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Identification and phase inversion of *Salmonella* flagellar antigens, using immuno-discs

Masatoshi FUJIHARA[1)]*, Yukino TAMAMURA[2)], Hiroyuki TABUCHI[1)] and Kaho UEGAKI[1)]

[1)]Hokkaido Hiyama Livestock Hygiene Service Center, 281-1 Tazawamachi, Esashi, Hiyamagun, Hokkaido 043-0023, Japan

[2)]Bacterial and Parasitic Disease Research Division, National Institute of Animal Health, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

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ABSTRACT. Disc immuno-immobilization is a simple method for typing the flagellar phase of *Salmonella enterica*. We re-examined this method using commercial antisera, which contains the preservative sodium azide. Originally prepared motility agar activates bacterial motility and renders *S. enterica* resistant to sodium azide, resulting in the formation of immuno-immobilization lines around reactive immuno-discs. Though disc immuno-immobilization serves both serotyping and phase inversion, this method is insufficient for the strains in which phase variation rarely occurs. Here, we devised a novel immuno-disc phase inversion method, and all *S. enterica* strains tested were identically typed. These methods would drastically simplify the task of *S. enterica* typing in clinical laboratories.

KEY WORDS: flagellar antigen, immuno-disc, *Salmonella enterica*

Salmonella enterica has traditionally been classified by serological identification of specific somatic and flagellar antigens [15]. Traditional serotyping of flagellar antigens in most cases requires a strain phase reversal, which is time consuming and skill-intensive. Recently, molecular PCR-based methods for direct identification of flagellar antigens have been developed [3, 4, 6]; however, applicable flagellar antigens are limited in these methods. Furthermore, PCR-based methods and serological determination will make a decisive difference in the identification of *S. enterica* serovars harboring two phases of flagellin-encoding genes with mutated flagellar motor protein, flagellar export apparatus [8, 9] or *hin* site-specific recombinase (responsible for phase variation) [7].

Mohit reported a disc immuno-immobilization method in 1968 [10]. In this method, immuno-discs saturated with antisera were placed at the periphery and *S. enterica* was spot-inoculated at the center of the motility agar. During incubation, antisera diffused around each disc in a decreasing concentration gradient, and *S. enterica* moved uniformly through the agar peripherally. When *S. enterica* serovars encountered antisera that reacted with their specific flagellar phase, monophasic serovars were completely immobilized but biphasic serovars expressing the opposite phase were not immobilized, resulting in the formation of semicircular immuno-immobilization lines. In the disc immuno-immobilization method, more than 10 types of antiserum can be examinable on one motility agar plate, which implies that most *S. enterica* serovars can be serotyped using a few motility agar plates. Furthermore, this method takes 36 hr and requires less than 1 hr of work for definitive typing and isolation of the two flagellar phases [10]. Since Mohit used originally prepared antisera, we examined the availability of commercial antisera containing sodium azide, a preservative, in disc immuno-immobilization methods. In addition, we devised immuno-disc phase inversion for strains in which phase variation rarely occurs.

MATERIALS AND METHODS

Evaluation of motility agar

Five motility agars were prepared. Compositions of each agar are shown in Table 1. *S.* Abortusequi Ne-ae, *S.* Dublin Ab-du and *S.* Typhimurium Ab-ty [5] were spot-inoculated on the center of each motility agar. Immuno-discs, prepared using filter paper (6 mm in diameter) with 10 μl of *Salmonella* H-G, i, en, p, x, 1 or 2 typing antisera (Denka Seiken Co., Ltd., Tokyo, Japan), were placed on the periphery of the motility agar and incubated at 37°C for 15–60 hr.

*Correspondence to: Fujihara, M.: fujihara.masatoshi@pref.hokkaido.lg.jp

Table 1. Composition of each motility agar (per 1 liter)

Motility agar	Basal medium	Added substances
A	Luria broth (SIGMA-ALDRICH, Inc., Tokyo, Japan)	Agar (3 g)
B[a)]	50% concentration of Rappaport broth (Eiken, Tokyo, Japan)	Casamino acid (5 g), agar (3 g)
C	5% concentration of Rappaport broth	Casamino acid (5 g), NaCl (3.6 g), agar (3 g)
D	5% concentration of Rappaport broth	Casamino acid (1 g), NaCl (3.6 g), agar (3 g)
E	Distilled water	Casamino acid (5 g), NaCl (4 g), agar (3 g)

a) Same composition as modified semisolid Rappaport agar (4).

