Automation of a Hemagglutination-Inhibition Test for Parainfluenza 3 Antibodies in Bovine Sera

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An automated hemagglutination-inhibition (HI) test for the "shipping fever" strain (SF-4) of parainfluenza 3 antibody in bovine sera was developed and compared to manual tube and microtiter test procedures. The automated system operating at 60 samples per hr provided the most test results per specified time period, and the manual tube test provided the least. The manual microtiter test and the automated system at 40 samples per hr, falling between the two above procedures, were comparable in the number of sera that could be titrated in 1 day by one technician. There was little difference between automated and manual test reproducibility when measured at the twofold titer one-dilution difference level. However, the automated system titrated a higher number of sera at the same titer on repeat runs than either of the manual test procedures. The automated one-quartile difference reproducibility (each twofold dilution subdivided into 4 units—"quartiles") was equal to the manual test one-dilution difference reproducibility. The standard deviation of the per cent variation from the mean of paired serum titers for 40-sample-per-hr runs ranged from ±3.49 to ±5.36%. The manual and automated systems were of comparable sensitivity in their detection of negative sera.

For serological investigations in which there is either a very high or a very low prevalence of antibodies, serological screening may require vast numbers of specimens to find statistically valid differences for any given characteristic. Many large-scale sero-epidemiological studies have not been instituted in the past because of laboratory limitations of time, space, or adequately trained personnel, or all three.

Parainfluenza of cattle presents a case in point. The epidemiological study of this disease is currently impeded by virtue of the high prevalence of antibodies (ranging from 50 to 86%) found in normal bovine populations (21).

Manual serological tests for parainfluenza antibody in cattle include complement fixation (CF), hemagglutination-inhibition (HI), and serum neutralization (SN) procedures, with the CF test being the least sensitive (1, 11). The HI test has been shown to correlate well with the SN test (1, 11) [which is generally recognized as a better indicator of protective immunity (14)] and is simpler and less expensive to conduct (11).

Automated systems are relatively new in the serology laboratory. There are several different automated tests in use in syphilis serology. These include CF procedures (6, 15), a flocculation test (with antigen adsorbed to charcoal particles; references 13, 17, 18), and a fluorescent treponemal antibody absorption test (G. W. Stout et al., Amer. Pub. Health Ass. Annu. Meeting, Detroit, Mich., 10 to 15 Nov. 1968). Recent developments have been made in CF procedures for the detection of Australia antigen (R. Vargues et al., E. Suba, and S. Vernace et al., 1970 Technicon Int. Congr., Advan. Automated Analysis, New York, 2 to 4 Nov. 1970). A diagnostic CF test for amebiasis (20) is under development at the Center for Disease Control in Atlanta, Ga. The automated CF test has also been used in the study of foot-and-mouth disease virus types and subtypes (16). Automated hemagglutination (HA) systems have been utilized in research associated with influenza viruses (9, 10). These systems have proved of great value to the pharmaceutical industry in the production of influenza vaccines (4, 7). An automated HI test was developed in England (3) for the detection of influenza antibodies.

1 Taken from a thesis submitted by Donald W. Webert to the University of Pennsylvania in partial fulfillment of the requirements for the Master of Medical Science (M.S. (Med.)) degree. Presented in part at the 2nd Technicon International Congress, Advances in Automated Analysis, New York, 1970.

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It is the purpose of this paper to present the procedures used and results obtained in the development of an automated serological HI system for the detection of parainfluenza antibodies in bovine sera and its comparison to accepted manual test procedures.

MATERIALS AND METHODS

Virus. Virus used in the parainfluenza 3 (PI-3) HI tests, both manual and automated, was the "shipping fever" (SF-4) strain obtained from G. Frank of the National Animal Disease Laboratory, Ames, Iowa. Viral antigen was prepared in monolayer cultures of bovine embryonic kidney cells.

Sera. A series of 51 bovine sera used in the manual and automated test procedures were obtained from the field services unit of the University of Pennsylvania School of Veterinary Medicine. An additional serum, titrated in each of the manual test runs, was used as the "standard" in the automated runs. Sera were inactivated at 56 C for 30 min and diluted 1:5 in sterile phosphate-buffered saline (PBS). Each specimen was divided into samples which were stored at -20 C until used.

Red blood (RB) cells. Bovine blood was collected in sterile Alsever's solution from calves or mature cattle at various local slaughterhouses. The RB cells were packed by centrifugation and washed three times in sterile PBS. The washed cells were resuspended in sterile Alsever's solution (in a 10 to 20% cell suspension) and stored at approximately 4C until used.

