. Although the activity was low against the two other pathogens, it was reduced in the same manner with the increase of temperature. These results were in agreement with the findings Bizani (2002)[22] who reported that the bacteriocin produced by a newly isolated *Bacillus* sp. strain 8A was stable at 80°C, but the activity was lost when the temperature reached 87°C.

Figure 4. Activity of heat treated bacteriocin produced by the isolate *Staphylococcus. warneri* against *Klebsiella. pneumoniae subsp. pneumoniae*

Figure 5. Activity of heat treated bacteriocin produced by the isolate *Staphylococcus. warneri* against *Klebsiella. pneumoniae subsp. Ozaenae*

Figure 6. Activity of heat treated bacteriocin produced by the isolate *Staphylococcus warneri* against *Staphylococcus aureus*

Figure 7. Activity of heat treated bacteriocin produced by the isolate *Staphylococcus warneri* against *Pseudomonas aeruginosa*

pH sensitivity of Bacteriocin

The bacteriocin produced by the isolate *Staphylococcus warneri* retains full activity against all respective pathogens (*Klebsiella pneumoniae subsp. pneumoniae*, *Klebsiella pneumoniae subsp. ozaenae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*) up to pH 7. But as presented in **Fig 8**, it exhibited stability up to pH 8 with some decrease in activity. In case of *Klebsiella pneumoniae subsp. pneumoniae*, the activity was reduced to half of its' highest activity and against *Klebsiella pneumoniae subsp. ozaenae* it decreased four-fold at pH 8. An investigation about bacteriocin characterization was made by Fatima (2013)[23] where bacteriocin produced from *Lactobacillus plantarum* and *Pediococcus pentosaceus* showed pH stability up to pH 2 to pH 6 which was closely related to our findings.

Figure 8. Activity (AU/ml) of *Staphylococcus warneri* produced bacteriocin subjected to different pH.

Figure 9. A- Bacteriocin sample treated with papain (Treatment) B- Bacteriocin sample without papain treatment (Control)

Enzyme sensitivity of bacteriocin

The bacteriocin samples from different isolates were exposed to the enzyme papain not only to determine its' sensitivity to enzyme but also to confirm the proteinaceous nature of bacteriocin. It was found that papain treated cell free supernatant did not show any antimicrobial activity (**Table 4 & Fig 9**), suggesting that the substances could be antimicrobial peptides. Our findings were in agreement with the report of Lauková (1993) [24] describing that bacteriocin-like substance produced by *Enterococcus faecium* CCM4231 which was sensitive to pronase.

Table 4. Activity (AU/ml) of bacteriocin produced by *Staphylococcus. warneri* after papain treatment

Bacteriocin	Bacteriocin activity (AU/ml)			
	Pathogen			
	<i>Klebsiella. pneumoniae</i> subsp. <i>pneumoniae</i>	<i>Klebsiella. pneumoniae</i> subsp. <i>ozaenae</i>	<i>Staphylococcus. aureus</i>	<i>Pseudomonas. aeruginosa</i>
Bacteriocin without papain	160	160	80	80
Papain treated bacteriocin	0	0	0	0

Table 5. Bacteriocin activity of *Staphylococcus. warneri* (AU/ml) against pathogens after in interface layer after chloroform extraction

Pathogen	Bacteriocin activity (AU/ml)
<i>Klebsiella. pneumoniae</i> subsp. <i>pneumoniae</i>	320*
<i>Klebsiella. pneumoniae</i> subsp. <i>ozaenae</i>	320*
<i>S. aureus</i>	160
<i>Pseudomonas aeruginosa</i>	160

Chloroform Extraction of Bacteriocin like substance

In our present investigation, we tried to extract bacteriocin from crude cell free supernatant. The extraction was performed according to Burianek and Yousef (2000) [20]. Then bacteriocin activity was measured in the interface layer, aqueous and organic phases. Highest bacteriocin activity was found in interface layer. Increased bacteriocin activity in the interface layer (**Table 5**) indicates that this method concentrates the bacteriocin at the interface between chloroform and the aqueous phases of the bacteriocin containing culture supernatant.

5. Conclusions

The study revealed that bacteriocin like substances produced by *Staphylococcus. Warneri* isolated from healthy human skin micro flora possesses a wide spectrum of inhibitory activity against some intestinal and skin pathogens. Therefore, it is better to say that the findings obtained from this journey to explore a potential alternative to antibiotic will contribute a lot in future research for controlling disease caused by drug resistant bacterial strains. To reach this final goal, further research including human trial with the produced bacteriocin is required to prevent the emergence of MDR pathogens and to improve the disease management system.

Acknowledgements

The researchers are grateful to The Chittagong Maa-Shishu O General Hospital, ICDDR, B, University of Chittagong and National Science and Technology (NST) Fellowship. Funding: (Ministry of Science and Technology, Bangladesh 39.012.002.01.03.019.2013-281)

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