Redetermination of flagellar antigens by disc immuno-immobilization method

Nineteen *S. enterica* serovars (27 wild strains isolated from livestock in Hokkaido [5], Chiba and Kanagawa prefectures and one purchased strain), were spot-inoculated at the center of motility agar D. Immuno-discs saturated with 28 antisera (H-a, b, c, d, eh, G, i, k, L, m, en, p, r, s, t, w, x, y, z, z_4, z_{10}, z_{13}, z_{15}, z_{29}, 1, 2, 5 and 7) (Supplementary Table S1) were placed on the periphery of the motility agar and incubated at 37°C.

Comparison of immuno-disc phase inversion and conventional phase inversion

Four *S.* Choleraesuis strains were spot-inoculated on motility agar D and overlaid with an immuno-disc that immobilized each strain (H-c or 1). Another immuno-disc saturated with the same antiserum was placed 3 cm apart from inoculation site. For conventional phase inversion, *S.* Choleraesuis was inoculated into a Craigie tube placed in SIM medium (Nissui Pharmaceutical, Tokyo, Japan) containing phase induction antisera H-c or 1 (contains no sodium azide) (Denka Seiken Co., Ltd.) according to the manufacturer's instructions. After incubation at 37°C, success or failure of phase inversion was identified by disc immuno-immobilization method. The same single colony of *S.* Choleraesuis was used for comparison between immuno-disc and conventional phase inversion method.

RESULTS

Performance of each motility agar

Figure 1 shows that *S. enterica* proliferated to the extent that it exceeded the titer of the antibody on motility agar A. Furthermore, sodium azide in commercial antisera seemed to induce formation of inhibition zones. On motility agar B–E, inhibition zones were absent and *S.* Typhimurium formed immuno-immobilization lines around H-i, 1 and 2 immuno-discs; however, *S.* Abortusequi and *S.* Dublin did not migrate on the motility agar B and E. These serovars were immobilized by H-en, x and H–G, p respectively on motility agar C and D. The immobilized area was wider on motility agar D than on C. While *S.* Typhimurium reached the immuno-discs within 15 hr, *S.* Abortusequi and *S.* Dublin took 60 and 40 hr, respectively, on motility agar D. It is noteworthy that the clarity of immuno-immobilization lines depends on the abundance ratios of flagellar antigens. Regardless of line thickness, semicircular lines around immuno-discs were considered specific immuno-immobilization lines.

Disc immuno-immobilization using motility agar D

Within 24 hr, all *S. enterica* serovars except for *S.* Abortusequi and *S.* Dublin encountered antisera that reacted with their specific flagellar phases. Antisera immobilizing each *S. enterica* strain are shown in Table 2. All monophasic serovars were completely immobilized (Fig. 2A), on the other hand, all biphasic serovars, except for three, formed immuno-immobilization lines around immuno-discs that reacted with their two flagellar phases (Fig. 2B). *S.* Paratyphi B and *S.* Mbandaka formed immuno-immobilization lines only around phase II immuno-discs; however, bacterial cells expressing phase I flagellin reached the immuno-discs and subcultured cells formed immuno-immobilization lines around the phase I immuno-disc (Fig. 2C and 2D).

Serotyping of S. Choleraesuis strains

Since *S.* Choleraesuis (four strains) was completely immobilized and the opposite phase immuno-disc(s) did not form immuno-immobilization lines by disc immuno-immobilization (Fig. 3A), *S.* Choleraesuis serotyping was conducted using two phase inversion methods. Figure 4 shows the principle of immuno-disc phase inversion. In this method, phase inverted *S.* Choleraesuis on motility agar D reached another immuno-disc (Fig. 3B) and subcultured cells were immobilized by opposite phase immuno-disc(s) (Fig. 3C). In case of no phase inversion, bacterial cells started to migrate after cell counts exceeded the titer of the antibody; however, they were completely immobilized by another immuno-disc, and a drop-shaped aseptic area was formed around the second immuno-disc (Fig. 3B lower left section). The number of successful phase inversions from five examinations is shown in Table 3. In immuno-disc phase inversion, cells that reached the other immuno-disc were observed from after 24 hr of incubation. After 48 hr of incubation, phase inversions were confirmed in all tests except for that of strain Ab-ch. On the contrary, in conventional phase inversion, phase inverted cells start swimming out from the Cragie tube after 15 hr of incubation. However, even after 48 hr of incubation, strain L2722 was phase inverted in only one out of five examinations.