During the preliminary phases of this study, it was found that the RB cells could not be maintained for more than 2 days in PBS without excessive lysis occurring. However, when they were maintained in Alsever's solution and were stored at 4 C (with gentle agitation to resuspend the cells daily), they were kept for up to 2 months without exhibiting any appreciable lysis. There was no detectable difference in sensitivity in these cells over this period of time in the automated system.

Manual HI tests. The manual HI tube test used was laboratory method 9-66 (Virology Section, Diagnostic Services, Animal Health Division, National Animal Disease Laboratory, Ames, Iowa).

The manual microtiter test was a modification of the procedure described by Lennette (12). PBS (0.05 ml) was dispensed into appropriate microtiter plate wells. After serum dilutions were completed (1:10 to 1:1280), 0.05 ml of antigen (4 HA units/0.05 ml) was added, and plates were incubated at room temperature for 1 hr. Then 0.025 ml of 0.5% bovine RB cells was added to appropriate wells. Plates were incubated at 4 C overnight (18 to 20 hr).

The highest dilution of serum completely inhibiting HA was considered the HI titer of that serum (± agglutination was considered as "complete" inhibition).

If the antigen control indicated that the antigen used in a test run was insufficient or in excess, the titers of all sera in that run were corrected by the procedure of Clarke and Casals (2). They stated that a regular reciprocal relationship exists between antigen units and HI titer.

In manual PI-3 HI tests with bovine RB cells, an antibody titer below 1:20 is considered "negative." This "level of significance" was used in all test results reported here (both manual and automated).

Automated HI test. Two basic automated HA systems (Technicon Corp., Tarrytown, N.Y.) were combined (Fig. 1-3). One-half of the system was used to test the sera for HI antibodies and the other half was used to test for nonspecific agglutination of RB
cells by the sera. Each serum sample was aspirated through two probes simultaneously. To determine what effect sample sequence might have on reproducibility, the same sequence of randomly selected specimens was run on 2 consecutive days on one channel of the system. This procedure was then repeated with a new, randomly selected sequence. Tests were run at the rate of 40 and 60 specimens per hr controlled by a 2:1 (sample-to-wash ratio) cam. The cam is a notched disc in the sampling unit which controls both the rate of sampling and the period of time that serum samples and intersample solutions are aspirated into the test system.

Serum samples were diluted 1:10 (1:2 dilution of the stored sera) and were treated with kaolin (Fisher Scientific Co., Fair Lawn, N.J.) to remove nonspecific inhibitors of agglutination (5). Approximately 1 ml of each treated serum was placed in a sampler cup. At the beginning of a run, a relatively high-titered serum was aspirated for 10 to 15 sec to act as a start-of-run marker. This was followed by 9 cups of a serially diluted "standard." Two samples of PBS were placed between the last standard sample and the first quality control (QC) specimen to allow the "carry-over" effect of the undiluted standard to wash out (Fig. 4). Following the first QC sample were 11 randomly selected "unknown" sera. These were followed by a PBS wash sample, a QC sample, 10 randomly selected unknowns, a PBS wash, etc., until all 51 unknowns had been tested. A PBS wash sample and a QC sample ended the sequence. All QC specimens were preceded by a PBS wash sample. This standardized the effect of the "preceding sample" on all the control specimens in all runs (Fig. 5).

When a weak positive serum specimen is sampled after a strongly positive specimen, the titer of the weak sample is often uninterpretable on the recorder. Such "sloughed" specimens were retitrated at the end of each run. Strongly positive sera, whose titers were above the linear region of the standard curve, were diluted 1:2 or 1:4 and were retitrated also. All reruns were periodically bracketed with PBS and QC specimens as they had been in the normal run period.

The description of the automated HI test procedure which follows is valid for both manifolds, with the exception that the serum HA control unit used no antigen. In the serum control unit, a solution of PBS with two drops of Tween 20 per liter (PBS + T20)
was pumped through the "antigen" line to maintain volumetric relationships in the two halves of the system.

