INFLUENCE OF THE HYDROGEN ION CONCENTRATION OF CULTURE MEDIA UPON THE DEVELOPMENT OF e, n, x FACTORS OF *SALMONELLA ABORTUS-EQUI* I.

Eiichi KATO

Laboratory of Veterinary Hygiene and Microbiology,
Faculty of Veterinary Medicine,
Hokkaido University, Sapporo, Japan
(Received for Publication, Oct. 22, 1954)

INTRODUCTION

According to Kauffmann-White Schema, *S. abortus-equi* has the antigenic formula 4, 12 :—, e, n, x. EDWARDS and BRUNER demonstrated phases 1 and 3 of this organism. NISI and KUMAGAI observed that 17.4% of the tested strains are inagglutinable in the presence of e, n, x serum and these inagglutinable strains have new phase 1 factors not yet known. They assumed that the freshly isolated strains usually appear in phase 1 and that the phase variation readily transforms into phase 2 through subcultures.

SAKAZAKI and MURASE reported that the strains directly isolated from gastric juice of aborted fetus do not react in the presence of e, n, x serum, however rabbit serum immunized with above cultures reveals e, n, x antibody to a certain extent. Thus the existence of e, n, x factors in the very fresh strains of *S. abortus-equi* is still in question.

The author tried to investigate this point and ascertained that the hydrogen ion concentration of broth for the preparation of H antigens has an important role in the development of flagellar antigens.

MATERIALS AND METHODS

1. Strains of *S. abortus-equi* Used for Experiments

The details of the strains are shown in table 1. Gastric contents of aborted fetus infected with *S. abortus-equi* were stored in the refrigerator at 4°C without contaminations during the course of the experiment. Stock strains were isolated at this laboratory and subcultures are made on the ordinary agar media at intervals of 60 days. The stock period of these strains covered from several months to 28 years. Each of the strains was confirmed to be S form by usual methods.

TABLE 1. *Strains of Salmonella abortus-equi Used for Experiments*

	Name of Strain	Source of Isolation	Date of Abortion or Isolation	Period of Preservation of Materials or Culture	Colony Form
Gastric Juice Strains	M 33	Gastric contents of aborted fetus	15/ II '54	35~265 days	Smooth
	M 34	"	21/ II '54	30~134 "	"
	M 35	"	21/ II '54	30~ 44 "	"
	M 36	"	19/ II '54	32~ 72 "	"
	M 39	"	2/ III '54	20~ 32 "	"
	M 40	"	5/ III '54	17~ 29 "	"
	M 41	"	24/ III '54	10~202 "	"
	M 42	"	28/ III '54	53~218 "	"
Stock Strains	Tôfû	"	10/ III '54	2.5 months	"
	Fleur	"	30/ III '53	1 year	"
	Kon	"	31/ III '53	1 "	"
	Toyozakura	"	9/ II '52	2 years	"
	Ikaku	"	17/ III '51	3 "	"
	Umehana	"	12/ IV '51	3 "	"
	Endô	"	17/ IV '50	4 "	"
	Botan	"	23/XII '48	5.5 "	"
	Konishiki	"	14/ II '47	7 "	"
	Seika	Unknown	4/ I '42	12 "	Smooth (Unstable)
	Urahoro	Gastr. contents of aborted fetus	15/XII '33	21 "	"
	Daikai	Fistulous wither	28/XII '33	21 "	"
	Sitakara	Gastr. contents of aborted fetus	30/ IV '26	28 "	"

2. Preparation of Broth

Broth was prepared from horse meat extract and the pH of the medium adjusted at values from 6.0 to 8.5.

3. Preparation of Antigen

One drop of gastric content containing pure abortion bacilli was planted into broth of various pH and cultivated for 18 hours. Then an equal volume of 0.6% formol saline was added to broth cultures and they were placed in the 37°C incubator for 1 hour. They were used as the flagellar antigen.

4. Methods of the Preparation of e, n, x Serum

The e, n, x serum was obtained as follows: Rabbits were immunized with

formalin-broth culture of S. *abortus-equi* (strain β 202). Heat-killed abortion bacilli (strain Shizuno) were used for absorbing the 0 agglutinins. The e, n, x agglutinin titre of this absorbed serum reached to 1 : 6400 and 0 titre was negative at a dilution of 1 : 100.

Tube agglutination: The e, n, x serum was diluted with 0.5% carbol saline in a geometric series, starting from a dilution 1 : 200 and up to 1 : 6400 and 0.25 ml of culture was added to 0.25 ml of diluted serum. The result of floccular agglutination was read after an hour in a waterbath at 50°C.