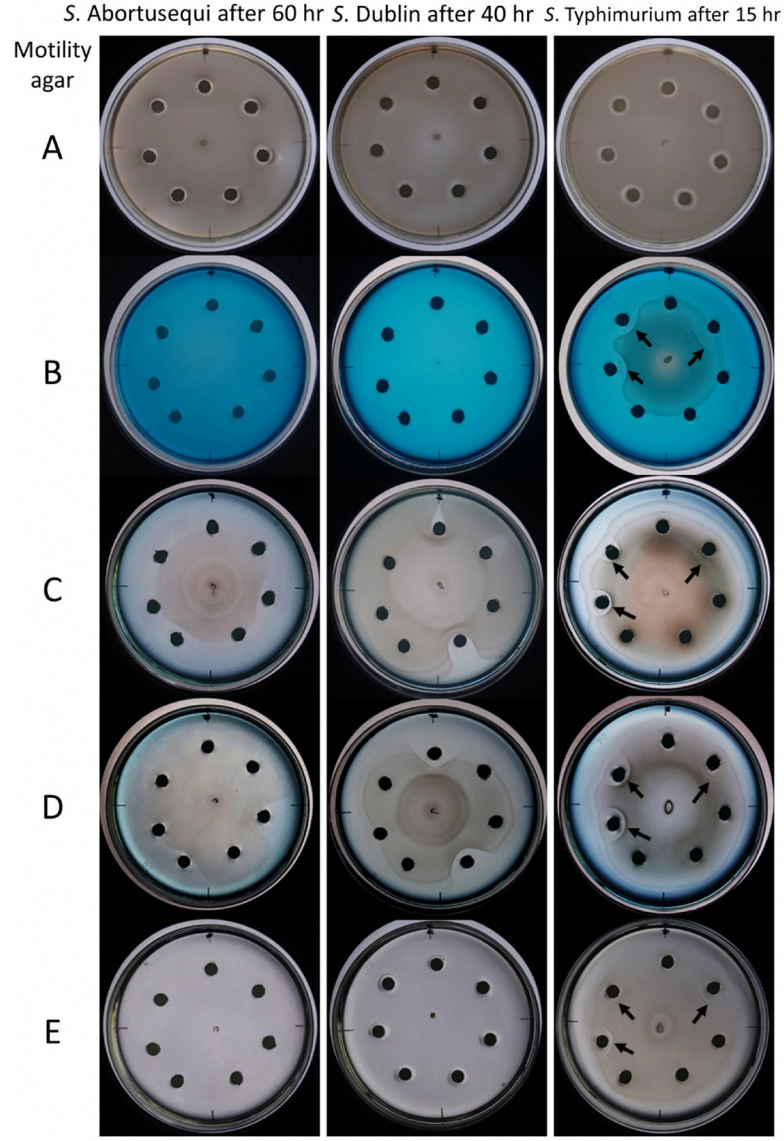

Fig. 1. Growth behaviors on each motility agar. *S.* Abortusequi Ne-ae, *S.* Dublin Ab-du and *S.* Typhimurium Ab-ty were inoculated at the center of each motility agar. Immuno-discs contained H-G, i, en, p, x, 1, and 2 antisera in a clockwise fashion from top. Arrows show immuno-immobilization lines.