Serum and antigen (see Fig. 3 for flow rates) were mixed and allowed to "react" for 8.5 min (Fig. 6A) before 2% bovine RB cells were added (Fig. 6B). An HA phase of 17.75 min at room temperature followed (Fig. 6C). PBS + T20 was then added to disperse any nonspecifically agglutinated RB cells (Fig. 6D). Agglutinated cells settled to the bottom of the moving fluid stream in a large horizontal glass coil (Fig. 6E). These cells were aspirated from the stream and discarded at two "T" fittings (Fig. 6F). Remaining nonagglutinated cells were lysed by the addition of Triton X-100, thereby releasing hemoglobin (Fig. 6G). The concentration of the hemoglobin was measured by a colorimeter and was recorded on a strip chart recorder (there was a separate colorimeter and recorder for each half of the system). The amount of hemoglobin measured reflected the amount of specific antibody in the serum sample being tested.

At the end of each run, the system was cleaned with either (i) 2.5% urea plus 0.05 M NaOH or (ii) 2% RBS 25 (Fisher Scientific Co.) at 50 C. Once a week
the system was flushed with dilute chromic acid solution. This was forced into the system with a syringe, thereby providing rapid dispersion throughout. After 5 min it was flushed out with distilled water. This was then pumped through all lines of the system for 30 min or more.

**Calculation of automated test titers.** At the end of each run, the “peak” values of the standards on the strip chart recorder were transposed from per cent transmission (%T) to optical density (OD). An OD value equal to that produced by the density of the
serum itself was subtracted from the standard curve peaks (Fig. 7). This was, in effect, diluted out at a 1:10 dilution. In the linear region of the corrected curve, the difference in OD values between two adjacent peaks was divided into equal parts (Fig. 8). This midpoint between two peaks was selected as the dividing point between adjacent twofold titers. To record results comparable to the manual twofold end points, any unknown peak value falling between two of these midpoints was recorded as possessing that particular twofold titer. Since automated clinical chemistry systems have generally been shown to provide much more precise and reproducible results than corresponding manual tests, the OD distance between two standard curve peaks for the automated HI system was further subdivided into four equal parts ("quartiles", Fig. 8). Unkn0wns were then also recorded with a quartile titer which could be directly compared to manual twofold titers.

An arbitrary set of "tolerance limits" was set for the QC specimens. A variation of 10% of the midpoint titer between the highest and lowest QC values in any given run was permitted above and below that midpoint. Any variation of a QC greater than this 10% limit would indicate an apparent baseline shift. Where this occurred, titers would be adjusted to compensate for the degree of drift. The maximum reproducibility to be expected from the automated system was determined by comparing the titers of the quality control samples within each run.

The reproducibility of twofold titers in paired and multiple replicate manual and automated runs was determined by the one-dilution difference method of Hall (8). The procedure was also adapted to the analysis of the reproducibility of quartile titers in the automated system.

**Method for evaluation of the automated system.** Test comparisons made in this study are summarized in Fig. 9. The manual tube test was used as the standard test procedure. The within-technician and between-technician reproducibility to be expected from this test system was determined first. The microtiter test was then compared to the tube test system. The reproducibility of the automated system was determined by comparisons between different sample sequences and sampling rates. The twofold and quartile titers were determined and compared within-system and to manual test twofold titers (solid lines in Fig. 9). The continuous titers for the automated system were calculated and their reproducibility within-system was determined (dashed lines in Fig. 9). The results obtained in the various test comparisons are found in the tables indicated by the numbers in parentheses in Fig. 9 or are in the text.

**RESULTS**

Comparison of manual and automated HI test systems. Table 1 indicates the number of sera that could be titrated in a day by using the test systems and procedures utilized in this study. Manual test results could not be read until after an incubation period of 18 to 20 hr. The time span from serum sample input to recorded-test output in the automated system was 37 min. It was found that satisfactory HA of bovine RB cells required approximately 20 min at room temperature (21°C), but only 7 min or less at 37°C.

A comparison of the amount of reagents used in the manual and automated test procedures
was made (Table 2). Reagents used in the associated HA titration and HI antigen controls for each run of sera are included in the table. The laboratory space needed for the two-channel automated system and the manual test systems was comparable.

**Evaluation of reproducibility of manual HI test results.** Eleven manual test runs were completed (six tube test and five microtiter test runs). In one tube test run the RB cell agglutination patterns were poorly formed. The results of this test run had to be discarded.

The reproducibility and agreement of duplicate runs of the 52 sera in test tubes are presented in Table 3 for each of two technicians. Two tube test runs of each technician were paired (four pairs of different technician runs) to demonstrate the agreement of test results between the two technicians (Table 4).