Experimental Results

1. H Agglutination of Broth (pH 6.7) Cultures of *S. abortus-equi* in the Presence of e, n, x Serum at Various Incubating Time

Gastric contents containing pure abortion bacilli were directly inoculated into broth of pH 6.7 and incubated at 37°C for 6 to 48 hours. Five stock strains were used as control. The agglutinability of these broth cultures was examined in the presence of e, n, x serum as described above. The results are shown in table 2.

Table 2. *H Agglutination of Broth Cultures (pH 6.7) at Various Incubation Times*

	Strains	Incubating Time in Hours					
		6	12	18	24	30	48
Gastric Juice Strains	M 33	0	3200	3200	6400	3200	3200
	M 34	0	3200	6400±	3200	6400±	3200
	M 35	1600	6400	3200	1600	3200	3200
	M 36	0	6400	6400	3200±	3200	3200
	M 39	0	6400	3200	6400±	3200	3200
	M 40	0	6400	3200	6400	3200	6400
Stock Strains	Kon	6400	3200	3200	6400	3200	3200
	Umehana	3200	3200	3200	3200	3200	6400
	Konishiki	6400	3200	6400	6400	6400	6400
	Seika	3200	3200	3200	6400	3200	6400
	Daikai	3200	3200	3200±	3200	6400±	3200

Note: H agglutination is started from a dilution of 1 : 400.
The dilution of serum in every following table is the same as in this table.
0: no agglutination at 1 : 400.

At 6 hours incubation, most of the gastric juice strains showed no agglutination except one slightly agglutinated strain. However, after 12 or more hours incubation, all of these strains developed to their maximum titres. On the other hand, broth cultures of all 5 stock strains developed their agglutinability to the maximum extent early, after 6 hours incubation.

2. Influence of the pH of Broth on the Development of Flagellar Antigens of *S. abortus-equi*

Seven gastric juice strains and 5 stock cultures were cultivated at 37°C for 18 hours in broth having a pH range from 6.1 to 8.3.

In order tc prepare antigens, an equal volumes of 0.6% formol saline was added to each broth culture as described above. The results of e, n, x titres of each strain are shown in table 3.

TABLE 3. *H Agglutination of the Gastric Juice Strains and Stock Cultures Grown in Broth of Various pH*

	Strains	pH of Broth					
		6.1	6.5	6.9	7.3	7.9	8.3
Gastric Juice Strains	M 33	3200	3200	6400±	3200	0	0
	M 34	3200	3200	3200	0	0	0
	M 35	3200	3200	3200	6400	0	0
	M 36	3200	3200	3200	0	0	0
	M 39	3200	3200	3200	0	0	0
	M 40	3200	6400	6400	0	0	0
	M 41	1600	6400±	3200	3200	0	0
Stock Strains	Kon	3200	6400±	6400	3200	0	0
	Umehana	3200	6400	3200	3200	3200	6400
	Konishiki	3200	6400	6400	6400	6400	6400
	Seika	3200	3200	3200	3200	3200	6400
	Daikai	6400	6400	6400	6400	6400	6400

As indicated in table 3, 7 fresh strains (gastric juice) showed H titre of 1:3200 or 1:6400 within the pH range of broth from pH 6.1 to 6.9. At pH 7.3, 4 strains out of 7 showed no agglutintion. At pH 7.9 and 8.3 none of the fresh strains were agglutinated.

On the other hand, all 5 stock cultures were agglutinated at dilutions of 1:3200~1:6400 at the pH range from 6.1 to 7.3. Even at pH 7.9 or above, 4 out of 5 cultures showed the same titres. One stock culture which failed to agglutinate at pH 7.9 or more had been stored for about 1 year. It is younger than the other stock cultures which are aged more than 3 years. These results seem to indicate that the development of e, n, x factors of very fresh strains of abortion bacilli is inhibited by alkaline reaction of the cultural media, whereas the stock cultures are adapted to develop these flagellar antigens even when they are grown in alkaline media.

3. Changes of Agglutinability of the Freshly Isolated Strains by Frequent Passages on Nutrient Agar

As mentioned above, the native abortion bacilli in gastric contents seem to

develop e, n, x antigens with difficulty in alkaline media. The author tried to examine the changes of agglutinability of these freshly isolated strains by drastic transferences on nutrient agar day by day. One loop of the abortion bacilli from agar slant cultures was inoculated into broth of the various pH values at every generation, and the agglutination was tested.