DISCUSSION

For disc immuno-immobilization using commercial serotyping antisera, motility agar needs to support bacterial migration and to make *Salmonella* resistance to the preservative sodium azide. As shown in Fig. 1, low concentration of Rappaport broth supports bacterial migration (motility agar C and D). Rappaport broth is a magnesium-rich medium and *S. enterica* down regulates flagella-mediated motility under low extracytoplasmic Mg^{2+} [1, 11]. Mg^{2+} and other consitituents of the Rappaport broth may enhance bacterial migration. On the other hand, sodium azide in commercial antisera induced formation of inhibition zones on motility agar A. Since inhibition zones were disappeared on motility agar B–E, casamino acids, which are known to increase resistance to certain stresses [2, 12], may also render *S. enterica* resistant to sodium azide. Motility agar C and D are applicable to disc immuno-immobilization; however, poorer nutrient of motility agar D decreased bacterial cell amount and the immobilized area became wider than motility agar C. Considering above, motility agar D was most suitable for disc immuno-immobilization.

Determination of flagellar antigens and phase inversion of most strains were easily completed using disc immuno-immobilization without non-specific reaction. However, certain *S.* Choleraesuis strains, which rarely undergo phase variation, required another phase inversion. Flagellar phase variation is known to occur at frequencies ranging from 10^{-5} to 10^{-3} per bacterium per generation [14], considering that two flagellar antigens are present in definite proportions. If the expression of one flagellin phase is considerably lower than that of the other, their motilities towards immuno-discs of opposite phase would be blocked by

Table 2. Results of disc immuno-immobilization

Organism	Strain	Flagellar antigen		Immobilized antisera
		Phase I	Phase II	
S. Abony	To-ab	b	e, n, x	H-b, en, x
S. Abortusequi	Ne-ae	-	e, n, x	H-en, x
S. Abortusequi	Ab-ae	-	e, n, x	H-en, x
S. Choleraesuis	Ab-ch	c	1, 5	H-1, 5
S. Choleraesuis	ATCC 10708	c	1, 5	H-1, 5
S. Choleraesuis	L-2150	c	1, 5	H-c, 1, 5
S. Choleraesuis	L-2454	c	1, 5	H-c
S. Choleraesuis	L-2722	c	1, 5	H-1, 5
S. Dublin	Ab-du	g, p	-	H-G, p
S. Dublin	To-du	g, p	-	H-G, p
S. Enteritidis	Ab-en	g, m	-	H-G, m
S. Enteritidis	To-en	g, m	-	H-G, m
S. Isangi	To-is	d	1, 5	H-d, 1, 5
S. Kedougou	Ne-ke	i	l, w	H-i, L, w
S. Livingstone	Ab-li	d	l, w	H-d, L, w
S. Mbandaka	Ne-mb	z_{10}	e, n, z_{15}	H-en[a)], z_{10}, z_{15}
S. Montevideo	To-mo	g, m, s	-	H-G, m, s
S. Newport	To-ne	e, h	1, 2	H-eh, 1, 2
S. Paratyphi B	Ne-pb	b	1, 2	H-b[a)], 1, 2
S. Ruiru	Ne-ru	y	e, n, x	H-en, y, x
S. Saintpaul	Ne-sa	e, h	1, 2	H-eh, 1, 2
S. Schwarzengrund	Ne-sc	d	1, 7	H-d, 1, 7
S. Senftenberg	Ne-se	g, s, t	-	H-G, s, t
S. Typhimurium	Ab-ty	i	1, 2	H-i, 1, 2
S. Typhimurium	Hi-ty	i	1, 2	H-i, 1, 2
S. Uganda	To-ug	l, z_{13}	1, 5	H-L, z_{13}, 1, 5
S. 4:i:-	Ab-4i	i	-	H-i
S. 4:i:-	Hi-4i	i	-	H-i

a) Phase inverted cells by disc immuno-immobilization were immobilized.