The first two of five microtiter test runs completed by technician B were 98.0% reproducible. These two runs were compared to each of the two tube test runs by the same technician (Table 5). The reproducibility of all five tube test runs was 90.0%, and that of the five microtiter runs was 86.2%.

As a screening procedure, 12 of the 52 sera titrated at less than 1:20 in 1 or more of the 10 repeat titrations. Nine of these sera titrated at this level in both manual test systems.

**Evaluation of reproducibility of automated tests.** A total of seven runs of 51 sera was made on

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### Table 1. Time required for a single technician to complete a titration run by using test tubes, microtiter plates, and the automated system

| Test type                  | No. of sera | No. of runs | Avg time (hr:min) | No. of tests per &hr-day (estimate) |
|----------------------------|-------------|-------------|-------------------|-------------------------------------|
| Manual tube test           | 52          | 5           | 7:29              | 60-70                               |
| Manual microtiter test     | 52          | 3           | 6:01              | 100-125                             |
| Automated (40 samples per hr) | 51          | 3           | 5:49              | 100-125                             |
| Automated (40 samples per hr) | 51          | 2           | 4:48              | 125-150                             |

* Includes time to prepare reagents and complete other pre- and postrun functions.
* Plus 9 standards, 9 quality control samples, 12 or more phosphate-buffered saline samples, to standardize the quality controls, and 21 to 29 serum sample reruns for 51 unknowns.
* This estimate would be higher if improved techniques reduced the number of reruns.

### Table 2. Comparison of the total amount of reagents required to titrate comparable runs of sera by using manual methods (52 sera) and automated methods (51 sera) with dilutions and units adjusted to the manual tube test level

| Method                      | Phosphate-buffered saline (ml) | Serum (ml), 1:5 dilution | Antigen: 10 HA units/ml | Red cells (ml), 0.04% | Red blood cell lysing agent (ml) |
|-----------------------------|---------------------------------|--------------------------|-------------------------|----------------------|----------------------------------|
| Manual tube test            | 190                             | 31.2                     | 120                     | 165                  | 0                                |
| Manual microtiter test      | 35                              | 5.2                      | 12                      | 18                   | 0                                |
| Automated test (40/hr)*     | 1,200                           | 6.3                      | 504                     | 275                  | 1,405                            |
| Automated test (60/hr)*     | 900                             | 4.4                      | 364                     | 196                  | 1,015                            |

* Manual test titrations were from 1:10 to 1:1,280 in twofold steps.
* Triton X-100.
* All figures include the amount of reagents required for standards, quality controls, reruns, etc.
TABLE 4. Distribution and agreement of repeat titrations in four pairs of manual hemagglutination-inhibition tube test runs by two different technicians

| Serum titer | Technician A | Technician B |
|-------------|-------------|-------------|
| <10         | 11          | 8           |
| 10          | 13          | 2           |
| 20          | 18          | 4           |
| 40          | 17          | 8           |
| 80          | 16          | 6           |
| 160         | 22          | 1           |
| 320         | 5           | 5           |
| 640         | 2           | 0           |

* Reproducibility is 100.0% at the one-dilution difference level.

TABLE 5. Distribution and agreement of duplicate titers for four paired runs between the manual microtiter and tube tests by one technician

| Serum titer | Tube test | Microtiter test |
|-------------|-----------|-----------------|
| <10         | 8         | 2               |
| 10          | 10        | 18              |
| 20          | 6         | 46              |
| 40          | 14        | 8               |
| 80          | 20        | 31              |
| 160         | 11        | 4               |
| 320         | 4         | 1               |
| 640         | 2         | 0               |

* Reproducibility is 99.5% at the one-dilution difference level.

TABLE 6. Reproducibility (by various parameters) of nine replicate quality control samples in each of seven runs of 51 sera with the automated hemagglutination inhibition system

| Run no. | Samples per hr | Mean titer | Midpoint titer* | Max variation | SDb | CVc |
|---------|----------------|------------|-----------------|---------------|-----|-----|
|         |                |            |                 | ± Titerd      | ± Per cent |     |
| 1       | 40             | 90.87      | 90.89           | 16.30         | 17.93 | 12.53 | 13.78 |
| 2       | 40             | 88.03      | 86.87           | 4.04          | 4.64  | 2.62  | 2.97  |
| 3       | 40             | 71.77      | 72.06           | 4.04          | 5.59  | 2.85  | 3.97  |
| 4       | 40             | 75.94      | 75.35           | 2.82          | 3.74  | 1.74  | 2.29  |
| 5       | 40             | 74.89      | 75.33           | 3.72          | 4.93  | 2.64  | 3.52  |
| 6       | 60             | 68.24      | 68.41           | 2.77          | 4.04  | 1.87  | 2.74  |
| 7       | 60             | 69.74      | 69.05           | 2.19          | 3.17  | 1.77  | 2.53  |