TABLE 4. *Changes of Agglutinability of the Freshly Isolated Strains from Gastric Contents by Frequent Transfers on Nutrient Agar*

STRAINS	pH OF BROTH	NUMBER OF PASSAGES ON NUTRIENT AGAR						
		1	2	3	4	5	10	15
M 33	6.0	1600	3200	3200	3200	3200	1600	3200
	6.8	1600	3200	1600	1600	3200	1600	3200
	7.3	3200	6400	0	400	0	3200±	3200
	7.9	0	0	0	0	0	1600	3200
	8.3	0	0	0	0	0	800	6400
M 34	6.0	0	3200	3200	3200	3200	1600	3200
	6.8	1600±	3200	1600	3200	1600	1600	3200
	7.3	0	6400	0	1600	0	3200	3200
	7.9	0	0	0	0	0	3200	3200
	8.3	0	0	0	0	0	1600	6400
M 36	6.0	3200	3200	1600	1600	3200	1600	3200
	6.8	1600	3200	1600	3200	1600	3200	3200
	7.3	0	3200	0	400	1600	3200	3200
	7.9	0	0	0	0	0	3200	3200
	8.3	0	0	0	0	0	3200	3200
M 41	6.0	3200	3200	3200	3200	3200	1600	3200
	6.8	1600	3200	1600	1600	1600	1600	3200
	7.3	3200	1600	0	0	0	3200	3200
	7.9	0	0	0	0	0	0	400±
	8.3	0	0	0	0	0	0	0
M 42	6.0	1600	3200	3200	3200	3200	3200±	3200
	6.8	1600	3200	3200±	1600	1600	1600	3200
	7.3	1600	3200	0	1600±	0	3200	3200
	7.9	0	0	0	0	0	1600	3200
	8.3	0	0	0	0	0	0	3200

As indicated in table 4, up to 5 transferences, most of the strains grown in broth of pH 7.3 or above are non-agglutinable. At the 10th transference, 4 out of 5 strains become agglutinable at pH 7.9 or 8.3 and at the 15th transference, these 4 strains showed higher agglutinability even at pH 8.3. However, 1 strain, M 41, remained inagglutinable at pH 7.9 and 8.3 after 15 transferences.

4. H Agglutination of the Resuspended Antigens in Formol Saline after Centrifuging of the Broth Cultures

It is uncertain whether the inagglutinability of gastric juice strains in alkaline broth is caused by the poor development of flagellar antigen or influenced by some physico-chemical properties of alkaline media. In order to clarify these points, the author carried out the following small experiment.

Gastric juice and stock strains were cultivated for 18 hours in broth of various pH values ranging from 6.1 to 8.3. After the centrifuge of broth cultures at 3,000 r. p. m. for 30 minutes, settled organisms were resuspended in two-fold quantities of 0.3% formol saline (pH 4.8). These resuspended antigens were tested in the presence of e, n, x serum.

TABLE 5. *H Agglutination of the Resuspended Antigens in Formol Saline after Centrifuge of Broth (Various pH) Cultures Incubated for 18 Hours*

	STRAINS	pH OF BROTH					
		6.1	6.5	6.8	7.1	7.9	8.5
Gastric Juice Strains	M 33	3200	1600	1600	1600±	0	0
	M 34	1600	1600	1600	1600	0	0
	M 41	1600	1600	1600	800	0	0
	M 42	1600	1600	3200	800	0	0
Stock Strains	Tôfû	3200±	1600	1600	1600±	0	0
	Fleur	1600	1600	1600±	1600	0	0
	Toyozakura	3200±	1600	1600	1600	1600	1600
	Ikaku	1600	1600	1600	1600	1600	0
	Endô	1600	1600	1600	1600	1600	1600
	Botan	3200±	1600	1600	1600	1600	1600
	Urahoro	1600	1600	800	1600	1600	1600
	Sitakara	1600	1600	3200±	1600	1600	1600

As indicated in table 5, the behavior of the resuspended antigens of gastric juice strains was the same as in the original broth cultures depending upon the pH of the culture media. That is to say, antigens resuspended from broth culture at the pH range from 6.1 to 6.8 were proved to be highly agglutinable and resuspensions from broth of pH 7.1 were less agglutinable. However, at pH 7.9 and 8.5 all gastric juice strains were non-agglutinable. Among the stock cultures, two strains of shorter stock periods showed the same behavior as above. The resuspensions of the older stock strains both from the alkaline and acid broth were agglutinable to the same degree.