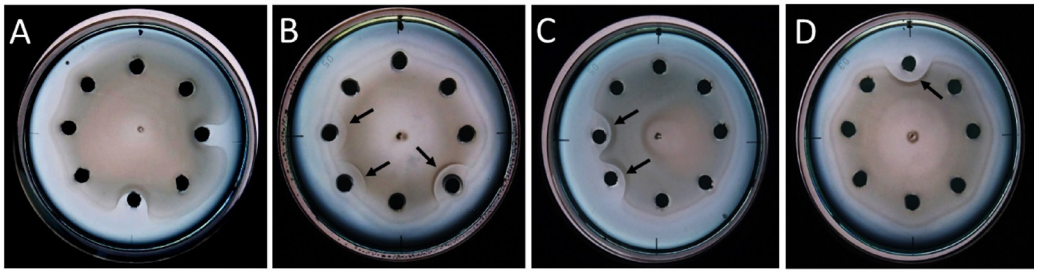

Fig. 2. Results of disc immuno-immobilization. Immuno-discs on motility agar D contained H-b, c, G, i, m, 1, 2 and 5 antisera in a clockwise fashion from top. Arrows show immuo-immobilization lines. (A) *S.* Enteritidis Ab-en was completely immobilized by the H-G and m immuno-discs. (B) *S.* Typhimurium Ab-ty formed immuno-immobilization lines around H-i, 1 and 2 immuno-discs. (C) *S.* Paratyphi B Ne-pb formed immuno-immobilization lines only around H-1 and 2 immuno-discs. (D) *S.* Paratyphi B picked from the inside of the immuno-immobilization lines formed by H-1 immuno-disc, formed immuno-immobilization lines around H-b.

agglutinated cells. Therefore, we devised an immuno-disc phase inversion method, in which phase-inverted *S. enterica* starts the precedent migration. Although *S. enterica*, which did not undergo phase variation, started to migrate after the antigen amount exceeded the antibody titer, bacterial cells were immobilized by another immuno-disc. Our data revealed that conventional phase inversion is superior in terms of speed; however, immuno-disc phase inversion showed either equal or better phase inversion ability. Two continuous inductions, escape from an overlaid immuno-disc, and penetration of another disc, may enhance phase inversion ability in this method. Furthermore, immuno-disc phase inversion is possible when antiserum containing sodium azide is used and it can substitute the conventional method.

Schrader *et al.* reported that Denka Seiken commercial antisera showed good performance in antigen detection of *S. enterica* [13]; we demonstrated that commercial antisera could be applied in the disc immuno-immobilization method using originally

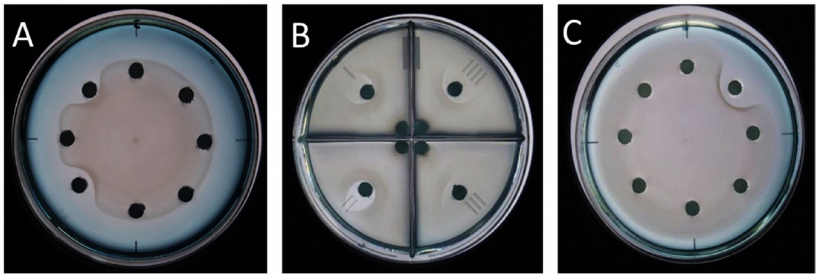

Fig. 3. Serotyping of *S.* Choleraesuis Ab-ch. Immuno-discs on motility agar D contained H-b, c, G, i, m, 1, 2 and 5 antisera in a clockwise fashion from top (A and C). (A) *S.* Choleraesuis was completely immobilized by H-1 and 5 immuno-discs. It did not form immuno-immobilization line around other immuno-discs including H-c by disc immuno-immobilization. (B) *S.* Choleraesuis was spot-inoculated under the inner H-1 immuno-discs. After incubation, *S.* Choleraeusuis reached the outer H-1 immuno-discs. Bacterial cells around outer immuno-discs are considered immuno-disc phase inverted. On the lower left section of the dish, *S.* Choleraesuis was completely immobilized and formed inhibition zones around outer immuno-disc. It was considered not to be phase inverted. (C) Immuno-disc phase inverted cells were completely immobilized by H-c immuno-disc.