* Midpoint between highest and lowest quality control titer. The same specimens were not used as the quality control sample in all runs. Therefore, differences in titer between runs does not necessarily reflect system variation from run to run.

b Standard deviation.

c Coefficient of variation = SD/mean × 100.
d Actual titer variation both above and below the midpoint of the nine replicate quality controls.

* Exceeds the ±10% quality control tolerance limits set for this study.
TABLE 7. Distribution and agreement of twofold titers in four replicate automated runs between two different sequences of 51 sera at 40 samples per hr

| Serum titer | Sequence 1 | Sequence 2 |
|-------------|------------|------------|
|              | <10        | 10         | 20         | 40         | 80         | 160        | 320        | 640        |
| <10          | 10         | 20         | 40         | 80         | 160        | 320        | 640        |
| <10          | 4          | 2          | 1          | 1          | 2          | 1          |
| 10           | 3          | 15         | 6          | 7          | 2          | 1          |
| 20           | 40         | 80         | 160        | 320        | 640        |
| 40           | 3          | 15         | 6          | 7          | 2          | 1          |
| 80           | 2          | 1          | 1          | 1          | 2          |
| 160          | 1          | 1          | 1          | 1          | 2          |
| 320          | 2          | 1          | 1          | 1          | 2          |
| 640          | 1          | 1          | 1          | 1          | 2          |

* Excludes results from 10 sera in each run in which all replicate titers were <10 or in which all four replicates were not interpretable. Reproducibility is 100.0% with 82.9% of the sera titrating at the same titer in all four replicates.

Table 7 shows the distribution and agreement of twofold titers for four sets of different-sequence paired runs at 40 samples per hr. Two of the 40-per-hr runs (one of each sample sequence) were followed on the same day by 60-per-hr runs (Fig. 5). The agreement and reproducibility of duplicate titers between the two runs at each speed are given in Table 8. The one-quartile difference reproducibility of these paired different-sequence runs at 40 and 60 samples per hr was 97.7% and 90.9%, respectively (100.0 and 95.4%, respectively, when only those results from the linear region of the curve were considered).

The two 40-per-hr runs and two 60-per-hr runs were paired against each other to determine if there was any difference between the results produced at the two different speeds on both a same-sequence and different-sequence basis. Reproducibility at the one-dilution difference level was found to be 100.0% in both cases. On a same-sequence basis, 75.8% of the serum pairs titrated at the same titer, whereas this occurred in 72.7% of the serum pairs on a different-sequence basis.

As a screening procedure, in the seven automated runs 12 of the sera were titrated at less than 1:20 in one or more of the runs.

**Evaluation of reproducibility between manual and automated systems.** Two manual tube test runs of each technician were compared to the four automated 40-sample-per-hr runs (Table 9).
Various paired and multiple test comparisons of six manual and five automated (40/hr) runs showed the reproducibility levels indicated in Table 10.

In the first seven manual runs completed and the seven automated runs, 13 sera were titrated one or more times at less than 1:20. Twelve of these sera were titrated at this level in both the manual and automated systems. Of the total of 91 individual titrations (13 sera × 7 titrations/serum), the manual and automated systems both titrated 35 samples at 1:20. A total of 56 specimens were negative (<1:20) by the automated system, with the manual systems titrating 52 specimens at <1:20 and 4 at 1:40.

Analysis of "continuous-titers." The variability of titers from "standardized" quality controls measured on the same day and the same channel of the automated system was presented in Table 4. The standard deviations ranged from ±1.74 to ±12.53 with a mean of ±3.72. The mean of the variation between paired "unknown" sera (expressed as a per cent of the mean titer of the pairs) and the corresponding standard deviations are shown in Table 11. As might be expected, the mean variation for 60-sample-per-hr different-sequence runs was greater than 40-sample-per-hr runs under the same conditions. The mean variation for 40-sample-per-hr different-sequence runs was greater than similar same-sequence runs only when analysis was confined to the linear portion of the standard curve.