TABLE 6. *H Agglutination of the Resuspensions in Formol Saline Mixed with an Equal Volume of Broth of Various pH*

pH OF INOCULATED BROTH	pH OF ADDITIONAL BROTH	GASTRIC JUICE STRAINS		STOCK STRAINS	
		M 33	M 36	Endô	Urahoro
6.1	6.1	3200	3200±	3200	3200
	6.5	3200±	3200	3200	3200
	6.8	1600	3200	3200	3200
	7.1	3200	1600	3200	3200
	7.9	6400	3200	3200	3200
	8.5	1600	1600	3200±	3200±
6.5	6.1	3200±	1600	3200±	3200±
	6.5	3200	3200	3200	3200
	6.8	3200±	3200	3200±	3200
	7.1	1600	1600	3200	1600
	7.9	3200	1600	3200	1600
	8.5	1600	3200±	3200	3200
6.9	6.1	3200	1600	3200	3200
	6.5	1600	1600	1600	3200
	6.8	3200	1600	3200±	3200
	7.1	3200	1600	3200	3200
	7.9	3200	3200	3200	3200
	8.5	1600	1600	3200	3200
7.3	6.1	1600	800	3200	3200
	6.5	1600	1600	1600	3200
	6.8	1600	0	1600	1600
	7.1	1600	0	1600	3200
	7.9	1600	1600	3200	3200
	8.5	800	800	3200	3200
7.9	6.1	0	0	3200±	3200
	6.5	0	0	3200	3200
	6.8	0	0	3200±	3200
	7.1	0	0	3200±	3200
	7.9	0	0	3200	3200
	8.5	0	0	3200	3200
8.3	6.1	0	0	3200	3200
	6.5	0	0	3200	3200
	6.8	0	0	3200	3200
	7.1	0	0	3200	3200
	7.9	0	0	3200±	3200
	8.5	0	0	3200	3200

5. H Agglutination of the Resuspensions in Formol Saline Mixed with an Equal Volume of Broth at Various pH Values

Two gastric juice strains and 2 stock cultures were cultivated in broth of various pH ranging from 6.1 to 8.5 for 18 hours, then centrifuged at 3,000 r. p. m. for 30 minutes. The settled organisms were resuspended in an equal volume of 0.3% formol saline (pH 4.8). The resuspensions were mixed with the equal volume of broth of various pH. These antigens also showed the same behavior as in the plain formol saline resuspensions without being influenced by the addition of broth.

6. Morphological and Serological Observations of Gastric Juice Strains Grown in Alkaline Broth

Samples of gastric juice containing pure abortion bacilli were directly inoculated into broth of pH values ranging from 6.6 to 8.0. After 18 hours incubation, the motility of the growing bacilli was observed by hanging drop preparations. They were motile in every broth of the various pH. By flagellar staining, there have been shown to occur several flagella in broth of pH 8.0 as well as in that of pH 6.6. As described above, at pH 7.9 or more, the broth cultures of gastric contents were not agglutinated by e, n, x serum. In order to demonstrate the possible variation of the flagellar antigen in alkaline media, 19 other factor sera such as a; c; d; f, g, t; e, h; k; l, v; l, w; m; p; q; s; t; y; z_5; l, 2; 1, 5; 1, 6; 5 were tested. None of them caused the agglutination of these alkaline broth cultures. Further studies are needed to clarify the mechanisms of the inagglutinable phenomenon of very fresh strains of abortion bacilli in alkaline media in the presence of e, n, x serum.

SUMMARY AND CONCLUSION

The author has attempted to investigate the influence of the pH of broth upon the development of flagellar antigens of the native form of *S. abortus-equi* directly cultivated in broth from the gastric juice of aborted fetus. The results obtained are summarized as follows:

1. The development of the flagellar antigens of the native form of *S. abortus-equi* when cultivated in broth directly from gastric juice, is strongly affected by the pH of the broth. They develop well in acid broth. In alkaline broth, at pH values 7.3—7.4, some of them develop their flagellar antigens to a slight extent, whereas at pH 7.8 or more, all of them fail to develop it.

2. The stock strains develop the flagellar antigens in alkaline media. The stock period or the number of passages on artificial culture media has a definite influence upon the development of H antigens. The longer the stock period, the more the strains are adapted to develop H antigens

in alkaline media. The stock strains being aged more than 4 years are stable for the production of e, n, x antigens in alkaline broth.

In view of these facts, it may be said that the hydrogen ion concentration of broth has an important role in the development of the flagellar antigens of *S. abortus-equi.*

The author wishes to express his cordial gratitude to Prof. K. HIRATO, the chief of this Laboratory, for his criticisms and review of this paper and also to Assist. Prof. K. SHIMIZU for his many kind advices.

REFERENCES

1) EDWARDS, P. R. & D. W. BRUNER (1939): *J. Bact.*, **38**, 63.
2) NISI, T. & T. KUMAGAI (1951): *Jap. J. Vet. Sci.*, **13**, 355 (Japanese).
3) SAKAZAKI, R. & N. MURASE (1954): *The 37th Meeting of Jap. Soc. Vet. Sci.*