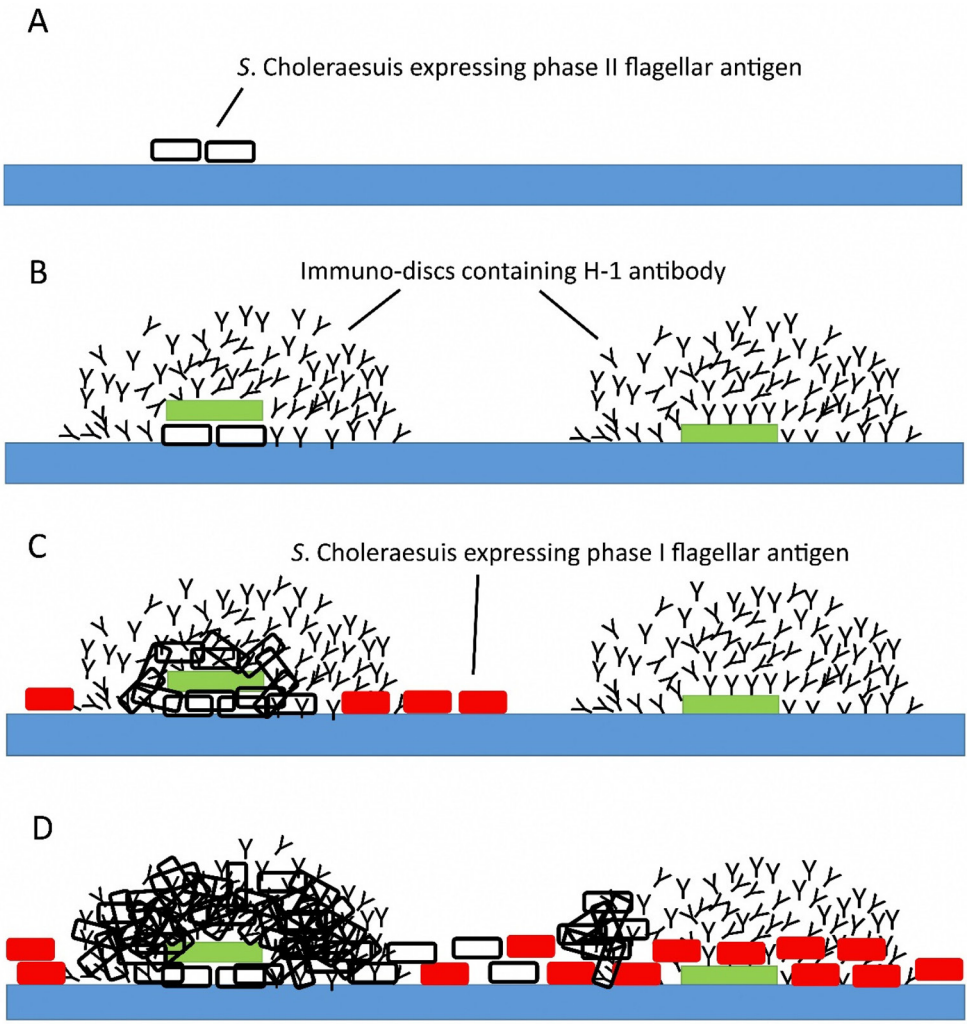

Fig. 4. The principle of immuno-disc phase inversion. (A) *S.* Choleraesuis was spot-inoculated on motility agar and (B) overlaid with a reactive immuno-disc. Another immuno-disc was separately placed. (C) *S.* Choleraesuis cells expressing phase II antigen are trapped by antibody. During growth, phase inverted *S.* Choleraesuis appeared and starts migration, (D) resulting to reach another immuno-disc. Bacterial cells expressing phase II antigen also starts to migrate after cell counts exceeded the titer of the antibody; however, they were immobilized by another immuno-disc.

Table 3. Phase inducted numbers (immuno-disc/conventional phase inversion) from five examinations

S. Choleraesuis strain	After 15 hr	After 24 hr	After 40 hr	After 48 hr
Ab-ch	0/0	1/2	4/4	4/4
ATCC 10708	0/4	3/5	5/5	5/5
L-2454	0/3	0/5	4/5	5/5
L-2722	0/0	0/0	4/1	5/1

prepared motility agar D. Disc immuno-immobilization, combined with immuno-disc phase inversion, is simple to interpret and all *S. enterica* strains tested in this study possessed flagellar antigens, which is consistent with the results obtained using conventional methods. Additionally, most serovars except for *S.* Abortusequi and *S.* Dublin were suitable for disc immuno-immobilization on modified semisolid Rappaport agar [5]. This indicates the possibility of simultaneous isolation and serotyping from contaminated materials (supplementary Fig. S1). These methods would drastically simplify the task of *S. enterica* typing in clinical laboratories.

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