**DISCUSSION**

The automated continuous-flow system (Technicon Corp.) is currently the major contributor to automated serology. Most of the discrete sample systems now used in automated clinical chemistry have not been reported to have been used in serology. These will probably contribute little to sero-epidemiology in the future, espe-

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**Table 10. Reproducibility of manual and 40-sample-per-hr automated hemagglutination inhibition test runs at different levels**

| Reproducibility level | Two runs/comparison | > Two runs/comparison |
|-----------------------|---------------------|-----------------------|
|                       | Manual (6) ^a       | Automated             | Manual (three runs) | Automated (four runs) |
|                       |                     | All pairs (4)         | Restricted pairs ^b (5) | All replicates (2) | Restricted replicates ^b (2) |
| Same titer (twofold dilutions) | 50.0–83.7 ^c | 79.5–88.9 | 90.9–100.0 | 22.0–48.0 | 66.7–80.0 | 86.4–90.0 |
| One-dilution difference ^d | 90.0–100.0 | 95.6–100.0 | 100.0 | 88.0–90.0 | 95.2–95.6 | 73.8–91.1 | 100.0 |
| One-quartile difference ^e | 90.0–97.7 | 95.4–100.0^f |

^a Number in parentheses is the number of comparisons represented in that column.

^b Only those results that fell on the central straight portion of the standard curve are included.

^c All values expressed as percentages.

^d The "accepted" reproducibility level of manual twofold dilution test procedures.

^e One-dilution difference encompasses a total of eight quartiles, whereas a one-quartile difference has a range of only two quartiles.

^f Four of the five paired runs were 100.0% reproducible at this level.

**Table 11. Per cent variation from the mean of paired serum titers of the same and different sequences at 40 and 60 samples per hr**

| Serum pairs ^a | Same sequence 40/hr | Different sequence |
|---------------|---------------------|--------------------|
|               | Sequence 1 | Sequence 2 | 40/HR | 60/HR |
|               | Mean | SD ^b | Mean | SD | Mean | SD | Mean | SD |
| 41 ^c | 4.03 | 5.15 | 4.41 | 4.20 | 4.29 | 3.49 | 6.77 | 5.45 |
| 22 ^c | 3.09 | 1.74 | 3.48 | 3.66 | 4.22 | 3.39 | 5.59 | 5.66 |

^a Number of duplicate sera with interpretable results, but not including pairs with both titers of <10 or pairs in which one result was uninterpretable.

^b Standard deviation.

^c All pairs were limited to the "straight" portion of the standard curve.
cially in programs involving large numbers (thousands) of serum specimens.

The study demonstrated the feasibility of combining individual HI units into multichannel HI systems as has been done with automated CF and clinical chemistry systems. These could be structured in two ways: (i) to titrate a serum specimen against several antigens simultaneously, and (ii) to titrate several serum specimens simultaneously against one antigen.

Vargosko (19) has estimated that, by using manual test procedures, 50% of the time of a serological laboratory technician is spent in performing clerical duties. The utilization of an automatic data acquisition system in conjunction with automated serological systems would eliminate most of the routine clerical duties. An on-line computer in a multichannel automated serology system would also be of great importance for system monitoring and control. This would provide immediate and automatic adjustment of test results whenever excessive baseline shift occurred.

Although it was quite conservative in the amount of undiluted serum used per test (0.02 ml), the automated system used antigen at a rate three to four times greater than the manual tube test and 30 to 40 times greater than the microtiter test system. This level of antigen consumption must be reduced and might be accomplished by one or more methods. Reagent concentrations and flow rates, or both, might be changed. Other methods would be to pump antigen into the HI system only when a serum specimen is aspirated or to increase the sampling rate without increasing reagent flow rates. A valve and control unit was developed during this study to regulate antigen flow in this manner. Although not used in this study, preliminary work with this unit showed it to be a satisfactory working system. The combination of sampling rate and sample-to-wash ratio would be a determining factor as to the applicability of such a procedure in any specific test system. The cost and availability of the antigen used in the test system would be another important factor to be considered.

In the 10 manual test runs carried out in the study, 60.8% of the sera titrated at one-dilution difference or less in all 10 replicates. The high degree of reproducibility consistently seen throughout the manual test phase of this study exceeded expectations. It can probably be attributed to the practice of adjusting the test results (2) in any of the runs in which the antigen control indicated that the amount of antigen used was not the optimum.

Since the quality control specimens were used in the automated test runs to determine the within-run system stability, they were subjected as closely as possible to the same prior-sample influence. Test results showed that inserting a PBS sample in front of each control specimen was a satisfactory procedure. An alternative method would be to use two quality controls in succession and read only the second one of each pair. The first QC of each pair would act as the "standardizing" prior sample. Tumbleson (Technicon Int. Congr., Advan. Automated Analysis, New York, 1970) has found this method to be the more stable of the two methods.

The calculation of automated twofold and quartile titers served basically one purpose: to provide a frame of reference for comparing the automated HI system directly to the currently accepted manual HI test procedures. These comparisons showed that when all test results in each automated run were included, the automated one-dilution difference reproducibility was only slightly higher than that of the manual test systems. However, if the per cent of replicates that titrated at the same titer in both manual and automated runs was compared, the automated system was found to be much more reproducible—especially when three or more runs were included (Table 10). All automated test comparisons showed 100.0% reproducibility at the one-dilution difference level if only those results from the linear portion of the curve were used, regardless of the number of runs in the comparison. In some paired runs of the automated system, the reproducibility was 100.0% at the same-titer level.

The one-quartile difference reproducibility of the automated tests was comparable to the one-dilution difference reproducibility of the manual tests. When restricted to the straight portion of the standard curve, the one-quartile reproducibility was 100.0% in four of five paired runs and in one of two multireplicate comparisons (Table 10). This high degree of reproducibility at the one-quartile difference level demonstrated the ability of the automated system to titrate sera much more precisely than is possible with the manual twofold procedure.

If the automated system standard curves are viewed as a series of discrete but contiguous points, it can be seen that antibody titers are definable to a much finer degree than either the twofold or quartile measuring systems allow. In fact, if the concept of dividing twofold titers into quartile titers is carried on into smaller and smaller units, a series of discrete points will be the result ("continuous titers"). This type of titer definition is not possible in manual test techniques, even if sera are titrated in fractional-fold dilutions. Furthermore, the inability of the
twofold titer measurement system to detect large fluctuations in automated test-run baselines is probably reflected in manual test systems. This might explain some of the problems experienced in manual serological test procedures. By providing continuous titers, the automated system makes as important a contribution to serology as it does with increased speed of analysis and reproducibility of titration results.

The ±10% tolerance limit established for automated system QC variation in this study (by using the continuous-titer measurement procedure) is a much narrower range than that which is accepted in the manual test procedures. The one-dilution difference accepted for "reproduced" repeat titrations in manual serological tests allows a variation of 50 or 100% of a given titer. That is, a test result with a titer of 1:160 may, on a subsequent run, be titrated at 1:80 (50% change) or 1:320 (100% change) and still be considered as a reproduced result. The ±10% tolerance limit at a titer of 1:160 confined repeat titrations to the range of 1:144 to 1:176 for a reproduced result. The greater reproducibility of the automated system may make it possible to reevaluate the serological definition of "evidence of infection" which is used in manual techniques (i.e., fourfold change in titer between acute- and convalescent-phase sera).

At 40 specimens per hr, sample sequence had little, if any, significant effect upon an individual serum titer when the practice of retitrating those sera which appeared to be adversely affected by a preceding sample was observed. Hopefully, mathematical correction of test results for sample interaction would be as satisfactory a procedure in serology as it is in those clinical chemistry systems which utilize on-line computer systems. This could preclude the necessity of retitrating sloughed samples.

In the automated test, the range of the standard deviations of the per cent variation from the mean titer of the serum pairs (40/hr runs) was twice as large when all test results in a run were included (±3.49 to ±5.36%) as when only those results on the central straight portion of the standard curves were measured (±1.74 to ±3.66%). When four replicate runs (at 40/hr) were analyzed, the standard deviations were equal to the highest value of the paired-run ranges (±5.41 and ±3.48%, respectively).

Comparison of the manual and automated procedures as screening test systems to detect positive sera (titers of 1:20 or greater) and negative sera (<1:20) indicated that the two types of systems were of comparable sensitivity. Automated systems will make it possible to screen large numbers of sera at a specific level of significance, especially in low prevalence antibody rate disease conditions. Screening runs can be carried out at a faster rate than titration runs in the automated system and would be of great value in determining which specimens have titers that are too low to be of further interest in a study.

Automated serological systems will provide the means for undertaking many long-range epidemiological studies of importance to both human and animal health. These may include long-range surveillance systems, specific disease eradication programs, etc. The results of this study show that automated continuous-flow HI systems are feasible.

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LITERATURE CITED

1. Abinanti, F. R., A. B. Hoerlein, R. L. Watson, and R. J. Huebner. 1961. Serologic studies of myxovirus para-influenza 3 in cattle and the prevalence of anti-bodies in bovines. J. Immunol. 86:505-511.
2. Clarke, D. H., and J. Casals. 1958. Techniques for hemagglutination-inhibition with arthropod-borne viruses. Amer. J. Trop. Med. Hyg. 7:561-573.
3. Cohen, A. 1966. Automatic technique for titration of influenza virus haemagglutination inhibitors. J. Clin. Pathol. 19:589-594.
4. Debellis, D. J., G. R. Erie, and J. F. Lawlis. 1968. Comparative studies of reagents used in automated CCA testing of influenza virus. Ann. N.Y. Acad. Sci. 153:582-594.
5. Frank, G. H. 1966. A hemagglutination-inhibition test for parainfluenza 3 virus antibodies, p. 59-66. Proc. 70th Ann. Meeting U. S. Livestock San. Ass.
6. Gaillon, R., J. Ripault, C. Studievic, and J. Daussot. 1967. Use of the Auto-Analyzer to perform serological tests for syphilis. Study of 1,009 sera using a complement fixation test with the cardiolipidic antigen. Int. Arch. Allergy Appl. Immunol. 32:278-281.
7. Gruenmeier, P. W., A. Gray, and A. Ferrari. 1965. Automated hemagglutination assays. Ann. N.Y. Acad. Sci. 134:809-818.
8. Hall, E. C., and M. B. Felkner. 1970. Reproducibility in the serological laboratory. Health Lab. Sci. 7:63-68.
9. Hebeka, E. K., F. B. Brandon, and J. Molteni. 1967. Assay of hemagglutination titer of influenza with the Auto-Analyzer. Appl. Microbiol. 15:956-958.
10. Hebeka, E. K., R. M. Walker, and W. B. Beadmore. 1968. Automated procedure for measuring antigenicity of extracted and intact influenza virus. Appl. Microbiol. 16:1699-1705.
11. Hoerlein, A. B., M. E. Mansfield, F. R. Abinanti, and R. J. Huebner. 1959. Studies of shipping fever in cattle. 1. Parainfluenza 3 virus antibodies in feeder calves. J. Amer. Vet. Med. Ass. 135:153-160.
12. Lennette, E. H. 1969. In E. H. Lennette and N. J. Schmidt (ed.), Diagnostic procedures for viral and rickettsial diseases, 4th ed., p. 52-58. American Public Health Association, Inc., New York.
13. McGrew, B. E., G. W. Stout, and V. H. Falcone. 1968.
Further studies of an automated flocculation test for syphilis. Amer. J. Med. Technol. 34:634-643.

14. Peacock, G. V. 1968. Comments on bovine parainfluenza-3 immunization procedures. J. Amer. Vet. Med. Ass. 152:879-880.

15. Pugh, V. W., and R. W. T. Gaze. 1966. The Reiter protein complement-fixation test using the AutoAnalyzer. J. Clin. Pathol. 19:595-599.

16. Roumiantzeff, M., and M. T. Fayet. 1969. Emploi de serums spécifiques d'éléments viraux dans les reactions de fixation du complément du virus de la fièvre aphteuse. Ann. Inst. Pasteur 116:833-851.

17. Schroeter, A. L., H. F. Taswell, and M. A. Sweatt. 1970. Adaptation of automated reagin test for syphilis to the Technicon multi-channel AutoAnalyzer for blood typing, p. 265-268. In Advances in automated analysis vol. 1. Medial, Inc., White Plains, N.Y.

18. Stevens, R. W., and E. Stroebel. 1970. The automateP reagin test: results compared with VDRL and FIA-ABS tests. Amer. J. Clin. Pathol. 53:32-34.

19. Vargosko, A. J. 1968. Developments in automation of microbiology. Lab. Manage. 6:23-27.

20. Walls, K. W., L. S. Hall, and G. R. Healy. 1970. Automated diagnostic complement fixation techniques in microbiology. I. Amebiasis, p. 259-264. In Advances in automated analysis, vol. 1. Medial, Inc., White Plains, N.Y.

21. Woods, G. T. 1968. The natural history of bovine myxovirus parainfluenza 3. J. Amer. Vet. Med. Ass. 152:771-777.